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Fibrosis, Vascular Activation, and Immune Abnormalities Resembling Systemic Sclerosis in Bleomycin-Treated Fli-1–Haploinsufficient Mice

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Abstract

Objective—Fli-1, a potential predisposing factor for systemic sclerosis (SSc), is constitutively down-regulated in the lesional skin of patients with SSc by an epigenetic mechanism. To investigate the impact of Fli-1 deficiency on the induction of an SSc phenotype in various cell types, we generated bleomycin-induced skin fibrosis in Fli-1^{+/-} mice and investigated the molecular mechanisms underlying its phenotypic alterations.

Methods—Messenger RNA (mRNA) levels and protein expression of target molecules were examined by quantitative reverse transcription–polymerase chain reaction and immunostaining. Transforming growth factor β (TGF β) bioassay was used to evaluate the activation of latent TGF β . The binding of Fli-1 to the target gene promoters was assessed with chromatin immunoprecipitation.

Results—Bleomycin induced more severe dermal fibrosis in Fli-1^{+/-} mice than in wild-type mice. Fli-1 haploinsufficiency activated dermal fibroblasts via the up-regulation of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins and activation of latent TGF β . Dermal fibrosis in Fli-1^{+/-} mice was also attributable to endothelial-to-mesenchymal transition, which is directly induced by Fli-1 deficiency and amplified by bleomycin. Th2/Th17-skewed inflammation and increased infiltration of mast cells and macrophages were seen, partly due to the altered expression of cell adhesion molecules in endothelial cells as well as the induction of the skin chemokines. Fli-1^{+/-} mouse macrophages preferentially differentiated into an M2 phenotype upon stimulation with interleukin-4 (IL-4) or IL-13.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Asano had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conclusion—Our findings provide strong evidence for the fundamental role of Fli-1 deficiency in inducing SSc-like phenotypic alterations in dermal fibroblasts, endothelial cells, and macrophages in a manner consistent with human disease.

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy, and fibrosis of the skin and certain internal organs (1). It is generally accepted that SSc is caused by the complex interplay between hereditary and environmental factors, leading to the accumulation of predisposing factors and the subsequent activation of endothelial cells, immune cells, and fibroblasts (2). Various combinations of predisposing factors may explain disease heterogeneity and a variety of organ involvement in SSc.

The transcription factor Fli-1, a member of the Ets transcription factor family, is epigenetically suppressed in SSc skin and SSc dermal fibroblasts and may represent such a predisposing factor for SSc (3). Fli-1 expression is decreased in nonlesional SSc skin in various cell types, including dermal fibroblasts, endothelial cells, and perivascular inflammatory cells, suggesting that down-regulation of Fli-1 is an early event preceding the development of fibrosis (4). The factors that might be involved in the down-regulation of Fli-1 include transforming growth factor β (TGF β) (4) and interferon- α (5) in addition to epigenetic mechanisms (3).

Fli-1 deficiency induces the SSc phenotype in dermal fibroblasts and dermal microvascular endothelial cells at the molecular level (4–6). In dermal fibroblasts, Fli-1 functions as a potent repressor of the *COL1A1* and *COL1A2* genes (4,6–9), and its deficiency modulates the expression of other fibrosis-related genes, resulting in myofibroblastic differentiation (10–15). In endothelial cells, Fli-1 deficiency suppresses the expression of genes regulating endothelial cell–endothelial cell and endothelial cell–pericyte interactions and up-regulates matrix metalloproteinase 9, thus promoting the remodeling of vascular basement membrane (16). Notably, endothelial cell–specific Fli-1–knockout mice reproduce the histologic and functional abnormalities characteristic of SSc vasculopathy (16). However, endothelial cell–specific Fli-1–knockout or Fli-1^{+/-} mice do not spontaneously develop apparent clinical symptoms of SSc.

Since the complexity of SSc cannot be entirely explained by genetic predisposition, epigenetic components such as Fli-1 deficiency potentially play a critical role in the development of this disease. The previous data regarding Fli-1–deficient mice support this idea and further suggest that some additional factors may synergize with Fli-1 deficiency to promote the development of clinically definite SSc. To assess this hypothesis, we generated a bleomycin-induced murine model of SSc using Fli-1^{+/-} mice and investigated the role of Fli-1 deficiency in the induction of the SSc phenotype by focusing on the key cell types involved in the pathogenesis of SSc.

MATERIALS AND METHODS

Mice

Eight-week-old female C57BL/6 mice were used. Mice were bred from Fli-1^{+/+} × Fli-1^{+/-} parents. The derivation of Fli-1^{+/-} mice has been described previously (17). All studies and procedures were approved by the Committee on Animal Experimentation of the University of Tokyo Graduate School of Medicine.

Bleomycin-induced murine model of SSc

Bleomycin (200 µg) dissolved in phosphate buffered saline (PBS) or control PBS alone was injected subcutaneously into a single location on the backs of the mice daily for 4 weeks (18).

Histologic assessment

On the day after the final injection, skin sections were obtained from the mice postmortem. Sections (6-µm thick) were stained with hematoxylin and eosin (H&E), Masson's trichrome, and toluidine blue. Dermal thickness was examined as previously described (19). Immunohistochemistry was performed using antibodies directed against α -smooth muscle actin (mouse clone 1A4; Sigma-Aldrich), connective tissue growth factor (CTGF/CCN2) (sc-14939; Santa Cruz Biotechnology), β 3 integrin (sc-6627; Santa Cruz Biotechnology), β 5 integrin (sc-5401; Santa Cruz Biotechnology), CD4 (rat clone 2H9; Relia Tech), CD8 (mouse clone 53-6.7; BD PharMingen), F4/80 (rat clone A3-1; AbD Serotec), and arginase 1 (sc-18531; Santa Cruz Biotechnology). For immunofluorescence, goat anti-VE-cadherin antibody (sc-6458; Santa Cruz Biotechnology), rabbit anti-fibroblast-specific protein 1 (anti-FSP-1) antibody (ab41532; Abcam), goat anti- β 3 integrin antibody (sc-6627; Santa Cruz Biotechnology), and goat anti- β 5 integrin antibody (sc-5401; Santa Cruz Biotechnology) were used as primary antibodies and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG antibody (sc-2090; Santa Cruz Biotechnology) and Alexa Fluor 555-conjugated donkey anti-goat IgG antibody (A21432; Invitrogen) were used as secondary antibodies. Coverslips were mounted by using Vectashield with DAPI (Vector), and staining was examined by using Biozero BZ-8000 (Keyence) at 495 nm (green), 565 nm (red), and 400 nm (blue). Ten random grids were evaluated under high magnification by 2 independent researchers (TT and YA) in a blinded manner. All experiments included at least 4 mice per study group.

Hydroxyproline measurement

Using QuickZyme Total Collagen Assay according to the recommendations of the manufacturer (QuickZyme Biosciences), 6-mm punch biopsy skin samples were hydrolyzed with 6N HCl, and collagen content was quantified. A standard hydroxyproline solution of 0–6 mg/ml was used to generate a standard curve.

Cell culture

Human dermal fibroblasts, human dermal microvascular endothelial cells (HDMECs), and peritoneal macrophages were prepared and maintained as previously described (8,16,20,21).

In some experiments, dermal fibro-blasts were treated with 2 ng/ml of TGF β 1 (R&D Systems) and/or 20 μ g/ml of RGD or RGE peptides (Takara Bio) for 24 hours, and peritoneal macrophages were stimulated with 20 ng/ml of mouse interleukin-4 (IL-4) or IL-13 (R&D Systems) for 24 hours.

Small interfering RNA (siRNA)

Cells were transfected with 10 nM Fli-1 siRNA or scrambled nonsilencing RNA (Santa Cruz Biotechnology) for 48 hours using HiPerFect transfection reagent (Qiagen) shortly after seeding. Thereafter, cells were serum starved for 24 hours and subsequently harvested.

RNA isolation and quantitative reverse transcription–polymerase chain reaction (PCR)

Total RNA was isolated from the lower back skin of mice and distal one-third of the forearm of SSc patients and healthy controls with RNeasy spin columns (Qiagen). One microgram of RNA was reverse transcribed using iScript cDNA Synthesis kits (Bio-Rad). Real-time quantitative PCR was carried out using SYBR Green PCR Master Mix (Life Technologies) on an ABI Prism 7000 system (Life Technologies) in triplicate. The messenger RNA (mRNA) levels were normalized to those of the GAPDH gene. The relative change in the levels of genes of interest was determined by the 2^{-C_t} method. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification. The primer sequences used are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>.

TGF β bioassay

To determine TGF β activation, transformed mink lung reporter cells (TMLCs) were cocultured with either scrambled nonsilencing RNA–transfected fibro-blasts or Fli-1 siRNA–transfected fibroblasts as described previously (22). TMLCs and test cells were mixed at a ratio of 1:1 and suspended at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum. These cells were plated at 200 μ l/well in 12-well plates and cultured for 24 hours. Cell lysates were prepared using Reporter Lysis buffer (Promega), and luciferase activity was determined using a Promega luciferase assay system. In experiments without cell–cell contact, similar cocultures were performed in 24-well plates with inserts designed for attachment-dependent cell culture (Millicell-PCF 3- μ m filter; Millipore), but 1.5×10^5 TMLCs and test cells were added to the lower and upper chambers, respectively. In some experiments, fibroblasts were treated with 2 ng/ml of TGF β 1 before coculture with TMLCs, and cocultures were conducted in the presence of 20 μ g/ml of RGD or RGE peptides.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using an EpiQuik ChIP kit (Epigentek). After reversal of crosslinking, the immunoprecipitated chromatin was amplified by PCR of specific regions of target genes. The amplified DNA products were resolved by agarose gel electrophoresis. The putative Fli-1 binding site was predicted by Tfsitescan. Primer sequences are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>.

Extraction of collagen from skin by the acetic acid method

Acetic acid extraction of collagen was performed as previously described (23,24). Skin punches (8 mm) were taken from the back of each mouse. Skin sections were then minced and incubated in 10 volumes of PBS overnight at 4°C with stirring. Tissue was harvested by centrifugation at 12,000g for 15 minutes and suspended in 10 volumes of cold 0.5M acetic acid with or without the addition of pepsin (1:10 ratio of pepsin to tissue wet weight). Extraction was performed overnight at 4°C with stirring, and the supernatant was dialyzed against 0.1M acetic acid. Next, the dialysates with pepsin added were treated with pepstatin A (Sigma-Aldrich), followed by lyophilization. Lyophilized proteins were resuspended in cold 0.1M acetic acid and were tumbled for ~20 hours. Equal aliquots from each sample were neutralized with 1M Tris base, boiled in sample buffer with the addition of 2-mercaptoethanol, resolved by 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and stained with Coomassie blue. Collagen levels were quantified using ImageJ software (National Institutes of Health).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism. One-way analysis of variance with Bonferroni post hoc test was used for multiple-group comparisons, and unpaired 2-tailed *t*-test was used for 2-group comparisons. For comparing 2 group values that did not follow Gaussian distribution, the 2-tailed Mann-Whitney U test was used. Within-group distributions are expressed as the mean ± SEM. *P* values less than 0.05 were considered significant.

RESULTS

Greater dermal thickness in bleomycin-treated Fli-1^{+/-} mice than in bleomycin-treated wild-type (WT) mice

Bleomycin injection for 4 weeks induced greater dermal thickness in Fli-1^{+/-} mice than in WT mice (2.4 fold versus 1.8 fold; *P* < 0.05), while dermal thickness was comparable between PBS-treated Fli-1^{+/-} mice and PBS-treated WT mice (Figure 1A). Masson's trichrome staining confirmed an increase in signal intensity in response to bleomycin treatment in both WT and Fli-1^{+/-} mice, but more importantly, a slight-to-moderate increase in the signal intensity was detected not only in bleomycin-treated Fli-1^{+/-} mice compared with bleomycin-treated WT mice, but also in PBS-treated Fli-1^{+/-} mice compared with PBS-treated WT mice (Figure 1B). This observation was confirmed by hydroxyproline assay (Figure 1B). Furthermore, the number of α -smooth muscle actin-positive cells showed the same trend, i.e., a significant elevation in both WT and Fli-1^{+/-} mice in response to bleomycin treatment and in Fli-1^{+/-} mice compared with WT mice after 4 weeks of PBS or bleomycin treatment (Figure 1C).

Thus, Fli-1 haploinsufficiency is sufficient to activate dermal fibroblasts in vivo and cooperatively establish tissue fibrosis together with additional factors induced by bleomycin. To elucidate the mechanism underlying this observation, we further investigated the impact of Fli-1 haploinsufficiency on fibroblasts, endothelial cells, and immune cells in vivo and in vitro. Since inflammation and dermal fibrosis peak on day 7 and day 28 after bleomycin

injection, respectively (18,25–27), mouse skin samples obtained on day 7 and day 28 were used in the subsequent experiments.

Fli-1 haploinsufficiency cooperates with bleomycin to activate latent TGF β through an α V β integrin–and α V β integrin–dependent mechanism, leading to CTGF induction

To assess the impact of Fli-1 haplo-insufficiency on dermal fibroblasts, we first focused on TGF β 1 and CTGF. Fli-1 haploinsufficiency significantly increased *Ctgf* mRNA levels while not affecting *Tgfb1* mRNA levels in murine skin after 4 weeks of bleomycin injection (Figure 2A). Furthermore, CTGF was mainly expressed in the dermal fibroblasts of bleomycin-treated mice, as confirmed by colocalization of CTGF with FSP-1, a marker of fibroblasts, in double immunofluorescence (results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>). Given that TGF β strongly induces CTGF expression in dermal fibroblasts and that CTGF is indispensable for the TGF β -dependent induction and maintenance of dermal fibrosis in vivo (28), we speculated that bleomycin may increase the sensitivity of Fli-1^{+/-} mouse fibroblasts to TGF β .

Since α V β 3 and α V β 5 integrins, receptors for the latent form of TGF β , increase the sensitivity of SSc dermal fibroblasts to TGF β by recruiting and activating latent TGF β on the cell surface (22,29–31), the impact of Fli-1 deficiency on the expression of these integrins was investigated in vivo and in vitro. Levels of mRNA for *Itgav*, *Itgb3*, and *Itgb5* were modestly, but significantly increased at baseline in Fli-1^{+/-} mice compared with WT mice, and bleomycin further potentiated expression of those genes, especially *Itgb3* and *Itgb5*, in Fli-1^{+/-} mice, while a smaller increase was observed in WT mice (Figure 2A). Immunostaining confirmed the elevated expression of these integrins, especially in dermal fibroblasts, at the protein level (results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>). Consistent with these findings, Fli-1 siRNA, which achieved an ~73% reduction in Fli-1 mRNA levels, significantly but moderately increased the levels of mRNA for *ITGAV*, *ITGB3*, and *ITGB5* compared with scrambled nonsilencing RNA in normal human dermal fibroblasts (1.3, 1.6, and 1.3-fold increase, respectively; $P < 0.05$). Furthermore, TGF β 1 induced the expression of mRNA for *ITGB3* and *ITGB5* to a greater extent in Fli-1 siRNA–treated fibroblasts compared with scrambled nonsilencing RNA–treated fibroblasts (2.3-fold and 1.9-fold increase, respectively; $P < 0.05$). In contrast, TGF β 1 did not change the fold increase in mRNA expression for *ITGAV* triggered by Fli-1 siRNA treatment (1.5-fold increase; $P = 0.34$), although there was a significant difference in mRNA expression for *ITGAV* between Fli-1 siRNA– and scrambled nonsilencing RNA–transfected dermal fibroblasts treated with TGF β 1 (Figure 2B).

To gain further insights into the Fli-1–dependent activation of latent TGF β , we used a TGF β bioassay. Fli-1 siRNA–treated or scrambled nonsilencing RNA–treated normal human dermal fibroblasts were cultured with TMLCs, a mink lung epithelial reporter cell line stably expressing a portion of the plasminogen activator inhibitor 1 promoter (32). When cultured as a mixture with fibroblasts, luciferase activity was significantly higher in TMLCs cocultured with Fli-1 siRNA–treated fibroblasts than in those cocultured with scrambled nonsilencing RNA–treated fibroblasts (1.20-fold increase; $P < 0.01$) (Figure 2C). In contrast, when coculture assays were conducted with inserts to separate TMLCs and fibroblasts while

allowing soluble molecules to pass, TMLCs cocultured with Fli-1 siRNA-treated fibroblasts showed significantly lower induction of luciferase activity than those cocultured with scrambled nonsilencing RNA-treated fibroblasts (0.78-fold decrease; $P < 0.05$) (Figure 2C).

These results, which are consistent with the findings of previous studies (22,30), indicate that a decrease in soluble activated TGF β in the cultured medium of Fli-1 siRNA-treated fibroblasts is likely due to the activation of endogenous latent TGF β and subsequent consumption by fibroblasts. Given that Fli-1 siRNA-dependent induction of luciferase activity was quite weak despite the high sensitivity of the TGF β bioassay, the modest increase in α V β 3 and α V β 5 expression by Fli-1 haploinsufficiency is likely to be insufficient for the induction of TGF β -dependent genes, including CTGF.

Since exogenous active TGF β 1 greatly increases these integrins in Fli-1 siRNA-treated fibroblasts, a TGF β bioassay was also performed with Fli-1 siRNA-treated or scrambled nonsilencing RNA-treated fibroblasts stimulated with active TGF β 1 before coculture. Notably, pretreatment with active TGF β 1 further enhanced luciferase activity in TMLCs cocultured with Fli-1 siRNA-treated fibroblasts compared with those cocultured with scrambled nonsilencing RNA-treated fibroblasts (2.04-fold increase; $P < 0.01$) (Figure 2C). Furthermore, an RGD peptide that blocks the binding of α V β 3 and α V β 5 to latent TGF β abolished elevated luciferase activity completely in TMLCs cocultured with Fli-1 siRNA-treated fibroblasts and also greatly reduced this activity in TMLCs cocultured with Fli-1 siRNA-treated fibroblasts prestimulated with active TGF β 1. In contrast, RGD treatment had a negligible effect on scrambled nonsilencing RNA-transfected fibroblasts pretreated with active TGF β 1 (Figure 2C), suggesting that the combination of Fli-1 siRNA and exogenous TGF β 1 activates latent TGF β in part through an integrin-mediated mechanism.

To elucidate the impact of integrin-dependent latent TGF β activation on CTGF expression, we further evaluated CTGF mRNA levels in Fli-1 siRNA-treated or scrambled nonsilencing RNA-treated normal human dermal fibroblasts stimulated with TGF β 1. TGF β 1 induced the expression of the CTGF gene to a greater extent in Fli-1 siRNA-treated fibroblasts than in scrambled nonsilencing RNA-treated fibroblasts, while CTGF mRNA levels were comparable without TGF β 1 treatment (Figure 2D). Furthermore, RGD peptide suppressed the expression of the CTGF gene in Fli-1 siRNA-treated fibroblasts stimulated with TGF β 1 to the levels comparable with those in scrambled nonsilencing RNA-treated fibroblasts stimulated with TGF β 1 (Figure 2D). These results indicate that gene silencing of Fli-1 augments exogenous TGF β -dependent CTGF induction through α V β 3 integrin- and α V β 5 integrin-dependent activation of latent TGF β in dermal fibroblasts. Overall, these experiments strongly suggest that bleomycin in cooperation with Fli-1 haploinsufficiency induces fibrogenic genes, including CTGF, at least partly through the integrin-mediated activation of latent TGF β .

Enhancement of endothelial-to-mesenchymal transition in response to bleomycin by Fli-1 haplo-insufficiency

Endothelial-to-mesenchymal transition is a mechanism underlying pathologic fibrosis, including bleomycin-induced pulmonary fibrosis (33–36). Therefore, we investigated whether Fli-1 haploinsufficiency facilitates bleomycin-induced endothelial-to-mesenchymal

transition by double immunofluorescence for FSP-1 and VE-cadherin (Figures 3A and B). Bleomycin-treated Fli-1^{+/-} mice showed a greater number of FSP-1/VE-cadherin double-positive cells distributed in the lower dermis than bleomycin-treated WT mice. Notably, FSP-1/VE-cadherin double-positive cells were detected in the dermis of PBS-treated Fli-1^{+/-} mice while absent in PBS-treated WT mice. In HDMECs, Fli-1 siRNA decreased the expression of mRNA for the endothelial marker VE-cadherin and increased the expression of mRNA for the mesenchymal markers *FSP1* and *ACTA2* and for *SNAIL*, an essential transcription factor regulating endothelial-to-mesenchymal transition (Figure 3C). Furthermore, Fli-1 occupied the promoter regions of the *FSP1* and *SNAIL* genes (Figure 3D) in addition to the previously reported VE-cadherin gene (16). Thus, Fli-1 deficiency directly contributes to endothelial-to-mesenchymal transition, and this process is further facilitated by bleomycin-induced factors.

Fli-1 haploinsufficiency modulates the expression of cell adhesion molecules related to a skewed T helper cell response

We previously demonstrated that the increased infiltration of mast cells and macrophages and the preferential polarization toward Th2 and Th17 phenotypes contribute to the development of bleomycin-induced dermal fibrosis in mice (37). Furthermore, we demonstrated that Th2/Th17 polarization depends on the expression of intercellular adhesion molecule 1 (ICAM-1) and glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) acting as positive regulators and P-selectin and E-selectin as negative regulators of these processes (37). Therefore, we next investigated the impact of Fli-1 on cell adhesion molecules in vivo and in vitro. The levels of mRNA for the *Icam1* and *Glycam1* genes were increased, while those of the *Selp* and *Sele* genes were decreased in bleomycin-treated Fli-1^{+/-} mice compared with bleomycin-treated WT mice on day 7 (Figure 4A). Furthermore, Fli-1 siRNA, which achieved an 89% reduction in *Fli1* mRNA levels, significantly increased the levels of mRNA for the *ICAM1* and *GlyCAM1* genes while decreasing those of the *SELP* and *SELE* genes in HDMECs (Figure 4B). Moreover, Fli-1 occupied the promoter regions of the *ICAM1*, *SELP*, and *SELE* genes (Figure 4C). Taken together, these results indicate that Fli-1 haploinsufficiency directly modulates the expression of cell adhesion molecules, leading to the promotion of profibrotic inflammatory cell infiltration and Th2/Th17-dominant immune polarization in bleomycin-treated mice.

Reproduction of the expression profiles of cytokines and chemokines characteristic of SSc by Fli-1 haploinsufficiency

We next examined the levels of mRNA for various cytokines and chemokines implicated in the pathogenesis of SSc in the lesional skin of mice. The levels of mRNA for *Il1b*, *Il4*, *Il6*, *Il10*, *Il17a*, *Ifng*, *Tnfa*, and *Mcp1* were significantly higher, and those of *Il12a* were significantly lower, in bleomycin-treated Fli-1^{+/-} mice than in bleomycin-treated WT mice on day 7 (Figure 5A), while the levels were comparable between PBS-treated Fli-1^{+/-} and WT mice (results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>). Thus, Fli-1 haploinsufficiency augments the expression levels of cytokines and chemokines characteristic of SSc in the lesional skin of bleomycin-treated mice.

Increased numbers of macrophages and mast cells and ratio of CD4+ to CD8+ T cells in the lesional skin of bleomycin-treated Fli-1^{+/-} mice

We next evaluated the phenotype of immune cells in the lesional skin of bleomycin-treated Fli-1^{+/-} mice. The number of macrophages and mast cells was markedly increased in bleomycin-treated Fli-1^{+/-} mice compared with bleomycin-treated WT mice on day 7 and day 28 (Figure 5B) (additional results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>). In contrast, the number of CD4+ and CD8+ cells was decreased on day 7 and day 28, while the CD4+:CD8+ ratio was significantly increased on day 7, in bleomycin-treated Fli-1^{+/-} mice compared with bleomycin-treated WT mice (Figure 5C) (additional results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>). Given that the numbers of macrophages, especially alternatively activated macrophages (M2 macrophages), and mast cells and the ratio of CD4+:CD8+ T cells are increased in the lesional skin of early diffuse cutaneous SSc (38,39), these data suggest that Fli-1 haploinsufficiency supports the development of an SSc-like immune response in the lesional skin of bleomycin-treated mice.

Fli-1 haploinsufficiency promotes M2 macrophage infiltration in the lesional skin of bleomycin-treated mice and M2 differentiation of peritoneal macrophages upon stimulation with IL-4 or IL-13

Since M2 differentiation of macrophages promotes tissue fibrosis in various pathologic conditions (40–42), including SSc (39,43,44), we next investigated if Fli-1 haploinsufficiency promotes M2 differentiation of macrophages. Levels of mRNA for the M2 macrophage markers *Arg1*, *Fizz1*, and *Ym1* were significantly elevated in Fli-1^{+/-} mice compared with WT mice on day 7 (Figure 6A), while comparable at baseline (results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>). Consistent with these findings, the number of arginase 1–positive macrophages in the lesional skin was significantly increased in Fli-1^{+/-} mice compared with WT mice on day 7 (Figure 6B). In addition, IL-4 and IL-13 induced polarization of macrophages toward the M2 phenotype to a greater extent in peritoneal macrophages isolated from Fli-1^{+/-} mice than in those from WT mice (Figure 6C). Collectively, these results indicate that Fli-1 haploinsufficiency promotes M2 macrophage infiltration in the lesional skin of bleomycin-treated mice by inducing M2 differentiation of macrophages via an intrinsic mechanism triggered by bleomycin.

DISCUSSION

In this study, we demonstrated that Fli-1 haplo-insufficiency exacerbates bleomycin-induced dermal fibrosis through multiple mechanisms, including induction of an SSc-like phenotype in dermal fibroblasts, endothelial cells, and macrophages; Th2/Th17-polarized inflammation; and increased mast cell infiltration. These results support the concept of the multifactorial nature of SSc and indicate that Fli-1 deficiency is a predisposing factor for this disease.

Fli-1 is a potent repressor of the *COL1A1* and *COL1A2* genes, and its down-regulation is a critical step in inducing matrix gene expression in dermal fibroblasts (4,6–10). In the skin of Fli-1^{+/-} mice, mRNA levels for the *Col1a2* gene were markedly increased, and the levels of

soluble type I collagen were also elevated (results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>), indicating that Fli-1 haploinsufficiency activates dermal fibroblasts in vivo. However, dermal thickness was comparable between Fli-1^{-/-} and WT mice. While counterintuitive, this observation is plausible because there was no difference between Fli-1^{+/-} and WT mice in CTGF expression, which is indispensable to establish and maintain dermal fibrosis in vivo (28,45,46). Supporting this notion, bleomycin increased CTGF expression as well as dermal thickness to a greater extent in Fli-1^{+/-} mice than in WT mice. Although the detailed mechanism underlying the bleomycin-dependent CTGF induction in Fli-1^{+/-} mouse dermal fibroblasts has not been fully elucidated, up-regulated expression of $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins and activation of latent TGF β may contribute to the enhanced expression of profibrotic genes, including CTGF. Indeed, it was recently reported that the αV -containing integrins collectively regulate the key profibrotic pathways during organ fibrosis (47).

Aberrant vascular activation plays a central role, together with inflammation and autoimmunity, in the initiation and maintenance of tissue fibrosis in SSc. Since endothelial cell-specific Fli-1-knockout mice reproduce the histologic abnormalities and increased vascular permeability characteristic of SSc, Fli-1 deficiency is also a potential predisposing factor for SSc vascular activation. To explore if Fli-1 deficiency links vascular events to tissue fibrosis in SSc, we evaluated the impact of Fli-1 haploinsufficiency on cell adhesion molecules regulating Th2/Th17-skewed inflammation and on endothelial-to-mesenchymal transition. Levels of the profibrotic cell adhesion molecules ICAM-1 and GlyCAM-1 were increased, while the antifibrotic cell adhesion molecules P-selectin and E-selectin were decreased in bleomycin-treated Fli-1^{+/-} mice, which theoretically promotes Th2/Th17-skewed immune polarization. Consistent with these findings, the expression levels of the *Il4*, *Il6*, *Il10*, and *Il17a* genes were elevated, while those of the *Il12a* gene were decreased in bleomycin-treated Fli-1^{+/-} mice on day 7.

Given that immune polarization in diffuse cutaneous SSc generally shifts from Th2/Th17 to Th1 in parallel with disease duration and that levels of mRNA for the *ICAM1* and *GlyCAM1* genes are relatively higher than those of the *SELP* and *SELE* genes in the skin of early diffuse cutaneous SSc (results are available online at <http://www.h.u-tokyo.ac.jp/der/pg237.html>), the altered expression of cell adhesion molecules in endothelial cells due to Fli-1 deficiency may contribute to the induction of inflammatory cell infiltration characteristic of SSc. Furthermore, in contrast to PBS-treated WT mice, endothelial-to-mesenchymal transition was observed in PBS-treated Fli-1^{+/-} mice and was induced to a greater extent in bleomycin-treated Fli-1^{+/-} mice than in bleomycin-treated WT mice. Importantly, key molecules regulating endothelial-to-mesenchymal transition, including VE-cadherin, FSP-1, and Snai-1, were shown to be the direct targets of Fli-1. Taken together, these findings indicate that Fli-1 deficiency potentially promotes the induction of a profibrotic phenotype in dermal microvascular endothelial cells, especially in the presence of certain environmental influences in SSc.

The monocyte/macrophage lineages are characterized by considerable diversity and plasticity, which is regulated by a complicated network of signaling molecules, transcription factors, and epigenetic mechanisms. The present study demonstrates that Fli-1

haploinsufficiency promotes the expansion of M2 macrophages in the lesional skin of bleomycin-treated mice and M2 differentiation of peritoneal macrophages in response to IL-4 or IL-13 stimulation in vitro, suggesting that Fli-1 haploinsufficiency could directly contribute to the differentiation of M2 macrophages. Given that M2 macrophages represent the predominant macrophage subset in the lesional skin of early diffuse cutaneous SSc (39), Fli-1 haploinsufficiency may also serve as a predisposing factor to induce the SSc phenotype in response to environmental influences in macrophages.

Another important observation in this study was that Fli-1 haploinsufficiency increased mast cell infiltration and the CD4⁺:CD8⁺ ratio of infiltrating lymphocytes upon bleomycin treatment in the lesional skin. In SSc patients, it has been demonstrated that mast cells are increased and serve as a major producer of TGF β in the lesional skin and that the CD4⁺:CD8⁺ ratio is increased in the peripheral blood and the lesional skin (38,48). Although the detailed mechanisms regulating mast cell infiltration and the significance of the elevated CD4⁺:CD8⁺ ratio are not well understood, the present finding further supports the idea that Fli-1 deficiency is also a predisposing factor integrating the induction of the SSc phenotype in various inflammatory cells.

In summary, this study provides strong evidence for the fundamental role of Fli-1 deficiency in inducing SSc-like phenotypic alterations in dermal fibroblasts, endothelial cells, and macrophages in a manner consistent with human disease. These results support the canonical idea that epigenetic reprogramming underlies pathogenic changes in SSc and implicate the Fli-1 deficiency-dependent pathway as a central mediator of this disease.

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Figure 1.

Exacerbation of bleomycin (BLM)-induced dermal fibrosis by Fli-1 haploinsufficiency. **A**, Left, Hematoxylin and eosin staining of representative skin sections from wild-type (WT) and Fli-1^{+/-} mice on day 28 after phosphate buffered saline (PBS) or bleomycin injection. Bars = 50 μm. Right, Dermal thickness. **B**, Left, Masson's trichrome staining of representative skin sections from WT and Fli-1^{+/-} mice on day 28 after PBS or bleomycin injection. Bars = 50 μm. Right, Collagen content, as measured by hydroxyproline assay. In **A** and **B**, values are the mean ± SEM (n = 4–8 mice per group). The relative ratio for each group is shown with the values for PBS-treated WT mice set at 1. **Arrows** indicate dermal thickness. **C**, Left, Representative skin histologic sections from WT and Fli-1^{+/-} mice treated with PBS or bleomycin. Bars = 50 μm. **Insets** show higher-magnification views of fibroblasts. Right, Relative number of myofibroblasts in the dermis. The number per high-power field is adjusted to that in PBS-treated WT mice, which is set at 1. Values are the mean ± SEM (n = 4–5 mice per group). * = *P* < 0.05; ** = *P* < 0.01; *** = *P* < 0.001.

Figure 2.

Amplification of bleomycin-induced expression of connective tissue growth factor (CTGF), α V β integrin, and α V β 5 integrin in Fli-1-haploinsufficient dermal fibroblasts. **A**, Expression of mRNA for the *Tgfb1*, *Ctgf*, *Itgav*, *Itgb3*, and *Itgb5* genes in the mouse skin tissue on day 28 after PBS or bleomycin injection (n = 10 mice per group). **B**, Expression of mRNA for the *ITGAV*, *ITGB3*, and *ITGB5* genes in scrambled nonsilencing RNA (SCR)–treated or Fli-1 small interfering RNA (siRNA)–transfected normal human dermal fibroblasts left untreated or treated with transforming growth factor β 1 (TGF β 1; n = 6 samples per group). **C**, Luciferase activity in transformed mink lung reporter cells (TMLCs) cocultured in the presence (left) or absence (middle) of cell–cell contact with scrambled nonsilencing RNA– or Fli-1 siRNA–transfected dermal fibroblasts left untreated or treated with TGF β 1 (n = 4–6 samples per group), and luciferase activity in TMLCs cocultured in the presence of RGD or RGE peptides with scrambled nonsilencing RNA– or Fli-1 siRNA–transfected dermal fibroblasts left untreated or treated with TGF β 1 (n = 6 samples per group) (right). **D**, Expression of mRNA for the *CTGF* gene in scrambled nonsilencing RNA– or Fli-1 siRNA–transfected normal human dermal fibroblasts left untreated or treated with TGF β 1 (n = 4 samples per group) (left) and in scrambled nonsilencing RNA– or Fli-1 siRNA–transfected dermal fibroblasts treated with TGF β 1 in the presence of RGD or RGE peptides (n = 3 samples per group) (right). Values are the mean $\pm$ SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. NS = not significant (see Figure 1 for other definitions).

Figure 3.

Endothelial-to-mesenchymal transition is directly induced by Fli-1 haploinsufficiency and further facilitated by bleomycin (BLM). **A**, Immunofluorescence staining for FSP-1 (green) and VE-cadherin (red) in skin samples from wild-type (WT) mice and *Fli-1*^{+/-} mice treated with phosphate buffered saline (PBS) or bleomycin. **Arrows** indicate FSP-1/VE-cadherin double-positive cells. **Insets** show a higher-magnification view of representative cells (an FSP-1 single-positive cell for PBS-treated WT mice and double-positive cells for bleomycin-treated WT mice and PBS- and bleomycin-treated *Fli-1*^{+/-} mice). Bar = 20 μ m. **B**, Number of FSP-1/VE-cadherin double-positive cells per field (counted under magnification $\times 200$). **C**, Expression of mRNA for the *VE-cadherin*, *ACTA2*, *FSP1*, and *SNAI1* genes in human dermal microvascular endothelial cells transfected with scrambled nonsilencing RNA or Fli-1 siRNA. In **B** and **C**, values are the mean $\pm$ SEM ($n = 4$ mice per group in **B** and 6 samples per group in **C**). * = $P < 0.05$. **D**, Chromatin immunoprecipitation analysis of Fli-1 binding to the promoters of the *FSP1* and *SNAI1* genes. See Figure 2 for other definitions. Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/doi/10.1002/art.38948/abstract>.

Figure 4.

Fli-1 haploinsufficiency modulates the expression of cell adhesion molecules in dermal microvascular endothelial cells, leading to the induction of Th2/Th17-skewed inflammation.

A, Levels of mRNA for the *Icam1*, *Glycam1*, *Selp*, and *Sele* genes in the skin of wild-type (WT) and *Fli1*^{+/-} mice treated with bleomycin for 7 days. **B**, Levels of mRNA for the *ICAM1*, *GlyCAM1*, *SELP*, and *SELE* genes in human dermal microvascular endothelial cells transfected with scrambled nonsilencing RNA (SCR) or *Fli1* small interfering RNA (siRNA). Values in **A** and **B** are the mean $\pm$ SEM (n = 10 mice per group in **A** and 6 samples per group in **B**). * = $P < 0.05$. **C**, Chromatin immunoprecipitation analysis of *Fli1* binding to the promoters of the *ICAM1*, *SELP*, and *SELE* genes.

Figure 5.

Fli-1 haploinsufficiency induces the expression profiles of cytokines and chemokines characteristic of systemic sclerosis (SSc) and promotes an SSc-like inflammatory infiltration in bleomycin-treated mice. **A**, Levels of mRNA for the *Il1b*, *Il4*, *Il6*, *Il10*, *Il12a*, *Il17a*, *Ifng*, *Tnfa*, and *Mcp1* genes in the skin of WT and *Fli-1*^{+/-} mice on day 7 after bleomycin injection ($n = 10$ mice per group). **B**, Number of macrophages, mast cells, CD8+ T cells, and CD4+ T cells in the lesional skin of PBS- and bleomycin-treated WT mice and *Fli-1*^{+/-} mice on days 7 and 28 ($n = 5$ mice per group). **C**, Ratio of CD4+ to CD8+ T cells in the lesional skin of PBS-treated and bleomycin-treated WT mice and *Fli-1*^{+/-} mice on days 7 and 28 ($n = 5$ mice per group). Cells were counted in 10 random grids in 400 $\times$ high-power fields (hpf). Values are the mean $\pm$ SEM. * = $P < 0.05$. See Figure 1 for other definitions.

Figure 6.

Fli-1 haploinsufficiency promotes M2 macrophage infiltration in the skin of bleomycin-treated mice and M2 differentiation of peritoneal macrophages upon interleukin-4 (IL-4) or IL-13 stimulation. **A**, Levels of mRNA for the *Arg1*, *Fizz1*, and *Ym1* genes in the skin of WT and *Fli-1*^{+/-} mice on day 7 after bleomycin injection (n = 10 mice per group). **B**, Number of arginase 1-positive macrophages in the skin of WT and *Fli-1*^{+/-} mice on day 7 and day 28 after injection with PBS or bleomycin (n = 5 mice per group). **C**, Levels of mRNA for the *Arg1*, *Fizz1*, and *Ym1* genes in peritoneal macrophages from WT and *Fli-1*^{+/-} mice treated with IL-4 or IL-13 (n = 4 mice per group). Values are the mean ± SEM. * = *P* < 0.05; ** = *P* < 0.01. hpf = high-power field (see Figure 1 for other definitions).