therapeutic utility. Lastly, there is no proof of a mechanism by which KGF protects against fibrosis. KGF mRNA expression in the lungs decreased after bleomycin administration. In contrast, collagen mRNA expression in the lungs increased after bleomycin administration, and the increase was significantly attenuated by the administration of Ad-KGF, which resulted in less severe fibrosis in the lungs. These results strongly suggest direct and/or indirect effects of KGF in preventing the pulmonary fibrosis caused by bleomycin.

In conclusion, administration of a KGF-expressing adenoviral vector to mice with preexisting bleomycin-induced pulmonary fibrosis reduced fibrosis of the lungs, improved respiratory function, and reduced mortality. These phenotypes were associated with KGF effects including alveolar epithelial cell proliferation, suppression of TGF-β1 production, and increased surfactant protein secretion. These observations suggest the therapeutic utility of the KGF-expressing adenoviral vector in pulmonary fibrosis.

Acknowledgements

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Figure legends

Fig. 1. Structure of Ad-KGF (AxCAmKGF) (i) and Ad-1w1 (Ax1w1) (ii).

The arrow indicates the direction of transcription. Solid triangles under the Ad genome represent deleted Ad sequences.

CAG, cytomegalovirus enhancer and chicken β-actin promoter; GpA, rabbit β-globin poly (A) signal; mKGF, mouse KGF cDNA (from Ref. #22, with permission).

Fig. 2. Survival rates after first bleomycin administration. Seven days after the administration of bleomycin, mice (n = 15 to 16 per group) were given (A) 1.0×10^8 PFU of Ad (Ad-KGF or Ad-1w1) (low dose) or (B) 1.0×10^9 PFU of Ad (high dose) intratracheally. Data are expressed as Kaplan-Meier product limit curves and were compared by log rank test. *: $p < 0.05$ vs. Saline group, #: $p < 0.05$ vs. 1w1 group. (A) When the low-dose vector was administered, the survival rate of the 1w1 group was the same as that of the Saline group, whereas the survival of the KGF group was significantly increased compared with that of the Saline and 1w1 groups. (B) When the high-dose vector was administered, the survival rate of the 1w1 group was significantly reduced compared with that of the Saline group; however, the survival rate of the KGF group was significantly increased compared with both the Saline

and 1w1 groups.

Fig. 3. Representative micrographs of lung tissue of mice given Ad-KGF alone. Shown are immunohistochemistry images for KGF (A) and SP-C (B) 1 week after Ad-KGF administration. High magnification images are shown in insets. Diffuse hyperplasia of cuboidal cells positive for KGF is seen in alveolar region. The cells are also positive for SP-C, suggesting that they have features of type II pneumocytes. Inflammation was minimal. Lung sections harvested 3 weeks after Ad-KGF administration are stained with H & E (C) or Masson's trichrome (collagen stained blue) (D). Hyperplasia of cuboidal cells still exists at 3 weeks, but there was no sign of fibrosis. Black bars: 50 μm.

Fig. 4. Representative micrographs of lung tissue stained with H&E. Masson's trichrome-stained images are shown in insets. Lungs from the naïve group are shown (A). Lungs taken 1 week after the administration of bleomycin show subpleural fibrosis (B). Two weeks after bleomycin treatment, lungs in the Saline (C), 1w1 (D), and KGF (E) groups had developed subpleural fibrosis. Four weeks after bleomycin treatment, expansion of fibrous areas towards the lung parenchyma was seen in the Saline (F), 1w1 (G), and KGF (H) groups; however, the area involved was significantly smaller in the KGF group than in the other two groups. Fibrosis did not progress much in the KGF group from 4 weeks (H) to 8 weeks (I). No mice in the Saline and 1w1 groups survived to 8 weeks. Black bar: 100 μm. (J) Quantitative analysis of lung fibrosis by Ashcroft score.

Each lung section was placed over 2 mm square grids, the lung morphology of each grid was scored, and the scores of all the grids were averaged. 1W is the score for the lungs taken 1 week after the administration of bleomycin and before any intratracheal (includes adenovirus vector) administration. 2W, 4W, and 8W are the scores for the lungs taken 2, 4, and 8 weeks after the administration of bleomycin and 1, 3, and 7 weeks after the vector administration. Values are mean ± SEM; n = 3-6 except at 2 weeks in the saline (n = 2) and KGF (n = 2) groups. *: $p<0.05$. N/A: no data were available because all mice had died by this time.

Fig. 5. Lung function tests.

(A) Quasi-static compliance (Cst) was measured under mechanical ventilation. (B) The lung volume (TLC - FRC) was calculated from a pressure-volume curve. Values are mean ± SEM; n = 3-5, except at 2 weeks (n = 2), and in the Saline group at 4 weeks (n = 2). *: $p<0.05$.

Fig. 6. Semi-quantitative analysis of KGF mRNA.

(A) Ad-KGF-derived KGF mRNA levels at 2, 4, and 8 weeks after bleomycin administration (1, 3, and 7 weeks after Ad-KGF administration, respectively) were quantitatively analyzed by RT-PCR and were normalized to that for β-actin. Values are mean ± SEM; n = 3 in each group.

(B) Total KGF (endogenous KGF + Ad-KGF derived KGF) mRNA levels were analyzed using real-time PCR and the values were normalized to that of the naïve group. Values are mean ± SEM; n = 3 in each group.

*: $p<0.05$. N/A: no data were available because all mice had died by this time.

Fig. 7. Immunohistochemistry for SP-C.

Lungs from the naïve group are shown (A). Two weeks after bleomycin treatment, lungs in the Saline (B) and 1w1 (C) groups had very few SP-C-positive cells, but there were many SP-C-positive cells in KGF group (D). Fewer SP-C positive cells per total number of lung cells were seen in the saline (E) and 1w1 (F) groups at 4 weeks. SP-C-positive cells were more prominent in the KGF group at 4 weeks (G). SP-C-positive cells were still dominant in the lungs at 8 weeks in the KGF group (H). No mice in the Saline and 1w1 groups survived to 8 weeks. Black bar: 50 μm. (I) SP-C-positive cells were counted in a blinded fashion and described as the percentage of SP-C-positive cells per total number of cells in the lungs. Values are mean ± SEM; n = 3-6, except at 2 weeks in the Saline (n = 2) and KGF (n = 2) groups. *: $p<0.05$. N/A: no data were available because all mice had died by this time.

Fig. 8. Levels of surfactant protein mRNA.

mRNA levels of SP-A, -B, -C, and -D after bleomycin administration were quantitatively analyzed by real-time PCR and the values were normalized to that for the naïve group. Values are mean ± SEM; n = 3 in each group.

Fig. 9. Protein concentrations of SP-D and TGF-β1.

Representative results of Western blotting for SP-D (A) and TGF-β1 (C) are shown. 2w, 4w, and 8w represent samples taken 2, 4, and 8 weeks, respectively, after bleomycin administration. Semi-quantitative analyses of SP-D proteins (B) and TGF-β1 (D) in lung homogenate are presented. The density and area of each signal obtained by Western blotting were analyzed using NIH Image. The results for quantitative SP-D or TGF-β1 protein were normalized to β-actin. Values are mean ± SEM; n = 3-4 except at 2 weeks in the saline (n = 2) and KGF (n = 2) groups.

*: $p < 0.05$. N/A: no data were available because all mice had died by this time.

Fig. 10. Expression of collagen mRNA in lungs.

mRNA expression of collagen 1a1 and collagen 3a1 after bleomycin administration were quantitatively analyzed by real-time PCR and the values were normalized to that for the naïve group. Values are mean ± SEM; n = 3 in each group.

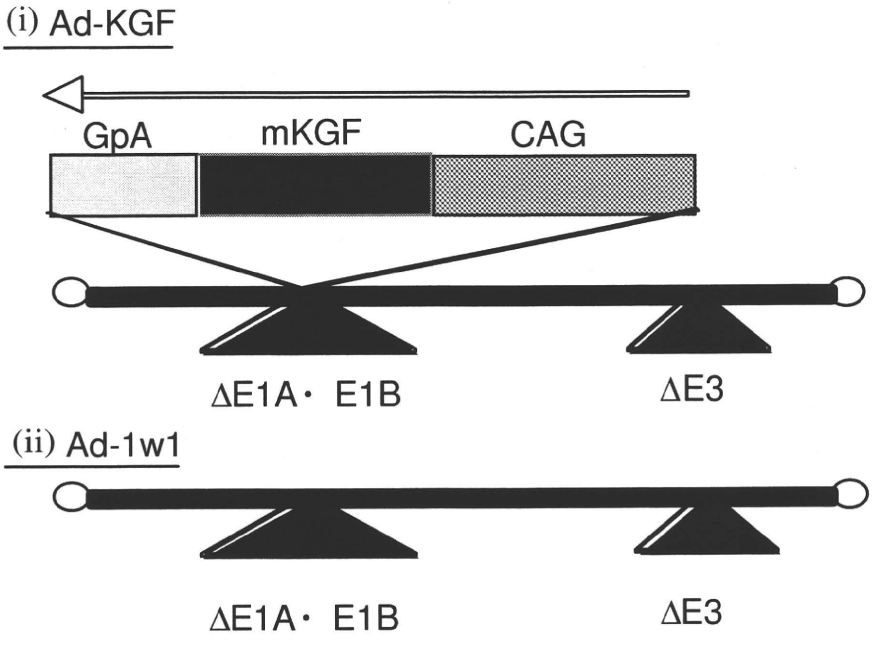

Fig. 1

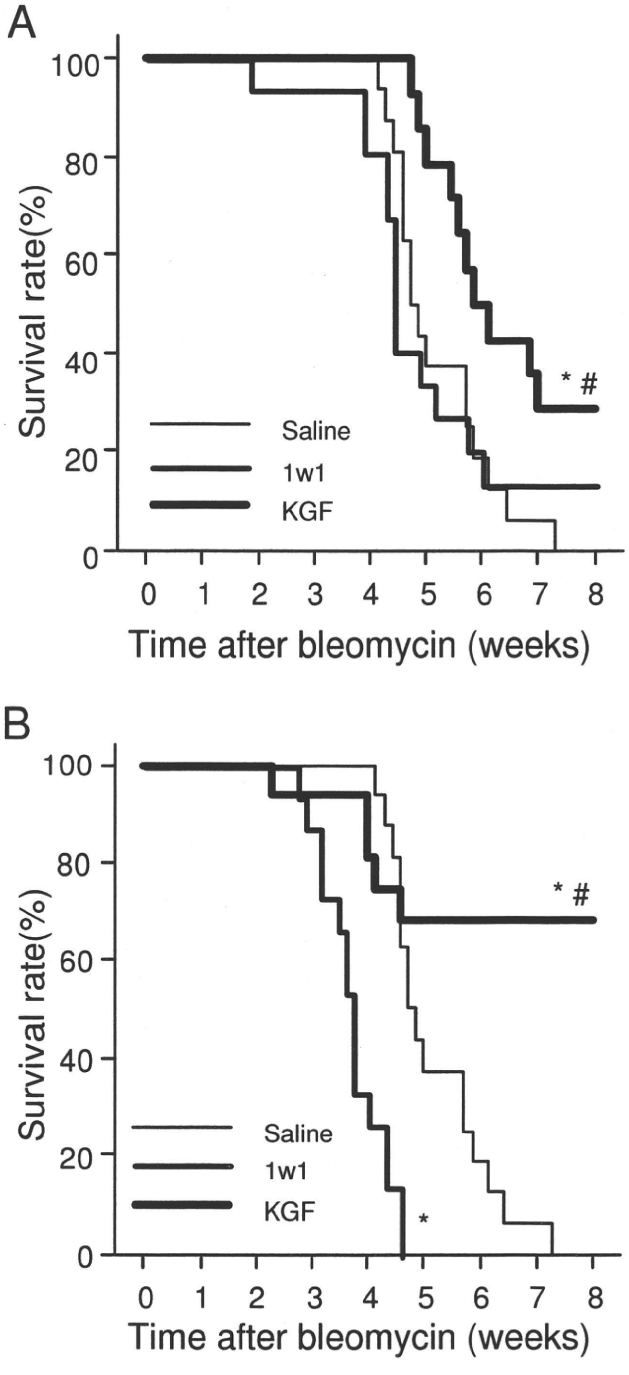
A
100
80
60
40
20
0
Survival rate(%)
Saline
1w1
KGF
* #
0 1 2 3 4 5 6 7 8
Time after bleomycin (weeks)
B
100
80
60
40
20
0
Survival rate(%)
Saline
1w1
KGF
* #
*
0 1 2 3 4 5 6 7 8
Time after bleomycin (weeks)

Fig. 2

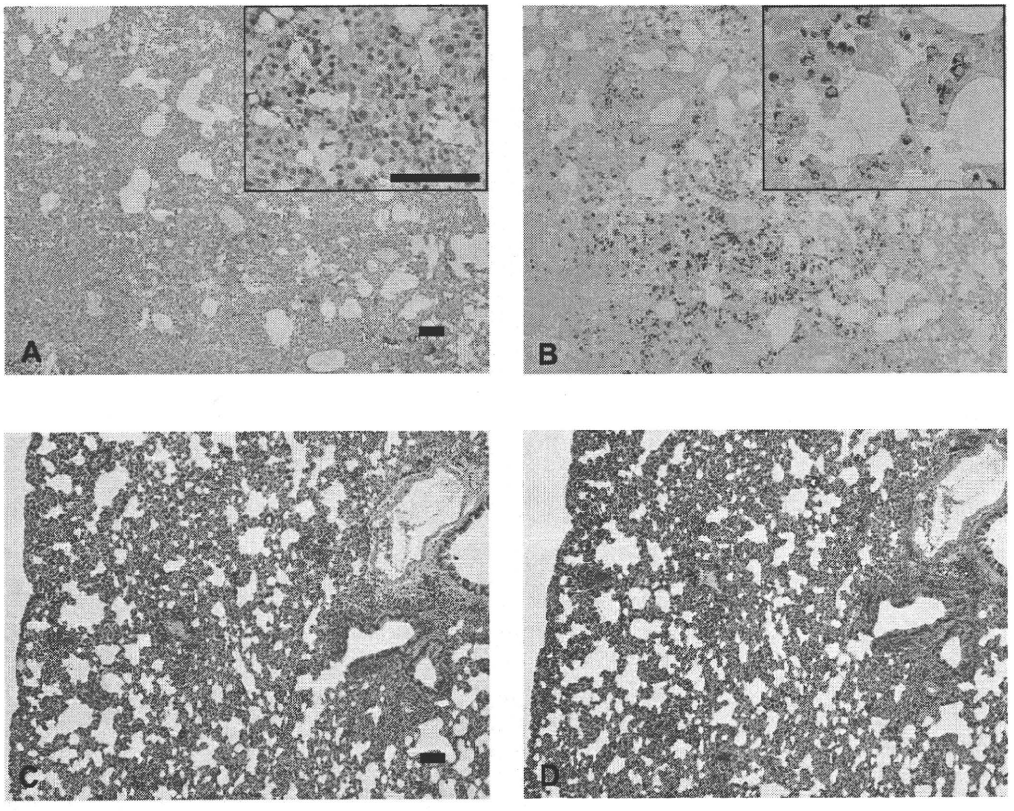

Fig. 3

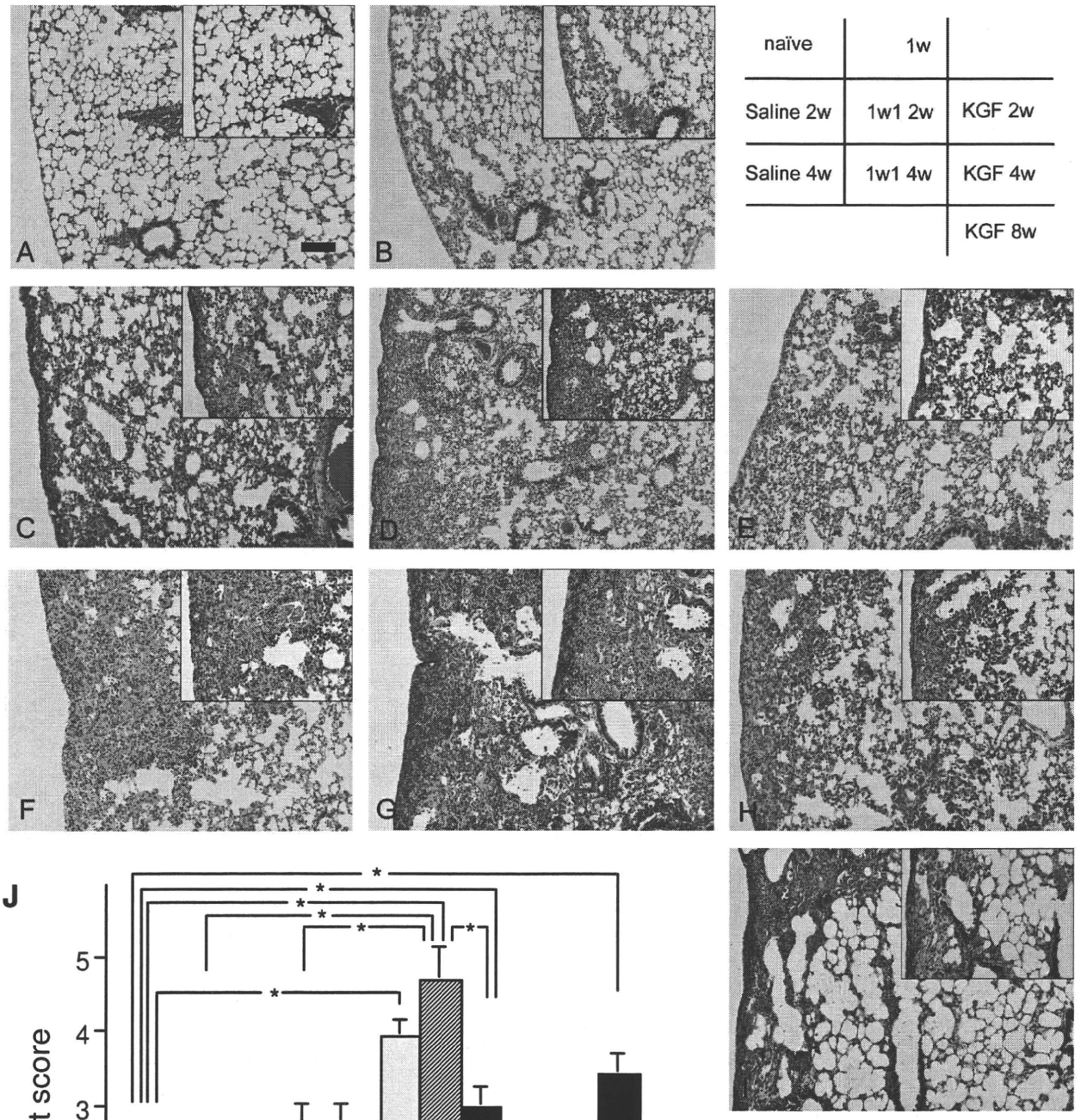
naïve
1w
Saline 2w
1w1 2w
KGF 2w
Saline 4w
1w1 4w
KGF 4w
KGF 8w
A
B
C
D
E
F
G
H
J
Ashcroft score
0
1
2
3
4
5
N/A N/A
naive
Saline
1w1
KGF
0 w
1 w
2 w
4 w
8 w

Fig. 4

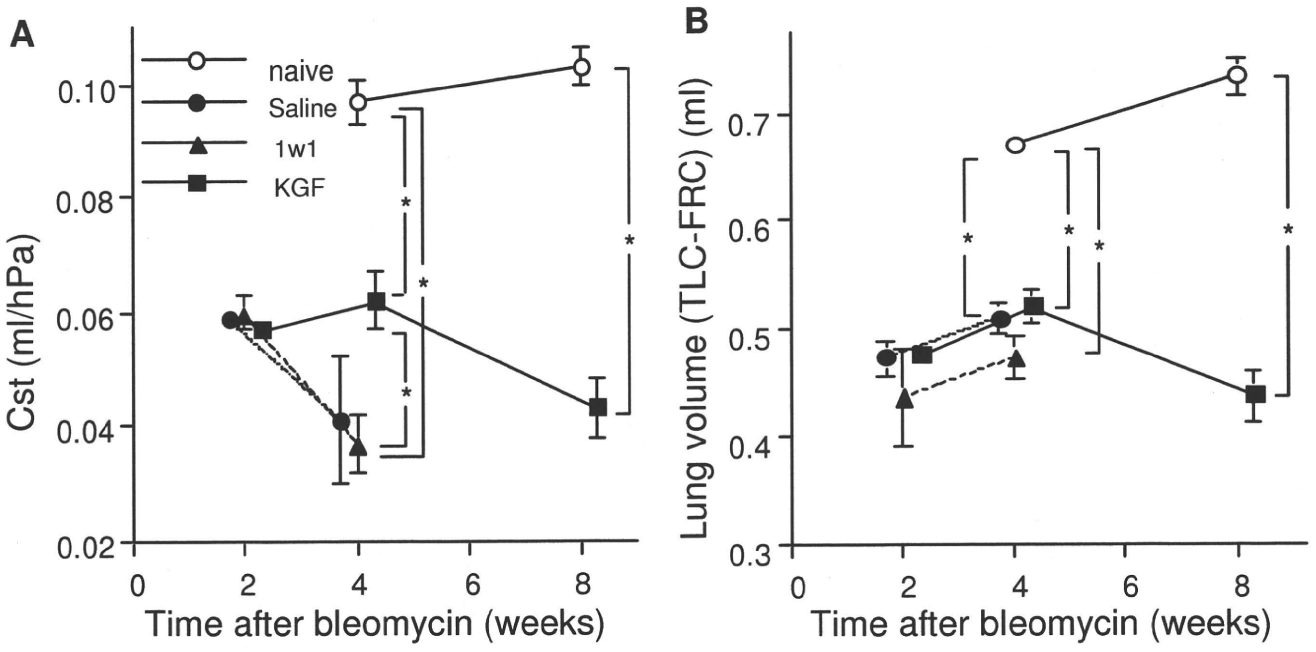
A
naive
Saline
1w1
KGF
Cst (ml/hPa)
0.10
0.08
0.06
0.04
0.02
0
2
4
6
8
Time after bleomycin (weeks)
B
Lung volume (TLC-FRC) (ml)
0.7
0.6
0.5
0.4
0.3
0
2
4
6
8
Time after bleomycin (weeks)

Fig. 5

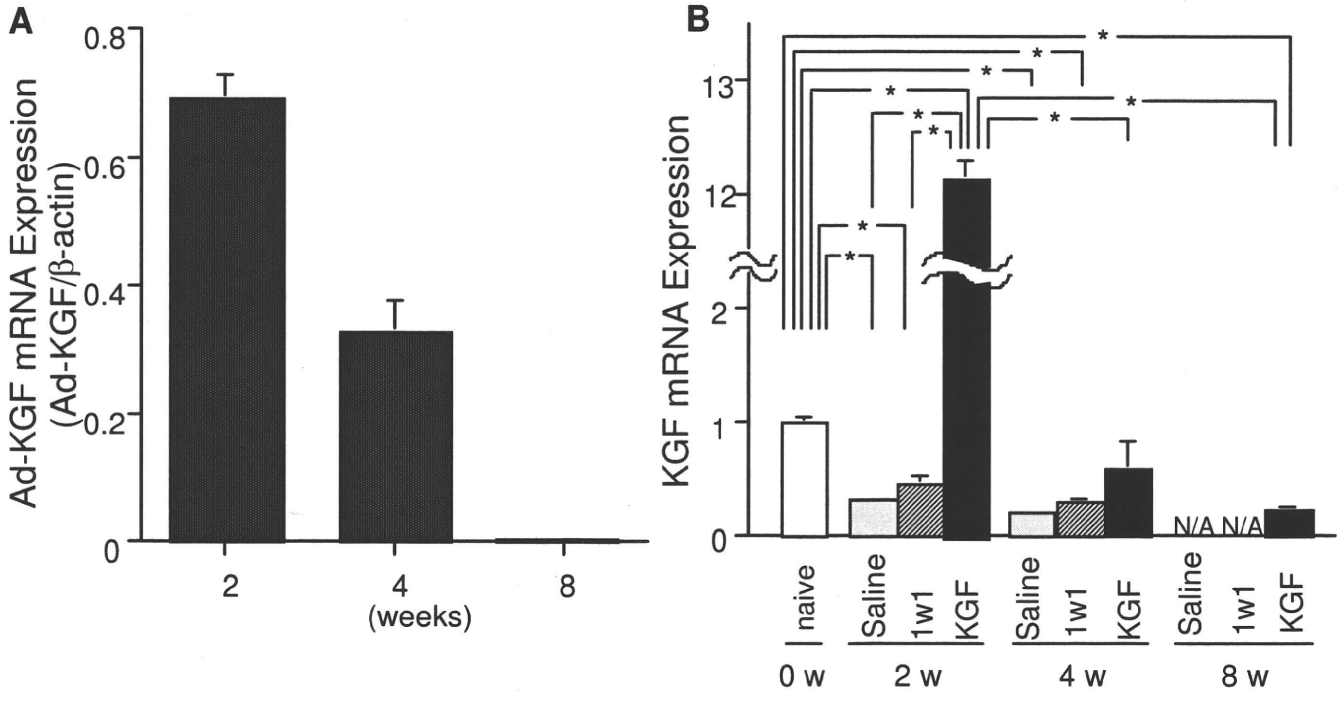
A
Ad-KGF mRNA Expression
(Ad-KGF/β-actin)
0.8
0.6
0.4
0.2
0
2
4
8
(weeks)
B
KGF mRNA Expression
13
12
2
1
0
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w

Fig. 6

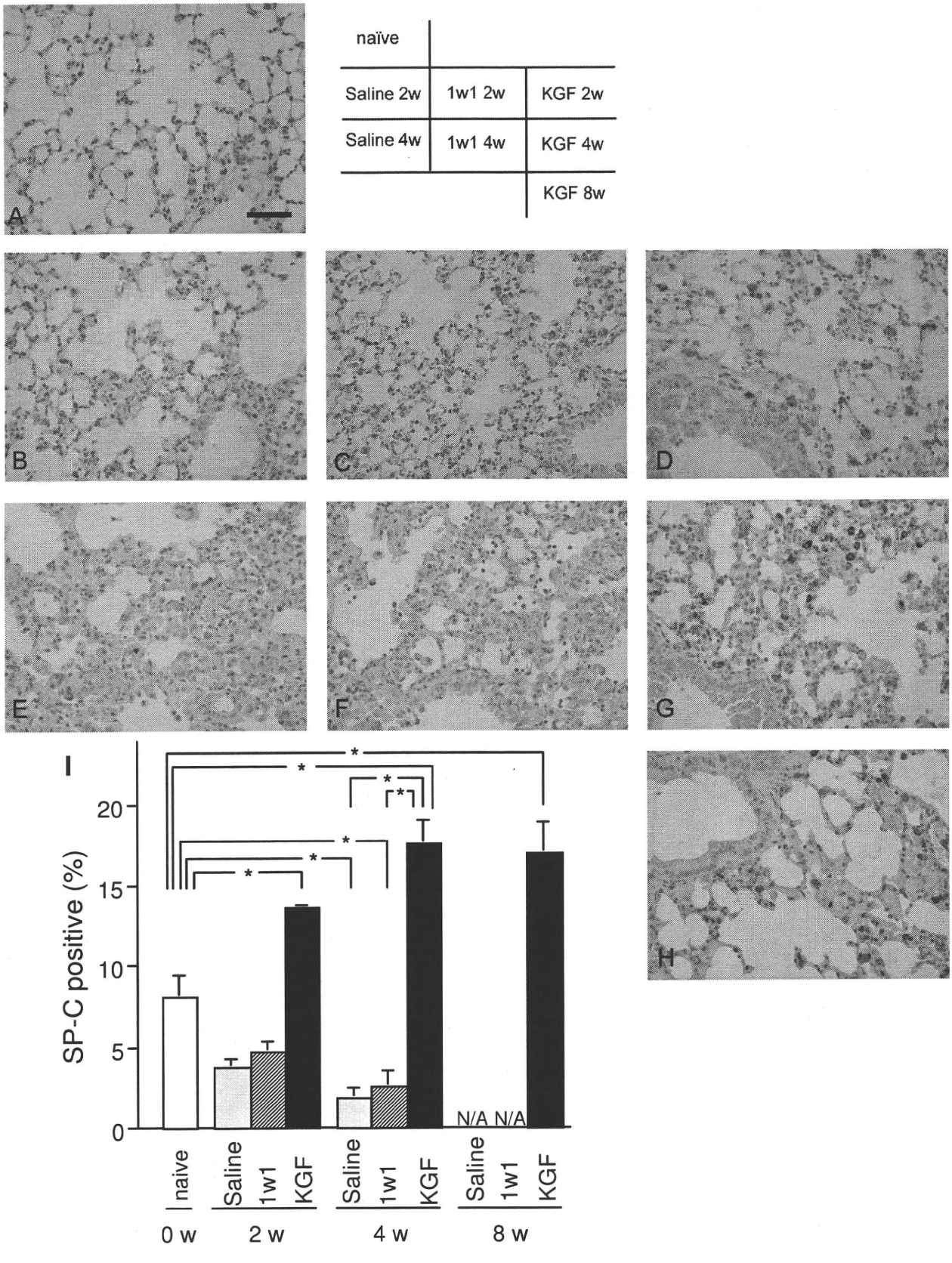
naïve
Saline 2w
1w1 2w
KGF 2w
Saline 4w
1w1 4w
KGF 4w
KGF 8w
A
B
C
D
E
F
G
H
I
SP-C positive (%)
0
5
10
15
20
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w

Fig. 7

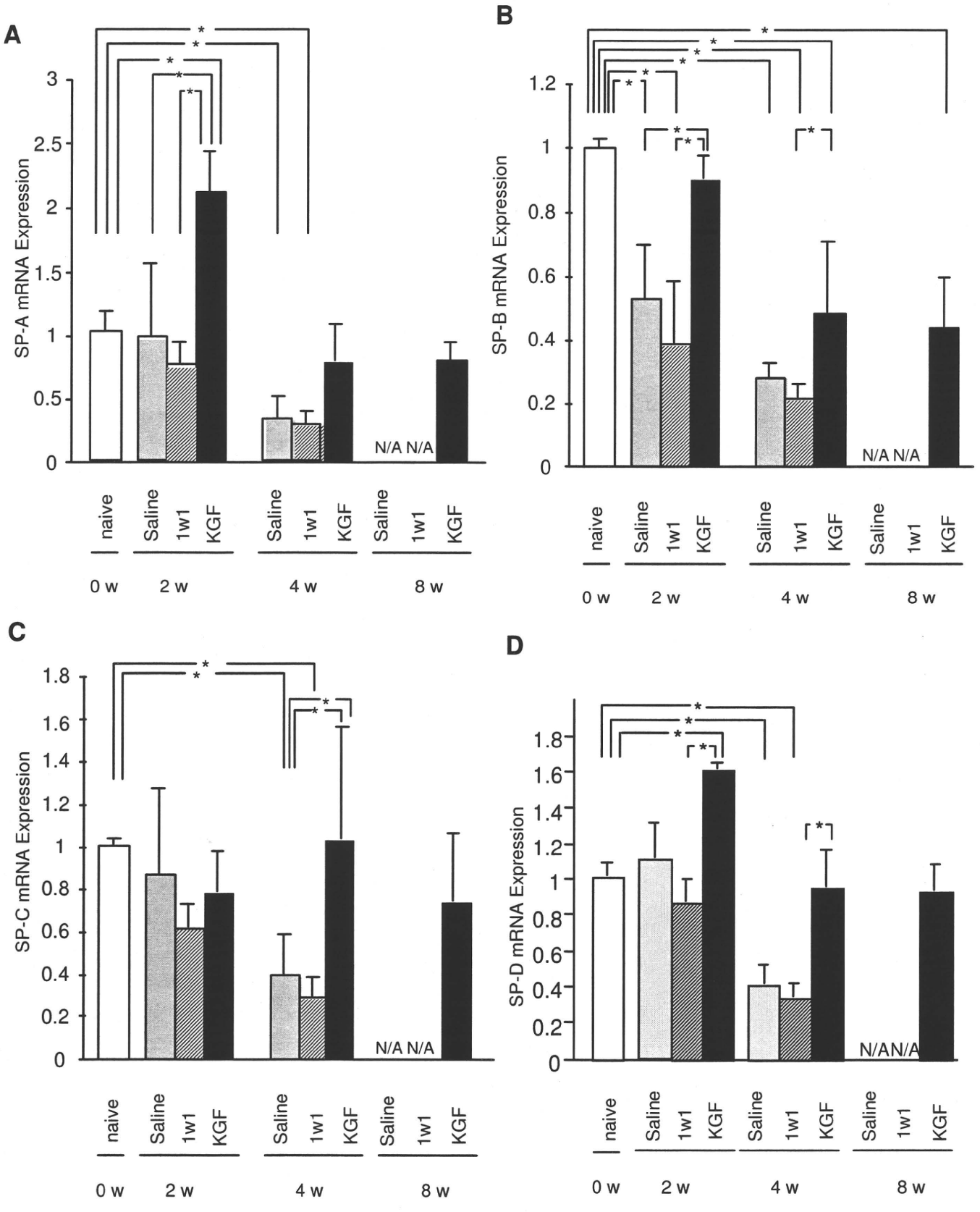
A
SP-A mRNA Expression
3
2.5
2
1.5
1
0.5
0
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w
B
SP-B mRNA Expression
1.2
1
0.8
0.6
0.4
0.2
0
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w
C
SP-C mRNA Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w
D
SP-D mRNA Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N/AN/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w

Fig. 8

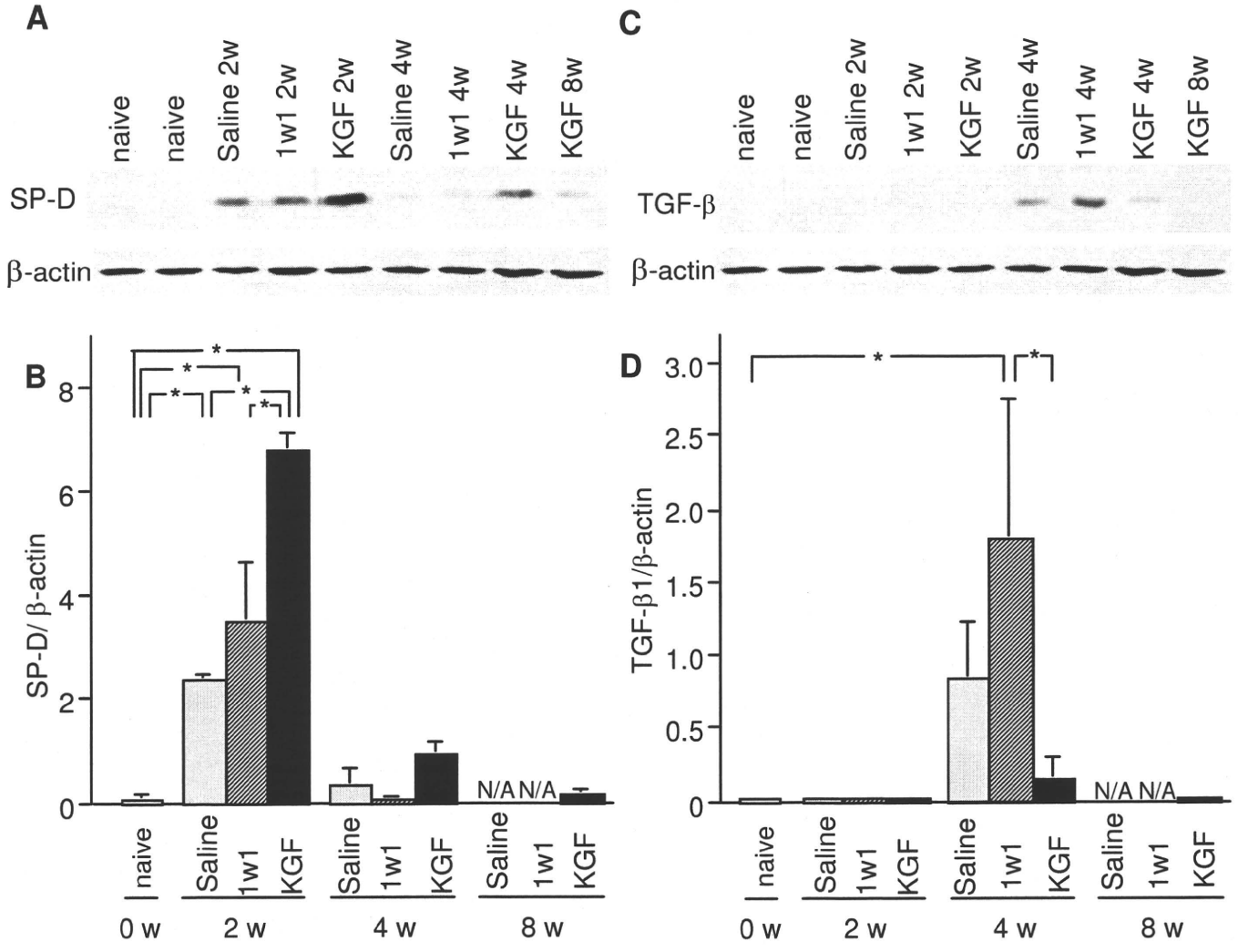
A
naive
naive
Saline 2w
1w1 2w
KGF 2w
Saline 4w
1w1 4w
KGF 4w
KGF 8w
SP-D
β-actin
C
naive
naive
Saline 2w
1w1 2w
KGF 2w
Saline 4w
1w1 4w
KGF 4w
KGF 8w
TGF-β
β-actin
B
SP-D/ β-actin
0
2
4
6
8
*
N/A N/A
naive
Saline
1w1
KGF
0 w
2 w
4 w
8 w
D
TGF-β1/β-actin
0
0.5
1.0
1.5
2.0
2.5
3.0
*
*
N/A N/A
naive
Saline
1w1
KGF
0 w
2 w
4 w
8 w

Fig. 9

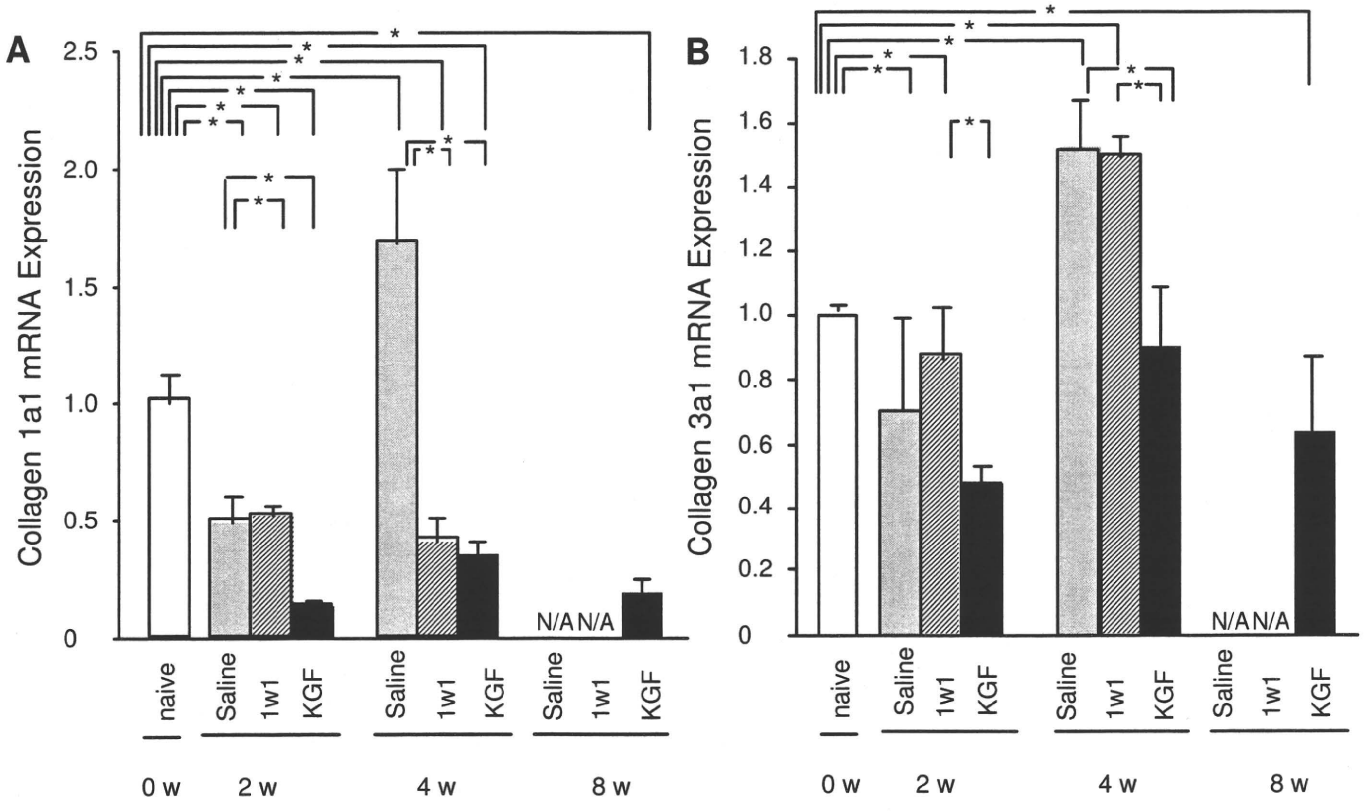
A
Collagen 1a1 mRNA Expression
2.5
2.0
1.5
1.0
0.5
0
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w
B
Collagen 3a1 mRNA Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
N/A N/A
naive
Saline
1w1
KGF
Saline
1w1
KGF
Saline
1w1
KGF
0 w
2 w
4 w
8 w

Fig. 10

Online data supplement

Keratinocyte growth factor gene transduction ameliorates pulmonary fibrosis induced by bleomycin in mice

Seiko Sakamoto[1], Takuya Yazawa[2], Yasuko Baba[1], Hanako Sato[2], Yumi Kanegae[3], Toyohiro Hirai[4], Izumu Saito[3], Takahisa Goto[1], Kiyoyasu Kurahashi[1]

Materials and Methods

Generation of recombinant adenovirus

We used a recombinant adenovirus (rAd) expressing murine KGF cDNA under the control of a potent CAG promoter (23) (AxCAmKGF denoted Ad-KGF in this paper), which was previously reported (22) (Fig. 1-i). The absence of contamination by replication-competent adenovirus (RCA) was confirmed by PCR of the E1 region sequence. Ax1w1, which bears no insert, including any promoter, cDNA, or poly(A) sequence (24), denoted Ad-1w1 in this paper was used as a control (Fig. 1-ii). Purified and concentrated viral stocks were prepared as described previously (E1).

Experimental protocol

All protocols for the animal experiments were approved by the Animal Research Committee of Yokohama City University (Yokohama, Japan). C57BL6 mice (male, 10 weeks) were purchased from Charles River Japan (Yokohama, Japan). Bleomycin was supplied by Nippon Kayaku (Tokyo, Japan). On Day 1, mice were anesthetized with isoflurane, xylazine, and ketamine, and a 7-day micro-osmotic pump (ALZET, 1007D, Durect, Cupertino, CA) containing 120 mg/kg of bleomycin was aseptically implanted into a subcutaneous space. On Day 8, mice were again anesthetized, and saline or 1.0 x 10^9 plaque-forming units (PFU) of an adenoviral vector, either Ad-KGF or Ad-1w1, was instilled intratracheally using a MicroSprayer (Penn-Century, Philadelphia, PA). In a survival trial, one group of mice received the lower dose of 1.0 x 10^8 PFU of the adenoviral vector. The choice of the 7-d interval between the start of bleomycin and administration of Ad-KGF was based on the study by Chaudhary *et al.* that demonstrated that the transition from the inflammatory to the fibrotic phase after bleomycin administration occurs in about 9 d in mice (E2). Our preliminary experiments showed that,

unlike pulmonary fibrosis in humans, which is mostly irreversible, pulmonary fibrosis induced by a single administration of bleomycin in mice was progressive up to 4 weeks but spontaneously tended to diminish thereafter. Therefore, a second osmotic pump containing 120 mg/kg of bleomycin was implanted on Day 29 to induce pulmonary fibrosis progressively for 8 weeks. Another set of animals (naïve group) received neither bleomycin nor the vector. On Day 56, mice were anesthetized, and pulmonary function was measured using a computer-controlled animal ventilator. Mice were then euthanized under deep anesthesia, and both lungs were harvested through a thoracotomy for histopathological examination, RNA extraction for PCR, and homogenization for Western blotting. One group of mice was used to evaluate the effects of adenovirus vectors on unaffected mice (without bleomycin pretreatment). Lungs of mice harvested 1 or 3 weeks after the administration of Ad-KGF were processed for histopathological examination.

Survival study

Mice were given bleomycin and the viral vector as described above (n = 15 to 16 in each group). Survival was then assessed for up to 8 weeks. Throughout this time period, the animals had free access to water and food.

Histopathological examination

Excised lungs were fixed with 4% paraformaldehyde in PBS for 48 h for histologic examination. The fixed lungs were embedded in paraffin. Four-micrometer-thick paraffin sections were dewaxed and stained with hematoxylin and eosin (H&E) for routine histopathological examination. To visualize collagen deposition clearly, we stained another set of sections with Masson's trichrome.

Histopathological quantification of fibrosis

The Ashcroft score was calculated for semiquantitative analysis of fibrotic change (25). In brief, each lung section was placed over 2 mm square grids. Grids that contained more than 50% alveolar tissue were selected and observed at a magnification of 100. Each field was individually assessed for the severity of fibrotic change and allotted a score from 0 (normal) to 8 (total fibrosis) using a predetermined scale of severity (25). After examination of the whole section, the average of the scores from all fields

was taken as the fibrosis score. Each specimen was scored independently by two investigators who were blinded to the treatment, and the mean of their individual scores was considered the fibrosis score of the sample.

Immunohistochemistry of lungs

Four-micrometer-thick paraffin sections were immunostained with KGF (FGF-7) antibody (sc7882, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-surfactant protein C (SP-C) antibody (sc-13979, Santa Cruz Biotechnology), followed by processing using an EnVision System (Dako, Glostrup, Denmark) according to the manufacturer's instruction. To detect KGF, the antigen in the sections was retrieved by autoclaving at 120°C for 15 min. Sections were briefly incubated with 0.03% hydrogen peroxide to eliminate endogenous peroxidase. The non-specific staining was blocked with a serum-free blocking reagent (X0909, Dako). The primary antibodies were diluted at 1:100 using Antibody Diluent (Dako). After washing in PBS, the sections were incubated for 30 min with a labeled polymer (K5027, EnVision System, Dako), and the signals were colorized with diaminobenzidine (DAB). Nuclei were counterstained with hematoxylin. Cells positive for SP-C were counted in three different parts of the section in a blinded manner and are presented as a percentage of the total number of cells in the field.

Measurement of pulmonary function

Mice were anesthetized with xylazine and ketamine, and the trachea was cannulated by a metallic needle (1.20 mm inner diameter, 4 mm long) through a tracheostomy. Mice were ventilated with a tidal volume of 8 ml/kg at a rate of 150 breaths/min, and pulmonary function was measured using a computer-controlled animal ventilator (flexiVENT, Scireq, Montreal, Canada). Quasi-static deflation pressure-volume (P-V) curves were collected with a positive end-expiratory pressure (PEEP) of 3 hPa to measure quasi-static compliance (Cst). Functional residual capacity (FRC) and total lung capacity (TLC) were defined as the lung volume at 3 hPa and 25 hPa, respectively (E3). The lung volume (TLC - FRC) was calculated as the volume of air required to inflate lungs from FRC to TLC.

Reverse transcriptase polymerase chain reaction (PCR)

Total RNA was extracted from the lungs using TRizol (Invitrogen, Carlsbad, CA) and cDNA synthesis