Amelioration of Dermal Fibrosis by Genetic Deletion or Pharmacologic Antagonism of Lysophosphatidic Acid Receptor 1 in a Mouse Model of Scleroderma

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Objective. Scleroderma (systemic sclerosis [SSc]), is characterized by progressive multiorgan fibrosis. We recently implicated lysophosphatidic acid (LPA) in the pathogenesis of pulmonary fibrosis. The purpose of the present study was to investigate the roles of LPA and two of its receptors, LPA1 and LPA2, in dermal fibrosis in a mouse model of SSc.

Methods. Wild type (WT), and LPA1-knockout (KO) and LPA2-KO mice were injected subcutaneously with bleomycin or phosphate buffered saline (PBS) once daily for 28 days. Dermal thickness, collagen content, and numbers of cells positive for α-smooth muscle actin (α-SMA) or phospho-Smad2 were determined in bleomycin-injected and PBS-injected skin. In separate experiments, a novel selective LPA1 antagonist AM095 or vehicle alone was administered by oral gavage to C57BL/6 mice that were challenged with 28 daily injections of bleomycin or PBS. AM095 or vehicle treatments were initiated concurrently with, or 7 or 14 days after, the initiation of bleomycin and PBS injections and continued to the end of the experiments. Dermal thickness and collagen content were determined in injected skin.

Results. The LPA1-KO mice were markedly resistant to bleomycin-induced increases in dermal thickness and collagen content, whereas the LPA2-KO mice were as susceptible as the WT mice. Bleomycin-induced increases in dermal α-SMA+ and phospho-Smad2+ cells were abrogated in LPA1-KO mice. Pharmacologic antagonism of LPA1 with AM095 significantly attenuated bleomycin-induced dermal fibrosis when administered according to either a preventive regimen or two therapeutic regimens.

Conclusion. These results suggest that LPA/LPA1 pathway inhibition has the potential to be an effective new therapeutic strategy for SSc, and that LPA1 is an attractive pharmacologic target in dermal fibrosis.

Scleroderma (systemic sclerosis [SSc]), is a potentially fatal autoimmune disease of unknown cause, characterized by progressive multiorgan fibrosis that is refractory to current therapies. Fibrogenesis in SSc is thought to result from tissue injury, followed by dysregulated wound healing (1). Discovery of the mediators that drive aberrant wound healing responses will hopefully
identify new therapeutic targets for SSc. We hypothesize that one such target is LPA₁, a receptor for lysophosphatidic acid (LPA).

LPA is a lipid mediator that signals through specific G protein–coupled receptors. Five high-affinity LPA receptors have been definitively established and designated LPA₁ to LPA₅; P2Y₅ is a lower affinity receptor that is likely to join the LPA receptor family as LPA₆ (2). Our laboratory recently implicated LPA/LPA₁ signaling in the pathogenesis of pulmonary fibrosis (3). We found that LPA₁-knockout (KO) mice were dramatically protected from bleomycin-induced pulmonary fibrosis and mortality and that LPA/LPA₁ signaling was responsible for the majority of fibroblast chemoattractant activity present in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis. LPA/LPA₂ signaling has also been implicated in pulmonary fibrosis. LPA/LPA₅ signaling can induce αvβ6 integrin-mediated activation of latent transforming growth factor β (TGFβ) by lung epithelial cells in culture (4), and TGFβ activation by this integrin is critically required for the development of bleomycin-induced lung fibrosis (5).

LPA may also be involved in the pathogenesis of SSc, as suggested by the recent demonstration that arachidonoyl (20:4) LPA levels are significantly higher in SSc patients’ serum versus healthy controls (6). Injured human skin has been shown to contain increased amounts of both LPA and cells expressing LPA₁ (7). We therefore investigated whether LPA signaling through either LPA₁ or LPA₂ is required for dermal fibrosis in the bleomycin model of scleroderma. In this model, repeated subcutaneous injections of the chemotherapeutic agent bleomycin results in dermal fibrosis that resembles scleroderma (8), with collagen deposition and both fibroblast and myofibroblast accumulation (9). Lesional skin also shows increased Smad2 and Smad3 phosphorylation (10), indicating activation of the TGFβ pathway, which is implicated in scleroderma (11,12). We found that bleomycin-induced increases in dermal thickness, collagen content, myofibroblast accumulation, and Smad2 phosphorylation were all markedly attenuated in LPA₁-KO mice. Bleomycin-induced dermal fibrosis was also significantly reduced in wild-type (WT) mice treated with the novel, orally bioavailable, LPA₁-selective antagonist AM095. In contrast, LPA₂-KO mice were not protected from bleomycin-induced dermal fibrosis. These results indicate that LPA₁/LPA₂ signaling contributes importantly to injury-induced dermal fibrosis.

MATERIALS AND METHODS

Animals. Experiments comparing LPA₁-KO and WT mice used offspring of mice heterozygous for the LPA₁ mutant allele, which were hybrids of the C57BL/6 and 129Sv/J genetic backgrounds (13). LPA₁-KO mice (generated in Dr. Jerold Chun’s Laboratory at The Scripps Research Institute) demonstrate impaired suckling in neonatal pups because of defective olfaction, which leads to increased neonatal death and reduced body size in survivors. Survivors also demonstrate craniofacial dysmorphism characterized by shorter snouts and more widely spaced eyes (13), but we have not noted any skin abnormalities at baseline.

Experiments comparing LPA₂-KO and WT mice used offspring of mice homozygous for the mutant LPA₂ allele in the BALB/c genetic background (14) and WT BALB/c mice (Charles River Laboratories). LPA₂-KO mice (also generated in Dr. Chun’s Laboratory) are born at the expected frequency and display no obvious phenotype abnormalities (14).

Experiments measuring plasma AM095 concentrations and comparing AM095-treated and vehicle-treated mice used WT C57BL/6 mice from Harlan Laboratories and the National Cancer Institute-Frederick Mouse Repository, respectively.

All experiments used sex- and age-matched mice at 6–8 weeks of age that were maintained in specific pathogen–free environments. All experiments were performed in accordance with National Institute of Health guidelines and with protocols approved by the Massachusetts General Hospital or the Amira Pharmaceuticals Institutional Animal Care and Use Committee.

Bleomycin injections and skin harvests. Bleomycin (Gensia Sicor) was dissolved in phosphate buffered saline (PBS) at 10 μg/ml and sterilized by filtration. Bleomycin or PBS (100 μl) was injected subcutaneously into 2 locations on the shaved back of LPA₁-KO, LPA₂-KO, or WT mice, once daily for 28 days. Mice were then killed, and full-thickness 6-mm punch biopsies were obtained from each injection site. One skin sample was fixed in 10% formalin and embedded in paraffin for histologic and immunohistochemical studies; the other was immediately frozen at −80°C for hydroxyproline analysis.

Histologic analysis and dermal thickness measurement. Multiple 5-μm sections of paraffin-embedded skin samples were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) or with Masson’s trichrome according to the standard protocols of our laboratory (3). Dermal thickness was determined with the use of photomicrographs (100× magnification) of H&E-stained sections, measuring the distance between the epidermal–dermal junction and the dermal–fat junction at 5 randomly selected sites per high-power field in 10 high-power fields per section.

Immunohistochemical analyses of α-smooth muscle actin (α-SMA) and phospho-Smad2. Multiple 5-μm sections of paraffin-embedded skin samples were cut onto ProbeOn Plus slides (Fisher Scientific), deparaffinized, and rehydrated. Immunolabeling of α-SMA and phospho-Smad2 was performed with primary rabbit anti-mouse α-SMA antibody (Abcam) and primary rabbit anti-mouse phospho-Smad2 antibody (Cell Signaling), respectively, using the MicroProbe staining system (Fisher Scientific) according to the manufacturer’s instructions. Appropriate biotinylated secondary antibodies
were used, followed by detection with an avidin–biotin–peroxidase complex development kit (Vector) and color development with aminoethylcarbazole (Dako). Cells positive for α-SMA and for phospho-Smad2 were then counted in 10 randomly selected, nonoverlapping high-power fields in dermal sections from WT and LPA_{1}-KO mice.

Hydroxyproline assay. Hydroxyproline content as a measure of skin collagen was determined using the standard protocol of our laboratory (15). Briefly, skin samples were homogenized in PBS and hydrolyzed overnight in 6N HCl at 120°C. A 25-μl aliquot was desiccated, resuspended in 25 μl of H_{2}O, and added to 0.5 ml of 1.4% chloramine T (Sigma), 10% n-propanol, and 0.5M sodium acetate, pH 6.0. After a 20-minute incubation at room temperature, 0.5 ml of Ehrlich’s solution (1M p-dimethylaminobenzaldehyde [Sigma] in 70% n-propanol, 20% perchloric acid) was added. After a 15-minute incubation at 65°C, absorbance was measured at 550 nm, and the hydroxyproline concentration was determined against a standard curve. Assay results were expressed as micrograms of hydroxyproline per 6-mm punch biopsy sample of skin.

Cell lines and culture. Human and mouse LPA_{1} and human LPA_{3} receptors were stably expressed in Chinese hamster ovary (CHO) cells (Invitrogen) and cultured in Ham’s F-12 medium with 10% fetal bovine serum (FBS) and 1 mg/ml of hygromycin B. Mouse LPA_{2} was stably expressed in human embryonic kidney (HEK) cells (Invitrogen) and cultured in Dulbecco’s modified Eagle’s medium with 10% FBS and 200 μg/ml of hygromycin B. Human and mouse LPA_{2} and LPA_{3} and human LPA_{4} were transiently expressed in rat neuroblastoma B103 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Calcium flux assay. LPA receptor–transfected cells were plated in 96-well poly-D-lysine–coated black-wall clear-bottomed plates (BD BioCoat) at 20,000–40,000 cells/well and cultured overnight in complete medium. Cells were then washed with PBS and cultured in serum-free medium either overnight (for stably expressing cells) or for 4 hours (for transient transfectants) prior to dye loading. On the day of the assay, cells were loaded for 1 hour at 37°C with 100 μl of FLIPR Calcium 4 dye (Molecular Devices) in Hanks’ balanced salt solution (HBSS) supplemented with 20 mM HEPES, 2 mM probenecid, and 0.3% fatty acid–free human serum albumin. Test compounds (in 25 μl) were then added to the cells and incubated at room temperature for 30 minutes. LPA (50 μl of 5× stock solutions prepared in HBSS with 20 mM HEPES and 0.3% fatty acid–free human serum albumin) was added after 15 seconds of baseline measurement. The final concentrations of LPA used were dependent on the receptor expressed: LPA_{1} and LPA_{3} assays used 10 nM LPA, LPA_{2} and LPA_{4} assays used 30 nM LPA, and LPA_{4} assay used 300 nM LPA. Intracellular calcium mobilization was measured using a FlexStation III (Molecular Devices). Inhibition curves were generated by plotting the percentage inhibition of calcium flux versus log_{10} of the concentration of compound. The 50% inhibition concentration (IC_{50}) was calculated by nonlinear regression using the sigmoidal dose-response (variable slope) equation in Prism 5 software (GraphPad Software).

Determination of AM095 concentrations in mouse plasma. C57BL/6 mice were administered the selective LPA_{1} antagonist AM095 by oral gavage (30 mg/kg) at time 0 and at 8 hours, and blood was collected by cardiac puncture under anesthesia into tubes containing sodium EDTA at 0, 4, 8, 9, 12, and 24 hours. Plasma samples were stored at −40°C prior to analysis of AM095 concentrations by liquid chromatography tandem mass spectrometry. Known amounts of AM095 were added to thawed mouse plasma to yield a concentration range from 0.8 ng/ml to 4,000 ng/ml. Plasma samples were precipitated using acetonitrile containing the internal standard buspirone. The analyte mixture (10 μl) was injected using a Leap PAL autosampler. Calibration curves were constructed by plotting the peak area ratio of analyzed peaks against known concentrations. The lower limit of quantitation was 1 ng/ml. The data were examined by linear regression analysis with 1/χ² weighting. The pharmacokinetic parameters of AM095 were calculated by noncompartmental analysis using WinNonlin Professional software (Pharsight). The maximum concentration (C_{max}) and the time to maximum concentration (T_{max}) were obtained directly from the measured data.

AM095 administration in the bleomycin model. The selective LPA_{1} antagonist AM095 was dissolved in sterile water, and a dose of 30 mg/kg of AM095 or sterile water alone (vehicle), was administered by oral gavage to each C57BL/6 mouse, twice daily on weekdays and once daily on weekends. AM095 was administered from the initiation of bleomycin challenge in a preventive regimen or beginning either 7 or 14 days after the initiation of bleomycin challenge in 2 therapeutic regimens. For all AM095 regimens, bleomycin or PBS was injected subcutaneously for 28 consecutive days, and skin samples were obtained at the completion of the experiment as described above.

Statistical analysis. Differences in dermal thickness, hydroxyproline content, and the numbers of α-SMA+ and phospho-Smad2+ cells between WT mice and LPA_{1}-KO or LPA_{1}-KO mice and between AM095-treated mice and vehicle-treated mice were tested for statistical significance by Student’s 2-tailed t-test, using Microsoft Excel software. P values less than 0.05 were considered significant.

RESULTS

Dependence of bleomycin-induced dermal fibrosis on LPA_{1}. Examination of H&E-stained skin sections of bleomycin- and PBS-challenged WT and LPA_{1}-KO mice demonstrated that LPA_{1}-KO mice were strikingly protected from bleomycin-induced dermal fibrosis (Figure 1A, upper panels). Compared with PBS-challenged mice, bleomycin-challenged WT mice demonstrated substantial thickening of the dermis, with densely packed connective tissue replacing the subcutaneous fat. These changes were markedly reduced in bleomycin-challenged LPA_{1}-KO mice. The substantial increase in dermal collagen induced by bleomycin in WT mice was also markedly attenuated in LPA_{1}-KO mice, as demonstrated by Masson’s trichrome staining (Figure 1A, lower panels).

To quantify the protection of LPA_{1}-KO mice against dermal fibrosis, we first assessed dermal thick-
ness in bleomycin- and PBS-challenged WT and LPA1-KO mice. The dermal thickness in bleomycin-challenged LPA1-KO mice was significantly reduced compared with that in WT mice (Figure 1B). Whereas bleomycin challenge increased the dermal thickness in WT mice by 56%, the dermal thickness in bleomycin-challenged LPA1-KO mice was only 5% greater than that in PBS-challenged LPA1-KO mice (Figure 1B). Genetic deletion of LPA1 therefore attenuated the increase in bleomycin-induced dermal thickness by 91%.

Biochemical assessment of skin collagen by measuring the hydroxyproline content confirmed the significant protection of LPA1-KO mice. Bleomycin challenge increased the amount of skin hydroxyproline by 31% in WT mice, but only by 3% in LPA1-KO mice (Figure 1C). Genetic deletion of LPA1 therefore attenuated the bleomycin-induced increase in hydroxyproline by 90%. This dramatic protection of LPA1-KO mice suggests that the LPA/LPA1 pathway contributes importantly to dermal fibrosis.

No requirement for LPA2 in bleomycin-induced dermal fibrosis. In contrast to the LPA1-KO mice, the LPA2-KO mice were not protected from bleomycin-induced dermal fibrosis. Bleomycin induced similar thickening of the dermis, with densely packed connective tissue, in LPA2-KO mice as in WT mice and similar increases in dermal collagen, as demonstrated in skin sections stained with H&E (Figure 2A, upper panels) and Masson’s trichrome (Figure 2A, lower panels), respectively. Dermal thickness and hydroxyproline content measurements confirmed that LPA2 deletion did not confer protection from bleomycin-induced fibrosis. Compared to the findings in PBS-challenged mice, bleomycin challenge increased dermal thickness by 46% in WT mice and by 50% in LPA2-KO mice (Figure 2B). Similarly, bleomycin increased the skin hydroxyproline content by 67% in WT mice and by 66% in LPA2-KO mice (Figure 2C). The lack of protection of LPA2-KO mice by bleomycin suggests that LPA2 signaling is not required for dermal fibrosis.

Dependence of bleomycin-induced dermal myofibroblast accumulation on LPA1. To begin to investigate the mechanism(s) through which LPA and LPA1 contribute to dermal fibrosis, we assessed two processes implicated in scleroderma, accumulation of myofibroblasts and activation of the TGFβ/Smad signaling pathway, in bleomycin-challenged WT and LPA1-KO mice. SSc fibrogenesis is associated with fibroblast differentiation into myofibroblasts, which secrete increased amounts of extracellular matrix components, including...
collagen (16). Myofibroblast differentiation is characterized by the acquisition of smooth muscle cell features, including &SMA expression (17).

To determine whether LPA/LPA1 signaling contributes to bleomycin-induced myofibroblast accumulation, we compared the number of &SMA+ cells in the dermis of bleomycin- and PBS-challenged WT and LPA1-KO mice. Bleomycin challenge substantially increased the number of &SMA+ cells in the dermis of WT mice but not LPA1-KO mice (Figure 3A). The number of &SMA+ cells increased by 70% in bleomycin-challenged WT mice, but only by 5% in LPA1-KO mice (Figure 3B), suggesting that LPA and LPA1 make important contributions to myofibroblast accumulation in dermal fibrosis.

Dependence of bleomycin-induced dermal Smad2 phosphorylation on LPA1. Myofibroblast differentiation and synthesis of matrix proteins are driven by TGFβ (18,19). By directing these key profibrotic processes, TGFβ is thought to play a central role in SSc fibrogenesis. When bound by active TGFβ, TGFβ receptors transmit signals through phosphorylation of cytoplasmic Smad proteins, which translocate to the nucleus and act as transcription factors (20). To determine whether LPA/LPA1 signaling contributes to the activation of the TGFβ signaling pathway following bleomycin challenge, we compared the number of cells with nuclear Smad2 phosphorylation in the dermis of bleomycin- and PBS-challenged WT and LPA1-KO mice.
Bleomycin challenge increased the number of nuclear phospho-Smad2 cells in the dermis of WT mice but not LPA1-KO mice (Figure 3C). The number of phospho-Smad2 cells increased by 81% in bleomycin-challenged WT mice, but did not increase at all in LPA1-KO mice (Figure 3D), suggesting that LPA and LPA1 may contribute to the activation of the TGFβ/Smad signaling pathway during the development of dermal fibrosis. Alternatively, the reduced number of nuclear phospho-Smad2 cells in bleomycin-challenged LPA1-KO mice could be at least partly attributable to reductions in the numbers of fibroblasts and myofibroblasts accumulating in the dermis of these mice. Reductions in fibroblast and myofibroblast numbers would reduce the number of cells present in the dermis that are able to respond to TGFβ by Smad phosphorylation.

Potent and selective LPA1 antagonism by AM095. To investigate the potential of LPA1 as a therapeutic agent for dermal fibrosis, we evaluated a potent new LPA1-selective antagonist, AM095 (sodium[4-[(R)-1-phenyl-ethoxy]carbonylamino]-isoaxazol-5-yl]-biphenyl-4-yl)-acetate) (Figure 4A). AM095 inhibited the LPA-induced calcium flux of CHO cells that had been stably transfectected with human or mouse LPA1 (Figure 4B). The IC_{50} for AM095 antagonism of LPA-induced calcium flux of human or mouse LPA1-transfected CHO cells was 0.025 μM and 0.023 μM, respectively (Table 1). In contrast, the IC_{50} for AM095 antagonism of LPA-induced calcium flux was >5 μM for CHO cells, HEK cells, or B103 cells transfected with 1 of the other 4 established human or mouse LPA receptors, demonstrating the selectivity of AM095 for LPA1 (Table 1).
The average plasma concentrations of AM095 produced in mice over a 24-hour period by the administration of two 30-mg/kg doses by oral gavage given 8 hours apart are shown in Figure 4C. The AM095 AUC value was 118.7 μg · hour/ml, with a plasma C_max of 6,200 nM (28 μg/ml) and a plasma C_min of 170 nM (0.08 μg/ml). Twice daily 30-mg/kg dosing by oral gavage was therefore used in all subsequent experiments, since this produced plasma AM095 concentrations that were greater than the IC50 for the LPA1 receptor throughout the treatment period.

Attenuation of bleomycin-induced dermal fibrosis by preventive and therapeutic AM095 regimens. Administration of AM095 from the initiation of bleomycin challenge in a preventive regimen attenuated bleomycin-induced dermal fibrosis, substantially mitigating the bleomycin-induced increases in dermal thickness and dermal collagen, as demonstrated in skin sections stained with H&E (Figure 5A, left panels) and Masson’s trichrome (Figure 5A, right panels) respectively.

Delayed administration of AM095 until after the initiation of bleomycin challenge was performed in two therapeutic regimens, one beginning on day 7 and the other beginning on day 14 after initiation of bleomycin challenge; these regimens also attenuated bleomycin-induced dermal fibrosis. Measurements of dermal thickness and hydroxyproline content indicated the protective efficacy of treatment according to all 3 regimens. Bleomycin challenge increased the dermal thickness of vehicle-treated mice by 82%, but by only 25%, 12%, and 32% for the preventive, therapeutic day 7, and therapeutic day 14 regimens, respectively, in AM095-treated mice (Figure 5B). Preventive pharmacologic inhibition of LPA1 therefore attenuated the bleomycin-induced increase in dermal thickness by 70%, while therapeutic pharmacologic inhibition of LPA1 begun on day 7 or on day 14 attenuated the bleomycin-induced increase in dermal thickness by 85% and 61%, respectively.

Similarly, bleomycin challenge increased the hydroxyproline content of skin from vehicle-treated mice by 117%, but by only 56%, 79%, or 86% for the preventive, therapeutic day 7, and therapeutic day 14 regimens, respectively, in AM095-treated mice (Figure 5C). Preventive inhibition of LPA1 therefore attenuated the bleomycin-induced increase in hydroxyproline by 52%, while therapeutic inhibition of LPA1 begun on day 7 or day 14 attenuated the bleomycin-induced increase in hydroxyproline by 32% and 26%, respectively. These data suggest an ongoing requirement for the LPA/LPA1
pathway in the maintenance of pathologic dermal fibrosis, suggesting that this pathway is a viable target for therapeutic intervention in fibrotic diseases of the skin.

**DISCUSSION**

Our results demonstrate that LPA$_1$ is required for the development of bleomycin-induced dermal fibrosis. Genetic deletion of this receptor or pharmacologic antagonism with a new orally bioavailable selective inhibitor protected mice from the increases in dermal thickness and collagen content produced in this model. In contrast, genetic deletion of LPA$_2$ did not confer protection against dermal fibrosis. Taken together, these data suggest that LPA signaling specifically through LPA$_1$ is critical for the development of skin fibrosis induced by tissue injury.

Although its precise cellular origin in biologic fluids and tissues has yet to be established, LPA production has been demonstrated in response to injury and has been shown to promote wound healing in multiple

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**Figure 5.** Attenuation of BLM-induced fibrosis by pharmacologic antagonism of lysophosphatidic acid receptor 1 (LPA$_1$). A, Skin sections from PBS- or BLM-challenged C57BL/6 mice treated with vehicle or with AM095 in a preventive regimen. Sections were stained with hematoxylin and eosin (left panels) and Masson’s trichrome (right panels). Bars = 100 μm. B, Dermal thickness of PBS- or BLM-challenged C57BL/6 mice treated with vehicle, preventive AM095, or therapeutic AM095 begun on day 7 (AM095 #1) or on day 14 (AM095 #2) after challenge with PBS or BLM. * = P < 0.0008; ** = P < 0.002; *** = P < 0.01. C, Skin hydroxyproline content in 6-mm punch biopsy skin samples obtained from the same mice as in A. * = P < 0.0005; ** = P < 0.02; *** = P < 0.02. Values in B and C are the mean ± SEM of 3 mice per treatment group for the dermal thickness studies and of 5 mice per group for the hydroxyproline content studies. Data for the PBS-challenged, vehicle-treated BLM-challenged, and preventive AM095 BLM-challenged groups were combined from 2 experiments; data for both therapeutic AM095 BLM-challenged groups were from 1 experiment. See Figure 1 for other definitions.
tissues, including the skin (7,21,22). Recurrent tissue injury and aberrant wound healing responses appear to contribute to the pathogenesis of multiple fibrotic diseases, including scleroderma (1,23), and arachidonyl (20:4) LPA levels were recently noted to be significantly higher in the serum of SSc patients as compared with healthy controls (6). We would therefore expect LPA levels to be increased in the skin during the development of injury-induced dermal fibrosis, both bleomycin-challenged mice and in SSc patients, although we have yet to investigate this.

Our initial investigations of the mechanism(s) through which LPA/LPA1 signaling contributes to dermal fibrosis revealed that in contrast to WT mice, bleomycin-challenged LPA1-KO mice failed to demonstrate two hallmarks of scleroderma--associated skin fibrosis: increased numbers of dermal myofibroblasts and increased numbers of dermal cells with nuclear Smad2 phosphorylation. These results suggest that LPA/LPA1 signaling is required for two central processes in scleroderma fibrogenesis: myofibroblast accumulation and TGFβ/Smad signaling pathway activation. We believe that attenuation of both of these interconnected fibrogenic processes in the absence of LPA1 accounts for the dramatic protection of LPA1-KO and AM095-treated mice from bleomycin-induced dermal fibrosis.

Myofibroblasts predominate in areas of increased collagen deposition in scleroderma lesional skin (24,25), where the number of myofibroblasts correlates with the severity of fibrosis (25). Promotion of myofibroblast accumulation by LPA/LPA1 signaling would therefore be expected to promote dermal fibrosis. LPA mediates multiple fibroblast activities that lead to the accumulation of these cells, including their recruitment and proliferation, and the prevention of their apoptosis (3,22,26–31). We hypothesize that reduced fibroblast accumulation in the absence of LPA/LPA1 signaling contributes to reduced myofibroblast accumulation in bleomycin-challenged LPA1-KO mice by reducing the pool of cells from which myofibroblasts differentiate. Our ability to evaluate this hypothesis in our immunohistochemical studies, however, was limited by difficulties in enumerating tissue fibroblasts, as opposed to myofibroblasts, by immunostaining, due to the lack of antigens specifically expressed by these cells.

Evidence from both mouse models and SSc patients indicates that TGFβ plays a central role in scleroderma fibrogenesis. Fibroblast-specific expression of a constitutively active TGFβ receptor is sufficient to recapitulate many features of scleroderma in mice, including dermal fibrosis (32), while inhibition of TGFβ signaling protects against dermal fibrosis in commonly used mouse models of scleroderma, including the bleomycin model (33). Gene expression profiling of lesional skin from scleroderma patients demonstrates increased expression of many TGFβ targets, similar to gene expression induced by treating normal fibroblasts with TGFβ (10,34,35). In previous studies, we found that LPA1 expression was not required for TGFβ downstream signaling in fibroblasts, since increases in procollagen type I α1 chain, fibronectin, and α-SMA expression induced by TGFβ were similar in WT and LPA1-deficient mouse lung fibroblasts (3).

TGFβ activity, however, is primarily regulated through the posttranslational activation of latent TGFβ complexes (36,37). The failure of phospho-Smad2+ cells to increase in bleomycin-challenged LPA1-KO mice therefore raises the possibility that LPA/LPA1 signaling may mediate TGFβ activation during the development of dermal fibrosis. Although decreased fibroblast and myofibroblast accumulation in bleomycin-challenged LPA1-KO mice could also have contributed to the reduced number of nuclear phospho-Smad2+ cells observed by decreasing the number of TGFβ-responsive cells present in the dermis, LPA has been reported to mediate TGFβ activation. LPA treatment of keratinocytes, as well as lung epithelial cells, has been shown to induce active TGFβ (4,38). While LPA signaling through LPA1 has been shown to induce αvβ6 integrin–dependent activation of latent TGFβ by lung epithelial cells in culture (4), our results suggest that LPA1 would be the receptor most likely to mediate LPA-induced TGFβ activation in the skin.

Activation of TGFβ by the epithelial cell–restricted αvβ6 integrin is required for the development of lung fibrosis in several animal models, including the bleomycin model of pulmonary fibrosis (5). In the lung, TGFβ-driven fibroblast activation and differentiation to myofibroblasts is therefore dependent on the activation of TGFβ by adjacent epithelial cells in a paracrine manner. In the skin of scleroderma patients however, activation of TGFβ by the fibroblasts themselves contributes to fibroblast activation and differentiation into myofibroblasts in an autocrine manner (39). The ability of scleroderma fibroblasts to activate TGFβ has been shown to result from their overexpression of 2 other αv-containing integrins that are capable of activating latent TGFβ, αvβ5 and αvβ3 (40,41). We therefore hypothesize that LPA signaling through LPA1 mediates TGFβ activation during the development of dermal
fibrosis through the αvβ5 and αvβ3 integrins expressed by skin fibroblasts.

As noted above, our laboratory recently implicated LPA/LPA1 signaling in the pathogenesis of pulmonary fibrosis (3). In addition, LPA has been implicated in renal and hepatic fibrogenesis. LPA1-KO mice were shown to be significantly protected in the unilateral ureteral obstruction model of renal tubulointerstitial fibrosis (42), and concentrations of circulating LPA correlated with the extent of hepatic fibrosis in the carbon tetrachloride rodent model of liver fibrosis (43). Including our results in this study, data now implicate the LPA/LPA1 pathway in the development of lung, kidney, liver, and skin fibrosis, suggesting that this pathway is of fundamental importance in the pathogenesis of fibrotic diseases associated with tissue injury. Additionally, the efficacy of a selective antagonist of LPA1 in our dermal fibrosis model provides preclinical support for targeting LPA1 in fibrotic diseases such as scleroderma.

In summary, we have shown that LPA signaling through LPA1, but not LPA2, is a critical requirement for the development of bleomycin-induced dermal fibrosis and for both myofibroblast accumulation and TGFβ/Smad signaling in this model. In addition to genetic deletion of LPA1, we found that pharmacologic inhibition of this receptor in both preventive and therapeutic regimens protected mice from dermal fibrosis. The ability of AM095 to attenuate dermal fibrosis when initiated after the onset of tissue injury in a therapeutic regimen suggests that antagonism of LPA1 may be effective in the treatment of patients with existing fibrosis, as would be needed for clinically useful antifibrotic drugs (40). Our results therefore indicate that LPA/LPA1 inhibition has the potential to be an effective new therapeutic strategy for scleroderma and that LPA1 is an attractive pharmacologic target for fibrosis.

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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. Tager 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.


Acquisition of data. Castelino, Bain, Brooks, King.

Analysis and interpretation of data. Castelino, Bain, King, Chun, Luster, Tager.

ROLE OF THE STUDY SPONSOR

Amira Pharmaceuticals facilitated the selectivity and pharmacokinetic studies of the LPA1 receptor antagonist reported herein. They reviewed and approved the manuscript prior to submission. The authors independently collected the data, interpreted the results, and had the final decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by Amira Pharmaceuticals.

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