(FLPe) containing four amino acid substitutions; these mutations were first identified in a cycling mutagenesis experiment performed using *Escherichia coli*.[20] Due to the protein's thermostability, FLPe has been reported to work as effectively as Cre in somatic cell lines[21–23] and in mouse,[24–26] one contrary report excepted.[27] However, in embryonic stem (ES) cell culture FLPe has been shown to be less efficient than Cre,[23] and so a mouse codon-optimized version of FLPe (FLPo) has been synthesized for use in ES cells.[28]

Another way to improve the virtual efficiency of FLP in mammalian cells is to increase the gene copy number of the transduced recombinase in the cells. The adenovirus vector (AdV) is one of the most efficient tools for gene transduction and expression in mammalian cells. Furthermore, Cre-expressing AdV has been widely applied for the regulation of gene expression and has been used as a "gene switch."[29–33] Although wtFLP is normally inefficient, wtFLP can excise 100% of the targets in mammalian cell cultures when expressed on AdV using a very potent CAG promoter.[34,35] Furthermore, FLP-driven excision was observed in the chromosomes of ES cell clones in nearly 100% of cells transformed with AdV without difficulty;[36] this efficiency in ES cells is extremely higher than that achieved by plasmid electroporation using FLPe or

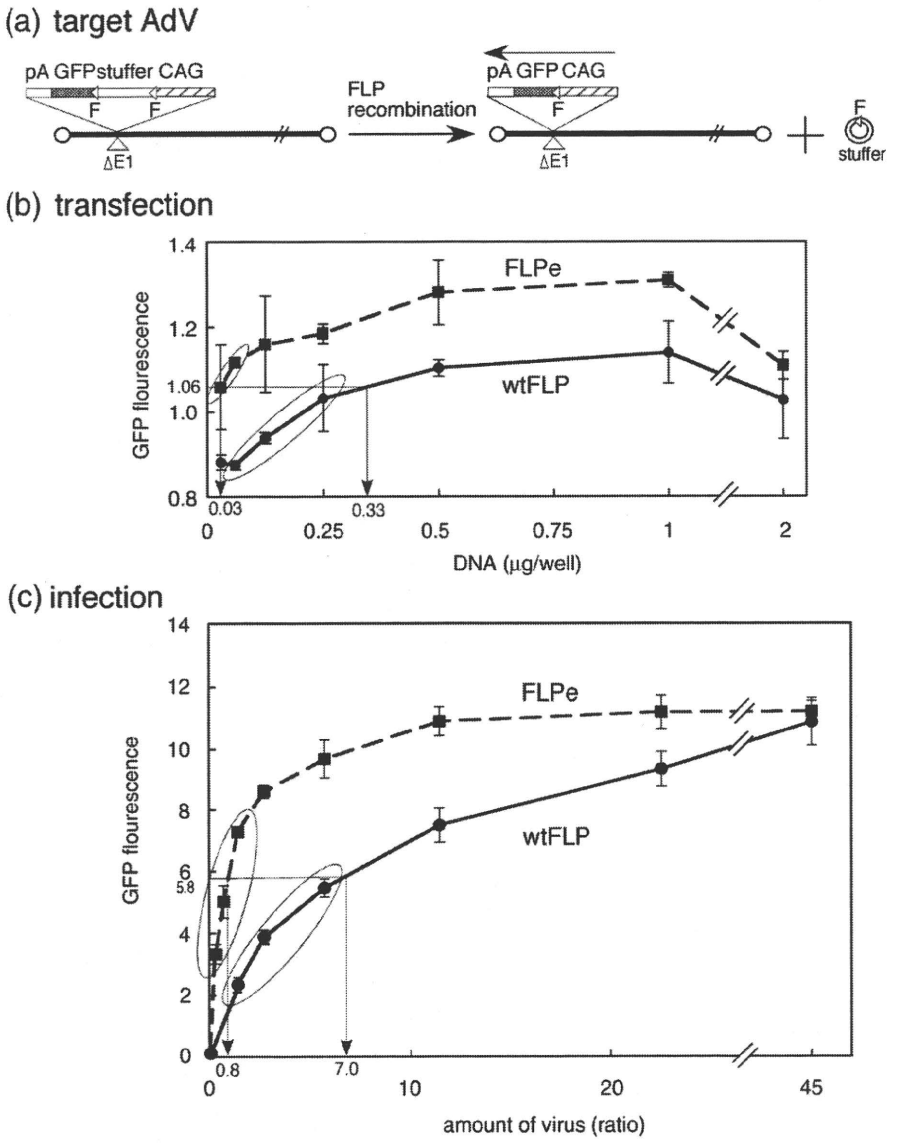

Fig. 1. Comparison of transduction between plasmid transfection and AdV infection. (a) Structure of the target AdV, AxCAFNFG. The stuffer consists of a neo gene and an SV40 pA sequence. CV-1 cells were infected with the target AdV AxCAFNFG. The wtFLP- or FLPe-expressing units needed to turn on GFP expression were transduced by the transfection of plasmids (b) or by infection with AdVs (c). pA, polyadenylation signal; F, FLP recognition site (FRT); E1, adenovirus E1 region. (b) Recombination efficiency using transfection. (c) Recombination efficiency using AdV. Each vertical line indicates 10× arbitrary units of GFP fluorescent intensity as measured using Fluoroskan AscentFL (Labsystems). The viral genome equivalent, indicated on the horizontal axis in (c) was defined using the amount of wtFLP genome at an MOI of 1 to represent a viral genome equivalent of 1 (see Materials and Methods). The areas defined as linear ranges are circled.

Cre. Using wtFLP-expressing AdV, Takashima *et al.* efficiently removed a gene encoding neomycin resistance from its chromosomal location in ES cells during generation of Cre-conditional knockout mice.[36] However, AdVs expressing wtFLP and FLPe have not been fully characterized.

Another important feature of AdV is that it enables a quantitative assay much superior to transfection-based assays, although no report has appeared to date showing such a comparison clearly. Using plasmids and AdVs, we precisely compared the enzymatic activities of wtFLP and FLPe in mammalian cells. We confirmed that FLPe showed higher recombination efficiency on a gene molar basis than did wtFLP with regard to both transfection and AdV expression. However, counter to expectations based on wtFLP- and FLPe-catalyzed recombination in *E. coli*,[20] FLPe was less efficient than wtFLP in catalyzing recombination in mammalian cells. To obtain more efficient translation in human cells, we generated an altered FLPe coding gene, "humanized" FLPe (hFLPe), in which the codon usage was optimized for use in humans. The efficacy of transiently expressed hFLPe in catalyzing recombination makes it highly suitable for transfection experiments.

Results

Recombination efficiency of wtFLP and FLPe using FLP-expressing plasmids and AdVs

To compare wtFLP and FLPe and measure their recombination efficiencies precisely, both transfection and AdV infection were used. CV-1 cells were infected with the target AdV AxCAFNFG (Fig. 1a), and plasmids expressing either wtFLP or FLPe were transfected. The target AdV possesses an FLP target composed of a CAG promoter, a stuffer sequence flanked by a pair of FRTs, the green fluorescent protein (GFP) gene, and a polyadenylation (pA) sequence, in that order (Fig. 1a). When recombination occurs, expression of the GFP reporter gene is turned on. Three days after transfection, the recombination efficiency was detected as GFP fluorescence. The total fluorescence intensity of FLPe-transduced cells was higher than that of wtFLP-transduced cells (Fig. 1b). To obtain a GFP fluorescence intensity of 1.06, at which fluorescent intensity scaled nearly linearly with input FLPe or wtFLP plasmid DNA, a total of 0.03 and 0.33 μg of the FLPe- and wtFLP-expressing plasmids were

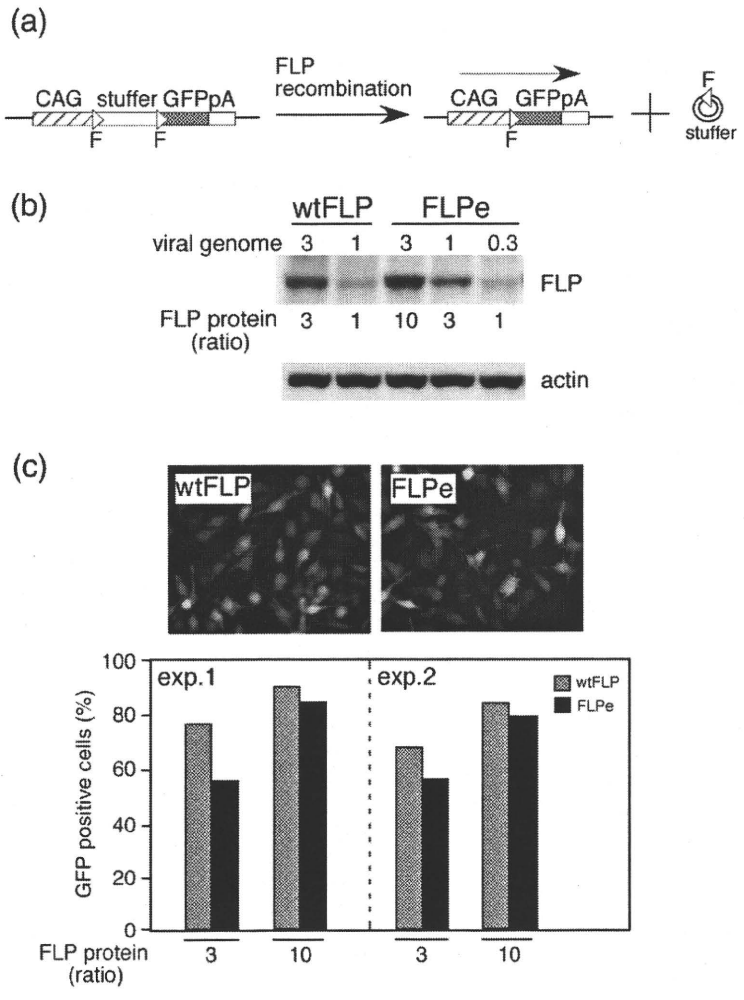

Fig. 2. Comparison between wtFLP- and FLPe-expressing AdVs in mammalian cells. (a) Structure of the chromosomally integrated target unit in CF cells. (b) Detection of FLP protein expressed in CF cells. Total protein was extracted from the infected cells, and FLP protein (top) and actin (bottom) were detected by Western blotting. At the bottom of each FLP band, the ratio of the amount of FLP protein is shown (see Materials and Methods). (c) Comparison of recombination efficiency. Top: fluorescent microscopic views of GFP-expressing cells 1 day after infection with the indicated FLP-expressing AdV. The cells were fixed 22 h after AdV infection, and the percentage of the GFP-expressing cells was measured using FACS (bottom, exp. 1, leftmost column). Exp. 1 and Exp. 2 were independent. 3 and 10 indicate the ratio of the amount of FLP protein when transduced as shown in (b). Because FACS counts very large number of cells, about 10,000 cells in each measurement, sampling effects were quite small.

required, respectively, showing that the recombination efficiency of FLPe was about 11-fold greater than that of wtFLP. Also, the linear response range upper cutoffs for FLPe and wtFLP input plasmid were approximately 0.1 and 0.25 μg, respectively, although the linear range was too narrow to compare precisely. These results were consistent with the previous *in vitro* results of Buchholz *et al.*[20] and *in vivo* results of Rodriguez *et al.*[26]

Next, wtFLP or FLPe was expressed through AdV infection instead of plasmid transfection (Fig. 1c). The recombination efficiency of FLPe was about nine times greater than that of wtFLP; that is, 0.8 and 7.0 viral genome equivalents of FLPe- and wtFLP-expressing AdVs, respectively, were required (estimated by Southern blot; see Materials and Methods) to obtain a GFP fluorescence of 5.8, where the amounts of both FLPe and wtFLP AdV vectors were within their linear ranges. The linear ranges of the FLPe- and wtFLP-expressing AdVs were less than 1.0 and 6.0 viral genome equivalents, respectively. These results show that both transfection and AdV infection provided similar quantitative measurements and that FLPe, on a gene molar basis, produced a fluorescent intensity that was about 10 times stronger than that produced by wtFLP. However, the AdV system gave much clearer results because the level of GFP expression after AdV infection was about 1 order of magnitude higher than that obtained using transfection, even when the maximum amount of FLPe-expressing plasmid was used. Furthermore, the linear response range for AdV infection was much wider than that for transfection; a comparison of Fig. 1b and c shows that transfection produced a linear response only for fluorescence readings between 0.85 and 1.15, while AdV achieved a linear response for readings between 1 and 8. Therefore, we concluded that AdV, a popular overexpression system, is also very useful for quantitatively analyzing precise measurements of enzymatic activity in mammalian cells. Therefore, the recombination efficiencies of wtFLP and FLPe were precisely compared with the use of the AdV system in all subsequent experiments.

Recombination efficiency of wtFLP and FLPe in an FRT-containing cell line

To compare the recombination efficiencies of the wtFLP and FLPe proteins on a molar basis in mammalian cells, wtFLP- and FLPe-expressing AdVs were infected into CF cells.[35] The CF cell line possesses a single copy of the FLP target unit integrated

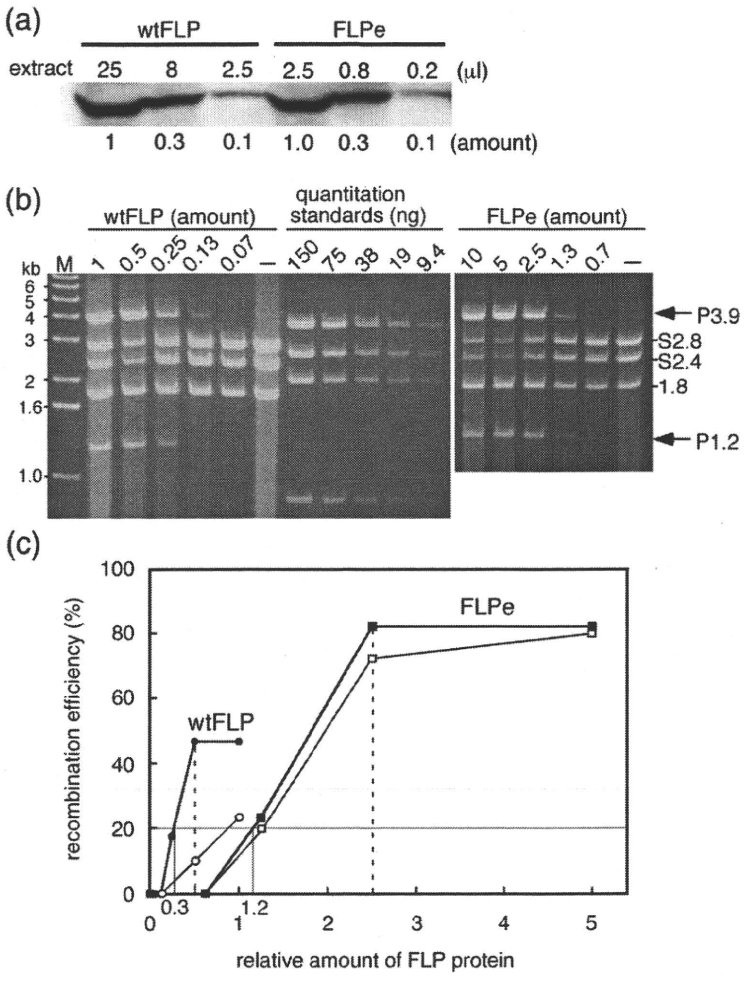

Fig. 3. *In vitro* recombination assay. (a) FLP protein contained in each extract was detected using Western blotting. The FLPe extract was diluted 10-fold with the reaction buffer. The amount of each lysate is shown above the band; the observed amounts of FLP protein are shown below the band. One aliquot unit was defined as the amount of FLP protein detected in the maximum reaction volume of wtFLP lysate (25 μL). (b) Quantification of *in vitro* recombination products. M, 1-kb Ladder marker (Gibco). The relative amounts of FLP protein are shown above the gel; the symbol '–' indicates the negative control. S, substrate; P, product. (c) Recombination efficiency of wtFLP (circle) and FLPe (square) in an *in vitro* assay at 30 °C (filled) and at 37 °C (open). The substrate DNA was reacted with wtFLP or FLPe *in vitro*, digested with FspI, and electrophoresed in an agarose gel. The original substrate DNA produced bands of 2.4 and 2.8 kb, while the recombined DNA generated bands of 3.9 and 1.2 kb.

in one of its chromosomes (Fig. 2a); GFP gene expression is turned on through the excision of the stuffer sequence mediated by FLP recombination. When the same amounts of FLPe- and wtFLP-expressing AdV were transduced into CF cells, the steady-state FLPe protein level was about threefold higher than that of wtFLP, as detected by Western blot analysis (Fig. 2b). This result is consistent with the thermostability of FLPe at 37 °C.

To examine the recombination efficiency of FLP, the GFP-expressing cells were observed with fluorescence microscopy (Fig. 2c, top panels). Notably, under conditions where the steady state amounts of FLPe and wtFLP enzymes were adjusted to identical intracellular levels, as determined by Western blot analysis, fewer GFP-expressing cells were observed in the visual field after FLPe transduction than after wtFLP transduction; this result was further confirmed quantitatively by fluorescence-activated cell sorter (FACS) analysis (Fig. 2c, bottom). The observation of fewer GFP-expressing cells after FLPe transduction than after wtFLP transduction was consistent in four pairwise comparisons over two independent experiments. One possible explanation for these results is that the recombination efficiency of the FLPe enzyme is less, not greater, on a molar basis than that of the wtFLP enzyme, with the activity of FLPe corresponding to about 70% of the activity of wtFLP in mammalian cells. This explanation also assumes that in a substantial percentage of cells, the expression of FLPe was insufficient to cause recombination even though the presence of FLPe protein was confirmed by Western blotting.

Recombination efficiency of FLP enzymes *in vitro*

Our result that the recombination efficiency of FLPe was lower on a protein molar basis than that of wtFLP in mammalian cells was unexpected because the opposite was believed to be true based on the results of a previous report using *E. coli*-produced FLPe and wtFLP *in vitro*.[20] Therefore, we decided to examine the recombination efficiencies of FLPe and wtFLP at their optimal temperature in an assay system using AdV-expressed FLPs. A comparative *in vitro* recombination experiment was performed at 30 °C using FLPe and wtFLP; recombination was performed with 1 μg of linearized pCAFNFG plasmid as a DNA substrate and the extracts of cells infected with AdVs expressing wtFLP or FLPe according to a previously described method.[34] Before assaying recombination, we measured the concentration of recombinase protein in the extracts by Western blotting, with the result that the FLPe extract contained a concentration of FLP protein that was about 10-fold higher than that of the wtFLP extract (Fig. 3a), presumably due to differences in thermostability. Therefore, the amounts of extract were normalized for FLP protein content by diluting the extract by an FLP reaction buffer. To measure recombination efficiency, the DNA products produced by recombination were quantified by comparing the corresponding bands with the intensity of a quantitation standard run on the same gel (Fig. 3b).

Lysate-dependent recombination was observed, reaching a plateau level of about 50% for wtFLP (Fig. 3c, filled circles). In contrast, recombination mediated by FLPe (filled squares) became detectable only after wtFLP-mediated recombination had reached its plateau value. Next, the recombination efficiencies were compared by titrating the amount of enzyme-normalized extract needed to achieve 20% recombination (Fig. 3c). The recombination activity of FLPe was only about one-fourth that of wtFLP (1.2 and 0.3 amounts corresponding to 1.2 and 0.3 μl of FLP protein-normalized extract, respectively, were required). These results showed that FLPe protein was considerably less efficient at recombination than the wtFLP protein when examined *in vitro* for enzymatic activity at their optimum temperature (30 °C). Note that wtFLP is thermolabile, while FLPe is thermostable; thus when the reaction starts with the same amount of each enzyme, the relative amount of wtFLP probably decreases over the course of the reaction. Therefore, if wtFLP is thermolabile even at 30 °C, the true recombination efficiency of wtFLP may be underestimated. Regardless, the activity of FLPe was lower than that of wtFLP at 30 °C. The recombination activity of FLPe was nearly the same at 30 and 37 °C (open squares), while the activity of wtFLP was extremely low at 37 °C (open circles). This result confirms the thermostability of the FLPe mutant and does not contradict the results described above.

Recombination efficiency of FLPe and hFLPe

To optimize the translational efficiency of the FLPe gene in mammalian cells, the FLPe gene was modified to coincide with mammalian codon usage. Because fully 19% of the FLPe coding sequence had to be altered, sixty-six 40-mer oligonucleotides were synthesized, annealed, and ligated to generate a humanized FLPe (hFLPe) gene (DNA Data Bank of Japan; AB434280). To measure the level of hFLPe gene expression in mammalian cells, FLPe- and hFLPe-expressing plasmids were transfected into the CF cell line and examined by Western blot analysis (Fig. 4). In fact, the amount of protein expressed by the hFLPe gene was about 10-fold greater than that expressed by the FLPe gene (Fig. 4a), suggesting that hFLPe was more efficiently translated in mammalian cells as a result of its optimized codon usage. Recombination efficiency was monitored as the fluorescent intensity of GFP expression. hFLPe was found to be more active; that is, the number of CF cells that became GFP-positive as a result of the hFLPe plasmid was about three times higher than the number that became GFP-positive as a result of the FLPe plasmid (Fig. 4b and c). The observation of only a threefold increase was probably attributable to the all-or-nothing nature of the assay; CF cells contain only a single copy of the recombination unit and cannot express GFP in a

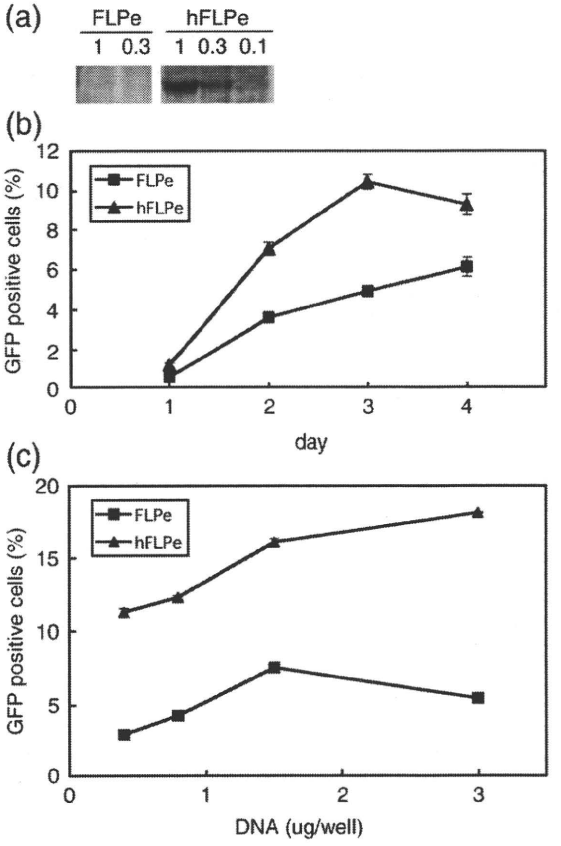

Fig. 4. Comparison of recombination efficiency between FLPe and hFLPe in mammalian cells. (a) Detection of FLP protein expressed in CF cells. Total protein was extracted from the transfected cells, and the FLP protein was detected by Western blotting. The relative amount of each cell extract is shown above the band. (b and c) Comparison of recombination efficiency. The cells were fixed after each plasmid was transfected, and the percentage of GFP-expressing cells was measured using FACS. Most error bars are difficult to see because the three measured values obtained by FACS were very similar.

dose-dependent manner. These results suggested that the hFLPe gene could successfully increase the translation of FLP in mammalian cells by about 10-fold compared with the FLPe gene. We also attempted to generate an hFLPe-expressing AdV to compare the expression levels of FLP and the recombination efficiency of FLPe and hFLPe further. However, only deleted viruses that did not express intact hFLPe were obtained in our attempts to construct an AdV vector expressing the hFLPe gene under the very potent CAG promoter.

Discussion

We observed that (i) an AdV system is useful for quantitative analysis involving precise activity measurements of enzymes expressed in mammalian cells, (ii) the recombination efficiency of FLPe is unexpectedly lower than that of wtFLP on a protein molar basis, (iii) hFLPe was generated, and in fact, hFLPe yielded substantially more recombined targets than did FLPe following transfection, and (iv) hFLPe-expressing AdV could not be generated, showing that it is difficult to generate an AdV construct expressing FLP showing high recombination efficiency; consequently, the FLPe-expressing AdV construct was the best AdV construct for FLP expression that could be produced in our study.

In general, the transfection of FLP-expressing plasmids is the most common method for comparing the recombination efficiencies of wtFLP and FLPe. Here, we showed that both plasmid transfection and AdV infection produced essentially identical results with regard to the characterization of wtFLP and FLPe recombination efficiencies; the recombination efficiency achieved by FLPe constructs was about 10-fold higher than that by wtFLP constructs. The second important result was that the values obtained by AdV constructs were several times higher than those obtained using transfection, and that AdV infection enables a more quantitative analysis to be performed. Furthermore, once FLP-expressing AdVs were prepared, AdV-mediated expression produced a linear range above the GFP background level that was severalfold greater than that produced by transfection, thus enabling more quantitative data to be obtained. Therefore, the AdV system appeared to be more suitable for characterizing enzymatic activity in mammalian cells. In the present study, the AdV system also compensated for the low activity of FLP and showed the differences in FLP recombination efficiencies more clearly. Therefore, in the present study at least, the results obtained using the AdV method were more reliable and more quantitative than those obtained using the transfection method. In general, the AdV method should enable one to obtain the superior quantitative data due to the above differences.

Here, we showed that on a protein molar basis, FLPe displayed lower recombination efficiency than did wtFLP at both 37 °C and its optimal temperature of 30 °C in an *in vitro* analysis (Figs. 2 and 3). Not only is the former observation novel, but the latter observation also apparently contradicts the results obtained by expression in *E. coli*,[20] where the purified FLPe protein catalyzed recombination more efficiently than did the purified wtFLP protein. In the previous study, the authors acknowledged the possibility that their results may have been influenced by their purification method, since sufficient active FLP enzyme was more difficult to obtain for wtFLP than for FLPe.[20] Because we were able to prepare a large amount of FLP enzyme from 293 cell extract using replicating AdV, we were able to avoid difficulties in the purification of wtFLP and were able to measure its recombination efficiency directly. In fact, it may be more reasonable to suggest that the four amino acid mutations in FLPe may have reduced recombination efficiency of the enzyme, compared with that of authentic wtFLP.

The observation that the recombination efficiency of FLPe on a protein molar basis was lower than

that of wtFLP at 30 °C was also true at 37 °C in mammalian cells (Fig. 2c). This finding was somewhat surprising, since the efficiency of FLPe has been demonstrated in mammalian cells,[21–23] in animals,[24–26] and in the present study (Fig. 1b and c). To explain this apparent contradiction, we hypothesize that the thermostability of FLPe outweighs the impact of the reduction in recombination efficiency of FLPe per protein. Fig. 1c clearly shows that FLPe-expressing AdV gave rise to more recombination than wtFLP-expressing AdV, although wtFLP-AdV has been previously applied to mammalian cells and has been shown to function efficiently in regulating transgene expression and catalyzing intentional chromosome rearrangement.[34–36] Because the apparent higher recombinant efficiency of FLPe was due solely to its higher steady-state levels resulting from its thermostability at 37 °C, we tried to enhance expressed levels of FLPe in mammals by generating hFLPe, in which the codon usage was optimized for expression in mammals. Approximately 19% of the FLPe coding nucleotides were substituted to produce the hFLPe gene. The codons most frequently used in humans were selected in all instances for the design of the hFLPe gene. No less than 14.4% of the gene sequence differed from that of FLPo,[28] another previously reported codon-optimized variant of FLPe. We were able to show that the expression of hFLPe increased by about 10-fold and that the hFLPe-expressing plasmid yielded about 2.3-fold more recombinants than did the FLPe-expressing plasmid in a transfection experiment. The lack of a greater increase seems to be due to the all-or-none nature of the assay in which the transfected CF cell line contained the FLP target unit present only in a single copy number. On account of its high expression, the hFLPe-expressing plasmid took only 2 days to yield the same extent of recombination attained by the FLPe-expressing plasmid 4 days after transfection (Fig. 4b). This quick, high-yield recombination obtained with hFLPe should be advantageous for various experiments, especially for application to ES cells, although no hFLPe-expressing AdV could be generated.

In fact, a limited deleterious effect was observed when wtFLP was expressed with the use of AdV in 293 cells during titer determination. When titration was performed with an end-point cytopathic effect assay in 293 cells,[37] cell death occurred much earlier than usual, and as a result, the wtFLP AdV acted as if its titer were about threefold higher than its actual value, as determined using Southern blot analysis to detect the transduced virus genome in CV-1 cells, for example (data not shown). This observation was specific for AdV expressing wtFLP and was rarely seen for AdV expressing-FLPe or Cre. Therefore, when we compared FLPe with wtFLP using AdV in the present study, Southern-blot-determined transduced viral genome equivalents were used instead of multiplicity of infection (MOI). We think that the wtFLP-expressing AdV may have had a very mild deleterious effect that was manifested only when the viral genome was amplified by more than 10,000 copies per cell in 293 cells during wtFLP-expressing AdV replication. Probably, this deleterious effect is so subtle that it is not discernible in typical plasmid transfection experiments or, more importantly, in the present study using FLPe, which shows lower recombination efficiency on a protein molar basis; nevertheless, such a deleterious effect magnified by the greater protein expression of hFLPe offers one potential explanation for the instability of hFLPe-expressing AdV. Therefore, if the subtle deleterious effect in 293 cells is correlated with the high molar recombination efficiency of wtFLP enzyme, the above observations could be consistently explained.

Our results showed that when one attempts to generate an effective FLP AdV, it is probably impossible to obtain a better FLP AdV than FLPe AdV in mammalian cells because the FLPe AdV had no perceptible deleterious effect and showed the best recombination efficiency practically achievable in mammalian cells. To obtain the same recombination efficiency in cells, FLPe AdV required a smaller viral dose than wtFLP AdV. The advantage of FLPe AdV in mammalian cells is that the recombination efficiency of FLPe is maintained at 37 °C not only due to its thermostability, but also owing to minimal deleterious effects on 293 cells during preparation, despite its lower recombination efficiency per enzyme molecule. wtFLP-expressing AdV was a less ideal choice in mammalian cells not only due to instability of wtFLP protein at 37 °C, but also due to deleterious effect of the construct in preparing AdV; finally, an hFLPe-expressing AdV could not be generated because the FLPe protein expression was too high.

Although an AdV expressing hFLPe under the control of the very potent CAG promoter could not be generated, hFLPe might be useful if it were to be expressed in an AdV containing a weaker promoter, such as a tissue-specific promoter for tissue-specific expression. Moreover, we showed here that the translational efficiency of the hFLPe-expressing plasmid was about 10-fold greater than that of an FLPe-expressing plasmid after transfection. Thus, we strongly recommend the use of hFLPe in situations where AdV infection is not suitable, such as in transfection experiments or studies using transgenic mice. A plasmid, pxCAhFLPe, expressing hFLPe gene under control of the CAG promoter, is available from RIKEN DNA bank†.

Materials and Methods

Cell lines and AdVs

Human embryo kidney 293 cell line[38] and monkey kidney cell line (CV-1) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal

† http://www.brc.riken.go.jp/lab/dna/ja/

calf serum. The CF cell line[35] derived from HeLa cells was cultured in the same culture medium with 10% fetal calf serum plus geneticin (0.75 mg/mL). After the cells had been infected with a recombinase-expressing AdV, they were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum without geneticin. The wtFLP-expressing AdV (AxCAFLP) and the target AdV (AxCAFNFG) were described previously.[34] The FLPe-expressing AdV (AxCAFLPe) was generated with the intact-genome transfection method.[39] Briefly, the FLPe recombinase gene was derived from pBSSK-FLPe, which was prepared from pCAGGS-FLPe (Gene Bridges). The FLPe gene used here contained an initiation codon that complies with Kozak's rule[40] and lacked a nuclear localization signal. The genomes of AxCAFLP and AxCAFLPe were identical, except for the FLP gene. The wtFLP and the FLPe recombinases were expressed under the control of a CAG promoter,[41] consisting of the cytomegalovirus IE enhancer, chicken β-actin promoter, and rabbit β-globin poly(A) signal. AxCAFNFG contained a CAFNFG unit, which was composed of a CAG promoter,[41] a first FRT sequence, a stuffer, another FRT sequence, the GFP gene, and GpA, in that order.

Quantitation of AdVs

The amount of each viral genome transduced to the cells was measured using a Southern blot analysis. When wtFLP-expressing AdV was used: the MOI did not correspond to the copy number: to obtain the same amount of genome, the wtFLP-expressing AdV required an MOI that was three times higher than that of any other AdV, including the FLPe-expressing virus. Thus, in this study, the expression of FLP and the recombination efficiency of each AdV were described not with MOIs but the amount of transduced viral genomes. In this study, a transduced viral genome equivalent of 1 corresponds to an MOI of 1 of wtFLP-expressing AdV and MOI of 0.3 of FLPe-expressing AdV and AxCAFNFG.

Complete synthesis of hFLPe Gene

To obtain an FLPe codon usage suitable for human cells,[42] 19% of the FLPe gene was substituted to produce the hFLPe gene. To generate the hFLPe gene, sixty-six 40-mer oligonucleotides were synthesized in a manner such that each oligonucleotide overlapped with two antisense oligonucleotides for a length of 20 nucleotides. These oligonucleotides were phosphorylated with T4 polynucleotide kinase, annealed with each other, and ligated. The hFLPe gene was synthesized in three parts, and the sequence was verified using nucleotide sequencing. The 1.3-kb full-length hFLPe sequence was then constructed by ligating the three fragments in correct order and orientation and cloning in pBluescript II SK(-) (Stratagene), yielding pBSSK-hFLPe. The hFLPe sequence is available at DNA Data Bank of Japan (AB434280).

FLP-expressing plasmids

pxCAwtFLP, pxCAFLPe, and pxCAhFLPe are pBR327-derived vectors containing wtFLP-, FLPe-, and hFLPe-expressing units, respectively, under the control of the CAG promoter. Each recombinase gene has an initiation codon that complies with Kozak's rule.[40] Three days after each plasmid was transfected into CV-1 cells (which had been infected with the target AdV, AxCAFNFG, 1 day before transfection) using Transfast (Promega), the culture medium was changed to Hanks' balanced salt solution, and the GFP fluorescent intensity was measured using Fluoroskan AscentFL (Labsystems). Each day after each plasmid had been transfected into the CF cells, the cells were washed twice with phosphate-buffered saline (PBS) (−). Then, the cells were collected, and the total DNA of a portion of the cell population was extracted; real-time PCR was performed to confirm that a similar amount of DNA had been transfected in the FLPe and hFLPe experiments (data not shown). The remaining portion of transfected cells was used for the detection of FLP protein and to count the number of GFP-positive cells by FACS analysis.

In vitro recombination assay

The recombinase-containing lysates were prepared as described previously.[34] In total, 2×10^7 of the 293 cells were infected with AxCAFLP and AxCAFLPe at an MOI of 75 and 5, respectively (an MOI of 1 corresponds to about 13 and 53 virus particles per cell, respectively). Twenty-two hours later, the cells were harvested by scraping and collected by centrifugation at 1000 rpm for 5 min at 4 °C using an Avanti HP-25I (Beckman-Coulter). The cell pellet was washed twice with PBS(−) and resuspended in 1 mL of FLP storage buffer [10% glycerol, 20 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), and 0.02 mM phenylmethylsulfonyl fluoride]. The cell suspension was then sonicated for 3 min (six cycles, 30 s each) using a Bioruptor II (CosmoBio; Tokyo, Japan) at maximum power (200 W) and was then immediately centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was stored at −80 °C.

The *in vitro* recombination assay in this study was performed as described previously.[34] Briefly, the reaction was started by mixing HindIII-linearized pCAFNFG with each lysate (2.5–25 μL) and then incubating for 30 min at 30 °C in a 50-μL volume of FLP reaction buffer [25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 5 mM DTT]. The reaction was terminated by phenol/chloroform extraction followed by two cycles of chloroform extraction and ethanol precipitation. The recovered DNA was then digested with FspI and the DNA digests were detected using ethidium bromide staining after agarose gel electrophoresis. The recombination efficiency was calculated by quantifying the density of the DNA bands on the photograph based on the densities of the quantification-standard DNA digests run on the same gel as described previously.[34] To compare the recombination efficiency of FLP and FLPe per FLP protein molecule, the FLPe lysate was diluted 10-fold with the FLP reaction buffer.

Detection and quantitative determination of GFP gene expression

A fluorescence microscope (IX70, Olympus) was used to observe GFP expression. To determine the percentage of GFP-expressing cells among the transfected cells and the infected cells, a flow cytometry analysis was performed. One day after infection, one-third of the cells in a 10-cm dish were fixed using 4% paraformaldehyde for 15 min at room temperature. The cells were washed twice with PBS (−), and their fluorescence emission was measured using a band-pass filter at 525 nm for GFP in a FACSCalibur with Cell Quest software (Becton Dickinson). A gate was set on a homogeneous cell population, as determined by the scatter characteristics, and 10,000 events were monitored.

The marker gates were set to calculate the percentage and the mean fluorescence intensity of the positive cells.

Polyclonal antibodies

FLP peptide was synthesized using a cysteine residue followed by the amino acid residues of RYPAWNGIIS-QEVLDYLSSYINRRI (R399–I423), including helices P′ and Q (underlined) at the C-terminal domain of the FLP protein.[43] Before immunization, the C terminus of the peptide was covalently conjugated with keyhole limpet hemocyanin (KLH) to increase its antigenicity. Polyclonal antisera against the peptide were raised in rabbits using the standard immunization protocol at Yanaihara Institute Inc. (Shizuoka, Japan). Briefly, two Japanese white rabbits (body weight, 2.5 kg) were subcutaneously immunized with 1.3 mg (for the initial immunization) and 0.65 mg (for the booster immunizations) of the peptide emulsified with an equal volume of Freund's complete adjuvant. The immunization was carried out five times at 2-week intervals. Ten days after the last immunization, blood was collected, allowed to coagulate at 37 °C for 2 h, and stored at 4 °C overnight. The sera were then recovered in the supernatant after centrifugation. Finally, the sera were affinity-purified using a HITrap NHS-activated column with an immobilized FLP peptide.

Western blotting

One day and 3 days after infection, one-third of a 10-cm dish of CF cells and six-well plate of 293 cells, respectively, were harvested and the total protein was extracted using NP-40 lysis buffer [50 mM Tris–HCl (pH 8.0), 0.15 M NaCl, 5 mM EDTA, 1% NP-40]. The lysates were mixed well in a rotator for 2 h at 4 °C, then centrifuged at 15,000 rpm for 5 min at 4 °C, and the supernatants were collected. Detection of the FLP protein in the lysate was performed for the *in vitro* assay using the lysate as it was. Western blotting was performed as described previously.[18] The membrane was incubated for 2 h at room temperature in the presence of an anti-FLP peptide rabbit polyclonal antibody diluted to 0.3 μg/mL with PBS–Tween, followed by incubation with biotin-conjugated goat anti-rabbit IgG (Bio Source) diluted to $1/10^5$ with PBS–Tween for 1 h at 4 °C. The membrane was then incubated for 1 h at 4 °C in the presence of horseradish-peroxidase-conjugated streptavidin (Bio Source) diluted to 1/70,000 with PBS–Tween. To reduce nonspecific binding, the antisera were incubated at 4 °C overnight with a membrane transferred with the lysate of CV-1 cells infected with the control virus AxCA1w1. The recovered supernatant was used as the source of the anti-FLP polyclonal antibodies. An anti-actin mouse monoclonal antibody (Neomarkers) diluted to $1/10^4$ with PBS–Tween was also detected on the same membrane to show equal loading.

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

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Abstract

Pulmonary fibrosis has high rates of mortality and morbidity, but there is no established therapy at present. We demonstrate here that bleomycin-induced pulmonary fibrosis in mice is ameliorated by intratracheal administration of keratinocyte growth factor (KGF)-expressing adenovirus vector (Ad-KGF). Progressive pulmonary fibrosis was created by continuous subcutaneous administration of 120 mg/kg of bleomycin subcutaneously using an osmotic pump twice from Day 1 to 7 and Day 29 to 35. The mice initially exhibited subpleural fibrosis, and then advanced fibrosis in the parenchyma of the lungs. These histopathological changes were accompanied by reduced lung compliance (0.041 ± 0.011 vs. 0.097 ± 0.004; $p < 0.001$), reduced messenger expression of surfactant proteins, and reduced KGF messenger expression in the lungs at 4 weeks compared with naïve group. Intratracheal instillation of Ad-KGF at 1 week after the first administration of bleomycin increased KGF mRNA expression in the lungs compared with the fibrosis-induced mice that received saline alone. The phenotype was associated with alveolar epithelial cell proliferation, increased pulmonary compliance (0.062 ± 0.005 vs. 0.041 ± 0.011; $p = 0.023$), and decreased mortality (survival rate on Day 56: 68.8% vs. 0%; $p = 0.002$), compared with those received only the saline vehicle. These observations suggest the therapeutic utility of a KGF-expressing adenoviral vector for pulmonary fibrosis.

Keywords:

keratinocyte growth factor, pulmonary fibrosis, gene therapy, adenovirus

Introduction

Pulmonary fibrosis is refractory to treatment regardless of its etiology. International consensus statements conclude that existing therapies for idiopathic pulmonary fibrosis (IPF) are of unproven benefit and emphasize the need to develop novel therapies (1). Development of pulmonary fibrosis depends heavily on alveolar epithelial injury and aberrant repair (2). Persistent inflammation, tissue necrosis, and infection lead to chronic myofibroblast activation and excessive accumulation of extra-cellular matrix components, which promote the formation of a permanent fibrotic scar (3). Modulation of the alveolar repair process is a potential therapeutic target.

Bleomycin, an antitumor antibiotic, binds to DNA and inhibits DNA-dependent RNA synthesis (4). One of the major side effects of this drug is pulmonary fibrosis (5). The pathogenesis of this side effect has been examined in animals (6, 7), and bleomycin has been used to create fibrosis in animal experimental models to elucidate a therapy for pulmonary fibrosis in patients.

Keratinocyte growth factor (KGF) is a potent mitogenic factor for alveolar epithelial cells whose specific receptor is expressed on epithelial cells (8). KGF has many properties that may be beneficial in treating pulmonary fibrosis. For example, KGF stimulates type II pneumocyte proliferation (9), increases surfactant protein production (10), increases alveolar epithelial fluid transportation (11), decreases apoptosis (12-16), and promotes DNA repair (17). However, KGF protein has so far demonstrated only limited effectiveness in various models of acute lung injury including hyperoxia-, radiation-, and bleomycin-induced lung injury (18-21). This is perhaps because KGF protein has a short duration of action. To overcome this issue, we previously used a KGF-expressing adenovirus vector (Ad-KGF) in a mouse model of hyperoxia-induced lung injury (22). In our previous study, we demonstrated that intratracheal administration of Ad-KGF resulted in over-expression of KGF for 7 days, attenuated lung injury, and decreased mortality when the animals were observed for up to 11 days after KGF transduction. In all the previous studies including ours, KGF was administered preventatively, *i.e.*, *before* the lung injury was created. It is yet unknown whether KGF is beneficial when administered *after* the insults to the lung have been incurred.

The purpose of this study was to elucidate whether Ad-KGF ameliorated bleomycin-induced pulmonary fibrosis when it was administered 7 days after bleomycin was started.

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**). 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**).

Experimental protocol

C57BL6 mice (male, 10 weeks) were used. On Day 1, mice were anesthetized with isoflurane, xylazine, and ketamine, and a 7-day micro-osmotic pump containing 120 mg/kg of bleomycin was aseptically implanted into a subcutaneous space. On Day 8, mice were again anesthetized, and saline or $1.0x10^9$ plaque-forming units (PFU) of an adenoviral vector was instilled intratracheally. In a survival trial, one group of mice received the lower dose of $1.0x10^8$ PFU of the adenoviral vector. A second osmotic pump containing 120 mg/kg of bleomycin was implanted on Day 29 to induce pulmonary fibrosis progressively for 8 weeks.

Survival study

Mice were given bleomycin and the viral vector as described above. Survival was then assessed for up to 8 weeks.

Histopathological examination

In addition to routine hematoxylin and eosin (H&E) staining, we stained sections with Masson's trichrome to visualize collagen deposition clearly.

Histopathological quantification of fibrosis

The Ashcroft score was calculated for semiquantitative analysis of fibrotic change (27).

Immunohistochemistry of lungs

Paraffin sections were immunostained with KGF (FGF-7) antibody and rabbit polyclonal anti-surfactant protein C (SP-C) antibody, followed by processing using an EnVision System. Cells positive for SP-C were counted 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 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.

Reverse transcriptase polymerase chain reaction (PCR)

Total RNA was extracted from the lungs and cDNA synthesis was performed; subsequent PCR reactions were performed using an RNA-LAPCR kit.

Real-time PCR

Total RNA was transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo-(dT). PCR amplification was performed on a Thermal Cycler Dice (Takara Bio) using SYBR Green I as a double-strand DNA-specific binding dye and continuous fluorescence monitoring according to the manufacturer's instructions.

Western blotting for transforming growth factor (TGF)-β1 and SP-D

One lobe of the right lung was homogenized with an elution buffer on ice. Standardized quantities of proteins were loaded onto SDS-PAGE gel and transferred electrophoretically onto nitrocellulose membranes.

Statistics

Data are reported as means and SEM for each group. Comparisons of multiple groups were made with a Tukey-Kramer *post hoc* test after analysis of variance (ANOVA). Survival rates are shown on the basis of Kaplan-Meier product limit curves, and the groups were compared by log rank test. A $p < 0.05$ was considered significant.

Results

Survival rates using two different vector doses

Mice administered bleomycin were instilled intratracheally with adenovirus vector on day 8. The survival rate of mice given the low dose (1.0×10^8 PFU/mouse) of Ad-1w1 was similar to that of the saline-administered control (**Fig. 2A**). Mice given the low dose of Ad-KGF had a significantly higher survival rate than the saline group (p=0.011) and low dose Ad-1w1 group (p=0.032), and mice given the high dose (1.0×10^9 PFU/mouse) of Ad-1w1 had a significantly lower survival rate than the saline group (p<0.001) (**Fig. 2B**). Mice given the high dose of Ad-KGF had a significantly higher survival rate compared with the saline group (p=0.002), and the percentage of surviving mice at eight weeks was the highest of all groups (68.8%). These observations suggest that (i) even the low dose of Ad-KGF

worked effectively for survival, (ii) the high dose of the adenoviral vector lacking KGF expression (Ad-1w1) had a significant negative or toxic effect on the survival of mice, but (iii) the high dose of Ad-KGF clearly overcame the negative effect of the vector on mice given bleomycin. Therefore, we selected the high dose of vector virus (1.0×10^9 PFU/mouse) for the subsequent experiments.

Effects of adenovirus vectors

The effects of KGF-over expression using Ad-KGF and the adverse effects related to the adenovirus and/or KGF over-expression, including inflammation and fibrous change of lungs were evaluated histopathlogically in the lungs of mice that were given Ad-KGF. Remarkable KGF expression in the lung cells was observed one week after the administration of Ad-KGF (**Fig. 3A**). Secretion of SP-C, a product of type II pneumocytes, was remarkable one week after the administration of Ad-KGF (**Fig. 3B**). H&E staining (**Fig. 3C**) as well as Masson's trichrome staining, which stains collagen fibers blue (**Fig. 3D**), of lungs harvested from mice 3 weeks after Ad-KGF administration demonstrated minimal fibrosis in the lungs in the chronic phase. The average Ashcroft score of these lungs was 0.7 ± 0.1, which was comparable to that of naïve mice.

Histopathological evaluation

The morphology of the lungs was evaluated with H&E and Masson's trichrome stain (insets) (**Fig. 4**). Mild fibrous changes in subpleural areas were seen in the lungs at 1 week after bleomycin administration when the vector was instilled into the lungs (**Fig. 4B**). Progressive expansion of fibrotic areas toward the lung parenchyma was seen in all groups. The fibrotic areas were larger at 4 weeks (**F, G, H**) than at 2 weeks (**C, D, E**), but the area involved was significantly smaller in the KGF group (**E, H**) than in the saline (**C, F**) and Ad-1w1 (**D, G**) groups. Lung fibrosis in the KGF group at 8 weeks (**I**) was similar to that in the KGF group at 4 weeks (**H**). Because all mice in the saline and Ad-1w1 groups died before 8 weeks, no lung tissue was available for comparison of these groups. These findings suggested that the progressive fibrosis caused by bleomycin is attenuated by Ad-KGF. Inflammatory cell infiltration associated with bleomycin administration was found in the subpleural stroma and alveolar walls (**Fig. E1**). Inflammatory cells consisted of lymphocytes and

neutrophils, and the intensity of infiltration was not considerably different among the saline, KGF, and 1w1 groups. Adenovirus-exposed lung tissue (KGF and 1w1 groups) revealed mild lymphocyte infiltration around the bronchioles as reported previously (**22**).

Ashcroft score

Lung fibrosis was quantitatively analyzed by Ashcroft score using sections stained with Masson's trichrome (**Fig. 4J**). Applying the rules described in the Methods section in the online data supplement, at least 12 grids of each specimen were evaluated. The Ashcroft score was significantly higher at 4 weeks than at 2 weeks in the 1w1 group. Less fibrosis was seen in the KGF group than the 1w1 group at 4 weeks. Although the Ashcroft score trended to increase from 4 weeks to 8 weeks in the KGF group, the change was not statistically significant.

Lung function test

Cst decreased in the groups that were administered bleomycin compared with that of the naïve group (**Fig. 5A**). Cst levels at 2 weeks were the same in the saline, 1w1, and KGF groups, but it had declined further at 4 weeks in the control and 1w1 groups, and Cst was then significantly greater ($p<0.01$) in the KGF group compared with that in the 1w1 group. The lung volume (TLC - FRC) was less in the saline, 1w1, and KGF groups compared with that in the naïve group (**Fig. 5B**).

Analysis of Ad-KGF mRNA in lungs

In the KGF group, vector-derived KGF mRNA was detectable at 2 weeks (1 week after vector administration), it had declined at 4 weeks, and then was not detectable at 8 weeks (**Fig. 6A**). Vector-derived KGF was not detectable in the other groups.

Analysis of total KGF mRNA in lungs

The alterations of total KGF (endogenous KGF + Ad-KGF-derived murine KGF) mRNA levels were analyzed using real-time PCR, and the values were normalized to that for the naïve group (**Fig. 6B**). The total KGF mRNA levels of saline and 1w1 groups were significantly reduced compared with the naïve group at 2 weeks. In contrast, the level of the KGF group at 2 weeks was about 12 times that of the naïve group and was also significantly

higher than that of the saline and 1w1 groups. The total KGF mRNA levels of the saline and 1w1 groups at 4 weeks were still significantly lower than that of the naïve group. The total KGF mRNA level of the KGF group at 4 weeks had declined to a level that was not significantly different from that of the naïve group, and was significantly lower than that of the KGF group at 2 weeks. At 8 weeks, the total KGF mRNA level in the KGF group had declined further and was significantly lower than that of the naïve group.

Immunohistochemistry of SP-C

Surfactant proteins are secreted by type II pneumocytes. We used SP-C as a specific marker for alveolar type II cells (26). While only a few cells were SP-C-positive in the naïve, saline and 1w1 groups at 2 weeks, there were many SP-C-positive cells in the KGF group at 2 weeks (**Fig. 7 A-D**). This was confirmed by blinded observation and quantification of the samples (**Fig. 7I**). As fibrosis progressed, at 4 weeks, there were fewer SP-C-positive cells per total number of lung cells in the saline and 1w1 groups, whereas there were more SP-C-positive cells in the KGF group compared with the naïve, saline and 1w1 groups (**Fig. 7E-G, I**). SP-C-positive cells were still dominant in the lungs at 8 weeks in the KGF group (**Fig. 7 H, I**). These observations suggest that bleomycin reduced the number of type II pneumocytes, and that Ad-KGF administration increased it significantly to an even higher level than it was in the naïve group.

Analysis of mRNA for surfactant proteins in lungs

mRNA expression of SP-A, -B, -C, and -D were analyzed using real-time PCR, the same method used for total KGF mRNA analysis. Basically, mRNA expression of the four isotypes of SP showed the same trend (**Fig.8**). The mRNA expression decreased in a time dependent manner in the saline and 1w1 groups. SP-A and SP-D mRNA had increased significantly at 2 weeks in the KGF group. The mRNA expression in the KGF group at 4 weeks remained at a level similar to that of the naïve group, and was higher than that in the saline and 1w1 groups.

Quantification of SP-D protein in lung homogenate

The SP-D protein concentration in lung homogenate was analyzed using

Western blotting (**Fig. 9A**). The intensities of SP-D signals were normalized by dividing them by those of β-actin (**Fig. 9B**). The expression of SP-D protein in the naïve group was barely detectable. SP-D proteins in the saline, 1w1, and KGF groups at 2 weeks had increased significantly compared with that in the naïve group. Among them, the increase in the KGF group was significantly greater than in the other groups. Although, SP-D protein expression had declined significantly in all groups at 4 weeks, it was still detectable in the KGF group at 8 weeks.

Quantitative analysis of TGF-β1

The TGF-β1 concentrations in lung homogenates were also analyzed using Western blotting (**Fig. 9C**). The intensity of the TGF-β1 signal was normalized by dividing it by that of β-actin (**Fig. 9D**). TGF-β1 was not detected in the lungs of the naïve group and all experimental groups at 2 weeks. TGF-β1 was detectable in all groups at 4 weeks, and the level in the 1w1 group was significantly greater than in the naïve group. TGF-β1 in the KGF group was significantly lower than that in the 1w1 group at 4 weeks. These findings suggest that the TGF-β1production induced by bleomycin and/or adenovirus vector administration was reduced by KGF.

Analysis of collagen mRNA expression in lungs

Collagen mRNA expression was analyzed using RT-PCR, the same method used for KGF mRNA analysis (above). (**Fig. 10**). The mRNA of collagen 1a1 in the saline group had decreased significantly at 2 weeks compared with that in the naïve group. The mRNA of collagen 1a1 in the KGF group had further decreased at 2 weeks and was lower than that in the saline group at 2 weeks. The mRNA expressions of collagen 1a1 and 3a1 in the saline group had increased significantly at 4 weeks and were significantly greater than that in the KGF group at 4 weeks.

Discussion

In the present study, we examined the therapeutic effects of a KGF-expressing adenovirus vector on bleomycin-induced pulmonary fibrosis. We demonstrated that KGF overexpression in the lungs resulting from intratracheal administration of Ad-KGF reduced collagen deposition in the lungs, improved respiratory function, and reduced mortality. These

changes occurred concomitantly with KGF effects including alveolar epithelial cell proliferation, suppression of TGF-β1 production, and increased SPs.

We allowed a 7-day interval from the initiation of bleomycin administration to the beginning of treatment with the Ad-KGF. Because histopathological observation of the lungs taken at 7 days after the administration of bleomycin revealed early fibrotic changes, it would be safe to conclude that we administered Ad-KGF after the pathological processes of fibrosis had started within the lungs. In order to elucidate a therapy for a disease in experimental models, it is important to test the therapy when the disease is present; however, the majority of studies in the literature were concerned with the preventative use of KGF or the use of KGF within a few days after intra-tracheal instillation of bleomycin when fibrosis was not yet established. For example, Deterding *et al.* demonstrated that while administration of KGF before intra-tracheal bleomycin administration reduced lung injury, KGF treatments at 24 and 48 hours after bleomycin administration did not prevent lung injury and fibrosis (**21**). The difference between their results and ours may be due to different study designs including the routes for administration of bleomycin (intratracheal vs. subcutaneous), the form of KGF administered (protein vs. vector), and the timing of the administration of KGF (1 and 2 days vs. 1 week after bleomycin). Morikawa et al. demonstrated KGF overexpression in the lungs of rats after instillation of a KGF-expressing adenovirus vector (**27**). However, the authors used unaffected animals and no information is available about the effect of KGF gene transduction on injured lungs. As far as we know, the present study is the first to demonstrate the *therapeutic* (as opposed to *preventative*) effects of KGF.

We speculate that the mechanism by which KGF ameliorated the bleomycin-induced lung fibrosis in the present study involves multiple complex functions of KGF. The main mechanism of lung fibrosis is incomplete repair of impaired lung tissues. The proliferation of alveolar epithelial cells induced by KGF might enhance the repair of the lungs (**11, 28**) and thus reduce the fibrosis (**Fig. 4**) because regenerative alveolar type II cells were found in the lungs of mice that were administered Ad-KGF (**Fig. 7**). These cells might restore damaged tissue to its normal condition. Second, KGF is known to suppress the production of TGF-β1, which is the most

potent regulator of connective tissue synthesis in several different organs (14). The reduction in the concentration of TGF-β1 in the lung homogenate in the group administered Ad-KGF (**Fig. 9C, D**) might be responsible for the reduction in the fibrous change in the lungs. Third, KGF increases the secretion of surfactant proteins (**10**). The predominant function of pulmonary surfactants is to reduce the surface tension at the alveolar air/liquid interface and thereby prevent lungs from collapsing at the end of expiration. In addition, the pulmonary surfactant system exhibits host-defense properties (**29**). Secreted surfactant proteins in mice in the KGF group may be involved in preventing lung injuries induced by bleomycin (**Figs. 8 and 9A, B**).

Although the high dose of the adenoviral vector (1.0×10^9 PFU) lacking KGF expression (Ad-1w1) had significant negative effects on the survival of mice, we employed this dose of adenovirus vector because the survival rate for mice given the higher dose of Ad-KGF (1.0×10^9 PFU) was greater than that for mice given the lower dose of Ad-KGF (1.0×10^8 PFU). Thus the KGF expression obtained by the higher dose of the vector was expected to overcome the negative effects of the vector on mice.

There are some limitations in the current study. First, bleomycin was administered twice (Days 1 to 7 and then Days 29 to 35) to prevent spontaneous improvements of pulmonary fibrosis. Therefore, one may argue that our results represent the prophylactic effects of KGF for the second administration of bleomycin, instead of the post-disease effects of the first administration. However, Ad-KGF instillation had improved the Ashcroft scores and pulmonary functions at 4 weeks (before the second bleomycin administration was started), suggesting that Ad-KGF reduced the impact of the first bleomycin administration. Second, we tested the effects of Ad-KGF at only one time point, which was 1 week after the first administration of bleomycin. Therefore, it is not clear whether Ad-KGF administered at later time points, when fibrosis is more advanced, would similarly exert beneficial effects. Third, it is not clear whether Ad-KGF reverses the fibrosis towards the physiological status. However, pulmonary fibrosis is a progressive disease, and treatments that reduce the speed of the pathological progression would be useful. In this respect, the beneficial effects of KGF due to instillation of Ad-KGF after the onset of fibrosis shown in the present study have