Estrogens Counteract the Profibrotic Effects of TGF-β and their Inhibition Exacerbates Experimental Dermal Fibrosis

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Systemic sclerosis primarily affects women. This sex bias raises the question on the role female hormones could play in the development of fibrosis, which is largely unknown. Our aim was to evaluate the effects of estrogens in the development of experimental dermal fibrosis, in the mouse models of bleomycin-induced dermal fibrosis and tight skin (Tsk-1) mice, and on the activation of dermal fibroblasts by transforming growth factor-β (TGF-β). Estrogen inhibition, obtained through gene inactivation for the estrogen receptor-α knockout or treatment with tamoxifen, exacerbated skin fibrosis in the bleomycin model and in the Tsk-1 mice. In the dermal fibroblasts, treatment with 17β-estradiol significantly decreased the stimulatory effects of TGF-β on collagen synthesis and myofibroblast differentiation, decreased the activation of canonical TGF-β signaling, and markedly reduced the expression of the TGF-β target genes. Tamoxifen reversed the inhibitory effects of estrogens by restoring Smad2/3 phosphorylation and TGF-β-induced collagen synthesis. Our results demonstrate a beneficial effect of estrogens in dermal fibrosis. Estrogens reduce the TGF-β-dependent activation of dermal fibroblasts, and estrogen inhibition leads to a more severe experimental dermal fibrosis. These findings are consistent with the prominent development of systemic sclerosis in postmenopausal women and the greater severity of the disease in men.


INTRODUCTION

Fibrosis results from an imbalance between the synthesis and degradation of the extracellular matrix components and leads to the scarring of various tissues. It is the hallmark of systemic sclerosis (SSc), which is a prototypical fibrotic disease that affects both the skin and internal organs (Gabrielli et al., 2009; Varga and Abraham, 2007; Wynn and Ramalingam, 2012). The accumulation of the extracellular matrix components in SSc occurs because of an increased production by activated fibroblasts (Varga and Trojanowska, 2008). The profibrotic cytokine transforming growth factor-β (TGF-β) is one of the key mediators of fibroblast activation in SSc (Garrett et al., 2017; Varga and Pasche, 2009; Varga et al., 2017). However, the intracellular signaling cascades by which this cytokine stimulates the production of the extracellular matrix remain unclear.

SSc shows a strong and well-established gender bias, with an overall ratio (women to men) of approximately 6:1, reaching 10:1 at the childbearing period (Elhai et al., 2016; Whitacre, 2001). Although SSc is more frequent in women, data from the European Scleroderma Trial and Research (EUSTAR) group have shown that men with SSc have a more severe phenotype, and the prognosis of SSc is worse in men (Elhai et al., 2016). Consistent with this observation, an analysis of the effect of gender on the severity of experimental dermal fibrosis showed that male Balb/c, C57BL/6, DBA/2 mice had a tendency to develop more pronounced fibrosis than the females (Ruzehaji et al., 2015). Despite the well-established sex bias in SSc, the molecular basis of this critical aspect remains poorly understood. In particular, the role of sex hormones in modulating fibrosis is not well defined. Indeed, data on the role of sex hormones in the development of fibrosis are complex and contradictory among the different tissues studied (Murase et al., 2012; Tofovic et al., 2009; Voltz et al., 2008; Zhang and Wu, 2013). More specifically in SSc, the available data on the role of sex hormones in the promotion of fibrosis are scarce and heterogeneous. Some studies have shown that estrogens induce fibroblast dysfunction and increase the production of extracellular matrix proteins, whereas some other studies have reported a decreased collagen production upon treatment with estradiol (Aida-Yasuoka et al., 2013; Kashnikova et al., 1991; Soldano et al., 2010).

Thus, to explore the implications of sex hormones in the pathogenesis of dermal fibrosis in more detail, our aim was to evaluate the effects of estrogens (i) in the development of experimental dermal fibrosis and (ii) on the pathological activation of dermal fibroblasts induced by TGF-β.
RESULTS

Increased susceptibility of ERKO-α mice to bleomycin-induced dermal fibrosis

To evaluate the role of estrogen inhibition in fibrosis, estrogen receptor-α knockout (ERKO-α) mice and control C57BL/6 female mice were challenged with bleomycin. Skin architecture and dermal thickness did not differ between the ERKO-α mice and C57BL/6 mice injected with NaCl (Figure 1a). Upon administration of bleomycin injections, the mean increase in the dermal thickness was 55% in the C57BL/6 mice, as compared with 87% in the ERKO-α mice. Thus, dermal thickening upon bleomycin challenge was increased by 21% in the ERKO-α mice compared with the C57BL/6 mice (P = 0.034) (Figure 1a and b).

Consistent with increased dermal thickening, an increased accumulation of collagen upon bleomycin challenge was observed on the trichrome-stained skin sections of the ERKO-α mice (Figure 1c). In addition, the hydroxyproline content of the skin (Figure 1d) and the number of myofibroblasts in the lesional skin of ERKO-α mice upon challenge with bleomycin were significantly increased by 39% (P = 0.042) and 36% (P = 0.010), respectively, as compared with the C57BL/6 mice (Figures 1d–f). To evaluate whether estrogen inhibition influences the outcome of bleomycin-induced fibrosis by regulating the infiltration of inflammatory cells, we quantified the number of T cells, B cells, and monocytes in the fibrotic skin. The T cell counts were significantly increased in the ERKO-α mice compared with the wild-type mice (Supplementary Figure S1a). Conversely, the number of B cells (Supplementary Figure S1b) and monocytes (Supplementary Figure S1c) was similar between the ERKO-α and wild-type mice, upon bleomycin challenge.

Tamoxifen enhances bleomycin-induced dermal fibrosis

To further investigate the effects of estrogen inhibition observed with the gene inactivation strategy, we evaluated the effects of the ER antagonist tamoxifen in the model of bleomycin-induced dermal fibrosis. In the mice challenged with NaCl, tamoxifen did not increase dermal thickness and collagen content compared with the mice treated intraperitoneally with corn oil (data not shown). In mice challenged with bleomycin, tamoxifen significantly increased dermal thickening by 28% (P = 0.008) compared with the mice injected with corn oil (Figure 2a and b). Analyses of the hydroxyproline content and of myofibroblast counts confirmed that the accumulation of collagen increased by 23% (P = 0.002), and the activation of fibroblasts increased by 22% (P = 0.024) upon treatment with tamoxifen (Figures 2c–f).
Estrogen inhibition enhances the activation of canonical Smad signaling in bleomycin-induced dermal fibrosis

Because estrogen inhibition led to a more severe dermal fibrosis, we aimed to assess whether these effects were mediated by increased TGF-β signaling. To this end, we analyzed the nuclear levels of phosphorylated Smad2/Smad3 (pSmad2/3) signaling in the lesional skin of the bleomycin-treated mice. As expected, the levels of pSmad2/3 increased upon challenge with bleomycin (Supplementary Figure S2a–d). Estrogen inhibition obtained by gene inactivation (ERKO-a mice) or by the use of tamoxifen further stimulated canonical Smad signaling with increased nuclear levels of pSmad2/3 compared with the wild type mice (Supplementary Figure S2a and b) or sham-treated controls (Supplementary Figure S2c and d).

Tamoxifen enhances skin fibrosis in the tight skin mouse model

Because we demonstrated some profibrotic effects of estrogen inhibition by tamoxifen in a model of inflammation-driven dermal fibrosis, we next aimed to confirm these effects in Tsk-1 mice, which represents a model of later and less inflammatory stages of SSc with the endogenous activation of fibroblasts. The hypodermal thickness was increased by 278% in the sham-treated Tsk-1 mice ($P < 0.001$) and by 364% in the tamoxifen-treated Tsk-1 mice ($P < 0.001$), compared with that of the control pa/pa mice. Thus, hypodermal thickening was increased by 31% in tamoxifen-treated Tsk-1 mice compared with sham-treated Tsk-1 mice ($P = 0.028$) (Figure 3a and b). Hydroxyproline content was also significantly increased in tamoxifen-treated Tsk-1 mice by 16% ($P = 0.015$) and 541% ($P < 0.001$) compared with sham-treated Tsk-1 mice and pa/pa mice, respectively (Figure 3c).

Estrogens abrogate the profibrotic effects of TGF-β in dermal fibroblasts

Because enhanced TGF-β signaling was observed through estrogen inhibition in experimental dermal fibrosis, we next aimed to determine whether estrogens could antagonize the profibrotic effects directly induced by TGF-β. Incubation of the SSc fibroblasts with 10 μg/ml of 17-β-estradiol for 24 hours did not modify collagen production and differentiation of the resting fibroblasts to myofibroblasts (Figure 4a–f). However, treatment of SSc fibroblasts with 17-β-estradiol for 24 hours markedly decreased the stimulatory effects of TGF-β on COL1A1 and COL1A2 mRNA levels (Figures 4a and b). COL1A1 and COL1A2 mRNA levels were reduced by 47% ($P < 0.001$) and 34% ($P = 0.025$), respectively, upon exposure to 17-β-estradiol. Consistent with the decreased mRNA levels, the release of collagen in the fibroblast culture...
supernatants was also significantly reduced by 43% \( (P = 0.023) \) (Figure 4c).

To study whether the effects of 17-β-estradiol were mediated by ER-α or ER-β, SSc dermal fibroblasts were treated with specific ER-α or ER-β inhibitors (MPP and PHTPP, respectively) at a concentration of \( 10^{-6} \) M. Treatment of SSc fibroblasts with MPP or PHTPP before the addition of 17-β-estradiol and stimulation with TGF-β was both associated with a similar non-significant reduction of collagen synthesis. These results support that the potent antifibrotic effects of 17-β-estradiol in the dermal fibroblasts require both ER-α and ER-β (Supplementary Figure S3a–c).

Regarding myofibroblast differentiation, the stimulatory effect of TGF-β on the expression of α-SMA mRNA was markedly reduced by 17-β-estradiol (Figure 4d). The inhibitory effects of 17-β-estradiol were confirmed at the protein level by immunofluorescence and western blot analyses after 24 hours of incubation with 17-β-estradiol (Figure 4e–g).

Similar findings were observed in control dermal fibroblasts subjected to TGF-β stimulation. Indeed, the treatment of control fibroblasts with 17-β-estradiol potently reduced TGF-β–induced collagen release (Supplementary Figure S4a) and the expression of α-SMA (Supplementary Figure S4b–d). Consistent with these findings, the expression of the estrogen receptors ERα and ERβ was not significantly different between the SSc and control fibroblasts (Supplementary Figure S4e and f).

**Estrogens inhibit the activation of canonical Smad signaling and reduce TGF-β target gene expression in SSc dermal fibroblasts**

Because we showed that 17-β-estradiol reduced the response of dermal fibroblasts to TGF-β stimulation, we next focused on the effect of 17-β-estradiol on canonical Smad-dependent and non-canonical signaling pathways. Western blot and immunofluorescence experiments showed that TGF-β caused a robust upregulation of pSmad2/3 in human SSc dermal fibroblasts (Figures 5a and 6a and b). A marked decrease in pSmad2/3 expression induced by 17-β-estradiol was observed after the incubation of human SSc fibroblasts with 17-β-estradiol for 24 hours, whereas the levels of the total Smad2/3 remained unchanged. (Figures 5a and 6b). In addition, in the presence of the proteasome inhibitor MG132 at a concentration of 20 μM, 17-β-estradiol failed to reduce the TGF-β–induced expression of pSmad 2/3 (Supplementary Figure S5a and b). Conversely, the phosphorylation of TGFBR1, p38, and p44/42 in SSc fibroblasts by TGF-β was not potently inhibited by treatment with 17-β-estradiol (Figures 5b–d). Taken together, these results support that estrogens decrease the profibrotic effects of TGF-β by specifically interfering with the canonical Smad-dependent pathway through a proteasome-dependent degradation of pSmad2/3.

We also examined whether estrogens affect the mRNA levels of the TGF-βSmad target genes plasminogen activator inhibitor-1 (PAI-1) and matrix metalloproteinase-9 (MMP-9). Consistent with the effects of the estrogens observed on pSmad2/3, the TGF-β–induced expression of PAI-1 and MMP-9 was significantly decreased in the SSc dermal fibroblasts after incubation with 17-β-estradiol for 24 hours by 49% \( (P = 0.010) \) and 56% \( (P < 0.001) \), respectively (Figure 6c and d).

**Tamoxifen does not directly activate SSc dermal fibroblasts but reverses the inhibitory effects of estrogens on TGF-β signaling and TGF-β–induced collagen synthesis**

Because tamoxifen worsens dermal fibrosis in two complementary animal models of SSc, we next aimed to determine whether tamoxifen could contribute to the pathologic activation of SSc fibroblasts. Treatment of SSc dermal fibroblasts...
with 5 μg/ml of tamoxifen for 24 hours did not increase the mRNA levels of COL1A1 and COL1A2 (Supplementary Figure S6a and b). Consistent with this observation, the release of collagen protein into the cell culture supernatant was not modified upon treatment with tamoxifen (Supplementary Figure S6c). Moreover, tamoxifen did not markedly amplify the profibrotic effects of TGF-β on collagen synthesis in the SSc dermal fibroblasts (Supplementary Figure S6a–c). Consistent with collagen production, tamoxifen failed to induce the differentiation of SSc fibroblasts into myofibroblasts. The mRNA levels (Supplementary Figure S6d) and protein expression (Supplementary Figure S6e and f) of α-SMA were not modified in the SSc fibroblasts upon tamoxifen treatment. In addition, tamoxifen did not significantly amplify the effects of TGF-β on α-SMA expression in the SSc dermal fibroblasts (Supplementary Figures S6d–f).

Based on the effects observed with tamoxifen on experimental dermal fibrosis, and given that tamoxifen did not enhance the effects of TGF-β in dermal fibroblasts, we hypothesized that tamoxifen could reverse the inhibitory effects of estrogens on TGF-β signaling and TGF-β–induced collagen synthesis. We first observed that tamoxifen did not modify the TGF-β–induced protein expression of pSmad2/3 and mRNA levels of PAI-1 and MMP-9 (Figure 6b–d).

However, tamoxifen had the capacity to counteract the inhibitory effects of estrogens on TGF-β signaling. The incubation of SSc dermal fibroblasts with tamoxifen 4 hours before treatment with 17-β-estradiol restored the TGF-β–induced phosphorylation of Smad2/3 (Figure 6a and b). In addition, the sensitivity of PAI-1 and MMP-9 to TGF-β was re-established in fibroblasts incubated with tamoxifen before treatment with 17-β-estradiol (Figure 6c and d).

Following these results, we sought to determine whether tamoxifen could reduce the inhibition mediated by estrogens on collagen synthesis. Indeed, 17-β-estradiol failed to decrease COL1A1 and mRNA levels in the SSc dermal fibroblasts pre-treated with tamoxifen (Supplementary Figure S7a and b). This result was confirmed with the measurement of the collagen release in cell culture supernatants (Supplementary Figure S7c).

**DISCUSSION**

The genetic or pharmacologic inhibition of estrogen signaling led to increased sensitivity to bleomycin-induced dermal fibrosis, with a similar extent for both strategies (a 20% increase of dermal thickness). This result is consistent with the prevention of bleomycin-induced fibrosis previously observed with 2-methoxyestradiol (Zhu et al., 2015).
Increased T-cell infiltrates observed in the lesional skin of ERKO-α mice support the hypothesis that the antifibrotic effects of estrogens in the early stages of fibrosis are, at least partially, driven by inflammation. The proinflammatory effects of estrogen inhibition seem preferentially mediated by T cells, rather than B cells and macrophages, as observed in experimental autoimmune encephalomyelitis (Offner and Polanczyk, 2006). Consistent with our results, estrogen inhibition was also associated with inflammation and fibrosis in the liver, leading to increased tumorigenesis (Wei et al., 2016).

On the other hand, the exacerbation of skin fibrosis observed in this study was also observed upon tamoxifen therapy in Tsk-1 mice, characterized by the persistent overproduction of extracellular matrix proteins occurring in the absence of inflammatory infiltrates (Beyer et al., 2010). Consistent with our results, estrogen inhibition was also associated with inflammation and fibrosis in the liver, leading to increased tumorigenesis (Wei et al., 2016).

Tamoxifen potently prevented the 17β-estradiol-induced decreased phosphorylation of Smad2/3 in SSc dermal fibroblasts, and the incubation of SSc dermal fibroblasts with tamoxifen before the 17β-estradiol treatment restored the profibrotic effects of TGF-β on collagen synthesis. Despite the previously reported elevation of TGF-β production upon tamoxifen therapy (Thomas-Golbanov et al., 2003), tamoxifen alone failed to amplify the TGF-β signaling or the transcription of the TGF-β target genes, as previously reported (Goto et al., 2011), supporting that estrogen stimulation is required for tamoxifen to exert its action of a competitive estrogen inhibitor.

Taken together, our findings support the potential beneficial effects of estrogens in different stages of experimental dermal fibrosis. This is consistent with the improvement of hepatic fibrosis through estrogen therapy, with the inhibition of hepatic stellate cell activation (Zhang and Wu, 2013). In lungs, the exacerbation of bleomycin-induced pulmonary hypertension and fibrosis has been observed in estrogen-
deficient rats (Tofovic et al., 2009), and male sex hormones have been shown to increase lung function impairment after bleomycin-induced pulmonary fibrosis (Voltz et al., 2008). On the other hand, estrogen inhibition with tamoxifen improved several fibrotic diseases, such as progressive renal disease, sclerosing cervicitis, or retroperitoneal fibrosis (Delleè et al., 2012; Ruffy et al., 2006; Savelli et al., 1997; Thomas-Golbanov et al., 2003). Moreover, estrogens were implicated in left ventricular fibrosis and diastolic dysfunction exacerbation in a rat model of metabolic syndrome (Murase et al., 2012). Taken together, these data support that estrogens may differentially modulate fibrosis in different organs, and the heterogeneity of the results strongly suggest a tissue specificity of estrogens and sex hormones in fibrosis.

In conclusion, our results support that estrogens utilize ERα stimulation to alleviate the activated profile of SSc dermal fibroblasts by inhibiting TGF-β signaling (Supplementary Figure S8). These antifibrotic effects of estrogens are repressed by the estrogen antagonist tamoxifen (Supplementary Figure S8). These data are consistent with the amplification of TGF-β signaling observed in vivo through estrogen inhibition in the bleomycin mouse model and Tsk-1 mice, leading to the exacerbation of fibrosis. Thus, our data indicate that estrogen inhibition amplifies the profibrotic effects of TGF-β in the mouse models of skin fibrosis. Our results are consistent with the prominent occurrence of SSc in postmenopausal women and the greater severity of the disease in men.

**MATERIAL AND METHODS**

**Animals**

Six-week-old female ERKO-α mice on a C57BL/6 background and 5-week-old female Tsk-1 strains were purchased from Jackson Laboratory (Bar Harbor, ME), whereas 6-week-old female wild-type C57BL/6 mice were purchased from Charles River (Chatillon-sur-Chalaronne, France). All the animals were bred and maintained at the animal care facilities of the Cochin Institute (Cochin Hospital, France) and Montrouge Dental University (Montrouge, France). All the experimental procedures were conducted in compliance with Animal Health regulations, and the local ethical committee approved all the animal experiments.

**Estrogen inhibition in the mouse model of bleomycin-induced experimental dermal fibrosis**

Skin fibrosis was induced in the ERKO-α and C57BL/6 mice by local injections of bleomycin for 3 weeks; 100 μl of bleomycin dissolved in 0.9% sodium chloride (NaCl) at a concentration of 0.5 mg/ml was administered every other day by subcutaneous injections in defined areas of 1 cm² at the upper back. Subcutaneous injections of 100 μl 0.9% NaCl were used as controls.

**Bleomycin-induced dermal fibrosis in ERKO-α mice.** Four different groups, consisting of two groups of ERKO-α mice (a total of 20 mice) and two groups of wild-type mice (a total of 20 mice) were analyzed. One group of ERKO-α mice (n = 12) and one group of wild-type mice (n = 12) were challenged with bleomycin, whereas the remaining two groups (n = 8 each) were injected with NaCl.
Evaluation of the effects of tamoxifen in bleomycin-induced dermal fibrosis. Following the use of a gene inactivation strategy with the use of mice deficient for ER-α, we used a complementary targeting molecular strategy using tamoxifen, an ER antagonist. Tamoxifen was diluted in corn oil and kept in dark glass tubes at 4 °C. Tamoxifen was administrated by intraperitoneal (IP) injections at a dose of 400 µg in a volume of 200 µl of corn oil every other day, as previously described (Shoeger et al., 2003). One group of 14 mice was challenged with bleomycin injections for 3 weeks and was treated in parallel with IP injections of tamoxifen at a dose of 400 µg (n = 7) every other day. Three control groups were used: one group challenged with bleomycin and injected IP with corn oil (n = 7), one challenged with NaCl and injected with tamoxifen at a dose of 400 µg (n = 7) every other day, and one challenged with NaCl and injected with corn oil (n = 7).

Effects of tamoxifen in the tight skin mouse model
One group of five Tsk-1 mice received IP injections of 400 µg of tamoxifen every other day for 5 weeks, whereas another group of five Tsk-1 mice received IP injections of corn oil. A third group of five pa/pa mice on the same genetic background not carrying the tsk1 mutation was used as controls. These mice also received IP injections of corn oil. Treatment was started at the age of 5 weeks, and the mice were killed after 5 weeks of treatment.

Histological analysis and Hydroxyproline assay
These techniques have been previously reported (Avouac et al., 2011, 2012, 2013, 2014; Desallais et al., 2014; Woessner and Boucek, 1961), and a full description is available in the Supplementary Materials.

Immunohistochemistry for α-SMA, CD3, CD22, CD68
Myofibroblasts were identified by staining for α-SMA as previously described (Rawson et al., 1965). Cells positive for α-SMA in the mouse skin sections were detected by incubation with monoclonal mouse anti-human antibodies against anti-α-SMA.

To quantify the numbers of infiltrating T cells, B cells, and monocytes, skin sections were incubated with polyclonal rabbit anti-mouse antibodies for CD3, CD22 (Abcam, Paris, France), or with monoclonal mouse anti-mouse antibodies against CD68 (Novus Biologicals, Littleton, CO).

Fibroblast cultures
Fibroblast cultures were obtained from the skin biopsy specimens of eight female patients with SSc who signed a written consent form approved by the local institutional review boards. All the patients with SSc fulfilled the criteria for the SSc subsets, as suggested by LeRoy et al. (1988).

Human fibroblasts were prepared by outgrowth cultures from skin biopsy specimens and cultured in Dulbecco’s modified Eagle’s medium—Ham’s F-12 containing 10% heat-inactivated fetal calf serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 2.5 µg/ml amphotericin-B (all from Invitrogen, Karlsruhe, Germany) as previously described (Avouac et al., 2012). In selected experiments, the fibroblasts were stimulated with recombinant TGF-β for 24 hours (10 ng/ml, PeproTech, Hamburg, Germany). Fibroblasts from passages 4 to 8 were used for the experiments. In addition, we used tamoxifen (5 µg/ml), solubilized in methanol, 17β-estradiol (10 µg/ml), solubilized in phosphate buffered saline, the proteasome inhibitor MG132 (10 or 20 µl) solubilized in DMSO, the ER-α inhibitor MPP (10⁻⁶ M) and the ER-β inhibitor PHTPP (10⁻⁷M) solubilized in DMSO (all from Sigma-Aldrich, Saint-Quentin Fallavier, France) to modulate estrogens in the dermal fibroblasts.

Quantitative real-time polymerase chain reaction and western blots
The full description of these techniques is available in the Supplementary Materials.

Quantification of soluble collagen protein
Total soluble collagen in cell culture supernatants was quantified using the SirCol collagen assay (Biocolor, Belfast, Northern Ireland), as previously described (Tomcik et al., 2014).

Immunofluorescence for phosphorylated smad-2/3 and α-smooth muscle actin (α-SMA)
The expression of pSmad2/3 and α-SMA was detected by staining with polyclonal goat anti-human antibodies against phospho-smad-2/3 (Santa Cruz Biotechnology [Santa Cruz, CA] reacts with human and mouse pSmad2/3) and monoclonal mouse anti-human antibodies against anti-α-SMA antibody (clone 1A4; Sigma-Aldrich), respectively (Avouac et al., 2012).

Donkey anti-goat Alexa Fluor 594 and donkey anti-mouse Alexa Fluor 594 conjugated antibodies were used as the secondary antibodies. Counterstaining was performed with DAPI (Santa Cruz Biotechnology) for 10 minutes at room temperature. The slides were then viewed by microscopy using appropriate fluorescence filters.

 Statistical analysis
All data are expressed as the mean values ± standard error of the mean. Multiple group comparisons were analyzed by using a post hoc Dunnett’s test. An unpaired or paired t test was used for a two-group comparison. P < 0.05 (all two-sided) was considered significant.

Data availability statement
The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request. All the data generated or analyzed during this study are included in this published article and its Supplementary Materials.

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CONFLICT OF INTEREST
JA has had a consultancy relationship and/or has received research funding in relationship with the treatment of systemic sclerosis from Actelion, Roche, Pfizer, and Bristol-Myers Squibb. YA has had a consultancy relationship and/or has received research funding in relationship with the treatment of systemic sclerosis from Actelion, Bayer, Biogen Idec, Bristol-Myers Squibb, Genentech/Roche, Inventiva, Medac, Pfizer, Sanofi/Genzyme, Servier, and UCB.

AUTHOR CONTRIBUTIONS
Conceptualization: JA, YA; Formal Analysis: SP, VG, LB, AC, BR, GB, ME; Investigation: SP, VG, LB, AC, BR, GB, ME; Resources: MLB; Writing -
SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.07.719.

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Animals

Six-week-old female estrogen receptor (ER)-α knockout mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). These mice are characterized by a complete lack of ERα proteins, leading to rudimentary reproductive structures and infertility. Five-week-old female tight skin-1 (Tsk-1) strains were also purchased from Jackson Laboratory, and 6-week-old female wild-type C57BL/6 mice were purchased from Charles River (Châtillon-sur-Chalaronne, France). The Tsk-1 phenotype is caused by a dominant mutation in the fibrillin-1 gene. Tsk-1 mice are characterized by the accumulation of collagen fibers in the hypodermis resulting in progressive hypodermal thickening. In contrast to bleomycin-induced fibrosis, inflammatory infiltrates are absent, and the aberrant activation of fibroblasts is not caused by the release of inflammatory mediators from leukocytes. Similar to SSc fibroblasts, the fibroblasts from Tsk-1 are endogenously activated with an increased release of collagen that persists for several passages in vitro.

Histological analysis

The lesional skin areas were excised, fixed in 4% formalin, and embedded in paraffin. Five-micron-thick sections were stained with hematoxylin and eosin. The dermal thickness was analyzed at ×100 magnification by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction at four sites from the lesional skin of each mouse (Avouac et al., 2012, 2014; Desallais et al., 2014). The hypodermal thickness in the Tsk-1 mice was determined by measuring the thickness of the subcutaneous connective tissue beneath the panniculus carnosus at four different sites of the upper back in each mouse (Avouac et al., 2011b; Desallais et al., 2014). Two independent examiners blinded to the treatment of the mice performed the evaluation. (JA and LB)

Hydroxyproline assay

The collagen content in the lesional skin samples was evaluated by the hydroxyproline assay (Avouac et al., 2011b, 2013; Woessner and Boucek, 1961). After the digestion of the punch biopsy specimens (3 mm diameter) in 6 M HCl for 3 hours at 120 °C, the pH of the samples was adjusted to 7. Afterward, the samples were mixed with 0.06 M chloramine T and incubated for 20 minutes at room temperature. Then, 3.15 M perchloric acid and 20% p-dimethylaminobenzaldehyde were added, and the samples were incubated for an additional 20 minutes at 60 °C. The absorbance was determined at 557 nm with a spectrophotometer. Trichrome staining was performed for the direct visualization of the collagen fibers.

Immunohistochemistry for α-SMA, CD3, CD22, and CD68

The skin sections were deparaffinized for immunohistochemistry, followed by incubation with 5% BSA in phosphate buffered saline for 1 hour to block non-specific binding and incubation with 3% H2O2 for 10 minutes to block endogenous peroxidase activity. The staining was visualized with aminothiocyanoacetazo using a peroxidase substrate kit (Vector, Burlingame, CA).

Myofibroblasts were identified by staining for α—smooth muscle actin (α-SMA) as previously described (Rawson et al., 1965). The cells positive for α-SMA in the mouse skin sections were detected by incubation with monoclonal mouse anti-human antibodies against anti—α-SMA. Polyclonal rabbit anti-mouse antibodies labeled with horseradish peroxidase were used as secondary antibodies for 1 hour at room temperature. Irrelevant isotype-matched antibodies served as controls. The number of myofibroblasts was determined at ×200 magnification using a Nikon Eclipse 80i microscope (Nikon, Champigny sur Marne, France). The myofibroblasts were defined as single, α-SMA-positive spindle-shaped cells. Two experienced examiners (JA and LB) performed myofibroblast quantification in six randomly selected high-power fields for each section. Three sections were evaluated for each slide (Avouac et al., 2011b, 2013).

To quantify the numbers of infiltrating T cells, B cells, and monocytes, the skin sections were incubated with polyclonal rabbit anti-mouse antibodies for CD3, CD22 (Abcam, Paris, France), or with monoclonal mouse anti-mouse antibodies against CD68 (Novus Biologicals, Littleton, CO). The T cells, B cells, and monocytes were counted in a blinded manner by two examiners (SP and JA) in six different sections of the lesional skin of each mouse.

Fibroblast cultures

Fibroblast cultures were obtained from the skin biopsy specimens of eight patients with SSc who signed a written consent form approved by the local institutional review boards. All patients with SSc fulfilled the criteria for SSc subsets as suggested by LeRoy et al. (1988). The median age of the patients with SSc was 48 years (range: 31-68 years), and their median disease duration was 7 years (range: 1-10 years); three had limited cutaneous disease and five diffuse cutaneous SSc. None of the patients were treated with immunosuppressive or other potentially disease-modifying drugs.

Human fibroblasts were prepared by outgrowth cultures from skin biopsy specimens and cultured in DMEM–Ham’s F-12 containing 10% heat-inactivated fetal calf serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 2.5 μg/ml amphotericin-B (all from Invitrogen, Karlsruhe, Germany) as described (Avouac et al., 2008). In selected experiments, the fibroblasts were stimulated with recombinant transforming growth factor-β for 24 hours (10 ng/ml, PeproTech, Hamburg, Germany). This latter concentration represents the standard concentration used for the stimulation of dermal fibroblasts and is based on the serum levels in the patients with SSc (Avouac et al., 2014). Fibroblasts from passages 4 to 8 were used for the experiments. In addition, we used tamoxifen, solubilized in methanol, and 17-β-estradiol, solubilized in phosphate buffered saline (both from Sigma-Aldrich, Saint-Quentin Fallavier, France), to modulate the estrogens in the dermal fibroblasts.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from dermal fibroblasts with the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). First-strand complementary DNA was synthesized from 2 μg of total RNA using random primers and Superscript II reverse transcriptase (200 units/μl;
Invitrogen, Carlsbad, CA). TaqMan quantitative reverse transcriptase–PCR (RT-PCR) was performed as previously described (Avouac et al., 2008). We used primer sequences (inventoried by Life Technologies, Saint Aubin, France) for human α1(I) procollagen (col1a1) (Hs00164004_m1), α2(I) procollagen (col1a2) (Hs00164099_m1), plasminogen activator inhibitor-1 (PAI-1) (Hs01126606_m1), and matrix metalloproteinase-9 (MMP-9) (Hs 00234579_m1). Hypoxanthine guanine phosphoribosyl-transferase was used as a housekeeping control (Hs99999909_m1). All PCRs were performed in triplicate. The relative mRNA levels for each sample were quantified using the Ct approach (fluorescence threshold), normalized to HPRT RNA as the standard.

Western blot analysis
Western blots were performed as previously described (Avouac et al., 2008, 2011a). The SSc dermal fibroblasts were homogenized and sonicated in RIPA buffer containing protease and phosphatase inhibitors, and 30 μg of protein was used. To detect, immunoblots were incubated overnight at 4 °C with monoclonal rabbit anti-human or mouse anti-human antibodies against α-SMA, Smad2/3, phosphorylated Smad-2/3 (pSmad2/3), p38, phospho-p38, p44/42, phospho-p44/42 (Cell Signaling, Leiden, The Netherlands), TGFBR1 (Abcam, Paris, France), and phosphorylated TGFBR1 (pTGFBR1, Thermo Fischer, Waltham, MA). Horseradish peroxidase–conjugated goat anti-rabbit or rabbit anti-mouse (Dako, Glostrup, Denmark) were used as secondary antibodies.

REFERENCES


Supplementary Figure S1. Estrogen inhibition increases T-cell infiltration into lesional skin. (a) Representative images of lesional skin sections stained for CD3 and positive CD3 T-cell counts. Bar = 20 μm. (b) Representative images of lesional skin sections stained for CD22 and positive CD22 B-cell counts. Bar = 20 μm. (c) Representative images of lesional skin sections stained for CD68 and positive CD68 monocyte counts. Bar = 20 μm. The control mice were injected with NaCl, and the value for these mice was defined as 1. The results from the other groups were normalized to this value. A total of 40 mice were used and analyzed (n = 8 in NaCl groups and n = 12 in bleomycin groups). Values are the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ****P < 0.0001 determined by a one-way analysis of variance followed by Dunnett’s multiple comparison test. ERKO, estrogen receptor knockout.
Supplementary Figure S2. Estrogen inhibition enhances the activation of canonical Smad signaling in experimental fibrosis. (a, c) Representative images of the skin section of immunostained skin tissue for pSmad 2/3 in the bleomycin-induced dermal fibrosis mouse model. Bar = 50 μm. (b, d) Quantification of the number of nuclei stained for pSmad 2/3 (n = 5 for each). The control mice were injected with NaCl, and the value for these mice was defined as 1. The results from the other groups were normalized to this value. A total of 35 mice were used and analyzed. Values are the mean ± standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001 determined by a one-way analysis of variance followed by Dunnett’s multiple comparison test. BLM, bleomycin; ERKO, estrogen receptor knockout; TAMOX, tamoxifen.

Supplementary Figure S3. ER-α and ER-β cooperate to attenuate the profibrotic effects of TGF-β. (a–c) SSc dermal fibroblasts. mRNA levels of Col1a1 (a), Col1a2 (b), and collagen release (c) upon TGF-β stimulation (10 ng/ml), treatment with 17-β-estradiol (10 μg/ml), ER-α inhibitor MPP (μM) or ER-β inhibitor PHTPP (μM) for 24 hours (n = 5 for each). MPP or PHTPP were added 2 hours before the addition of TGF-β and 17-β-estradiol at 24 hours. All the data are shown as the mean ± standard error of the mean of three independent experiments of each group. *P < 0.05, **P < 0.01 determined by a one-way analysis of variance followed by Dunnett’s multiple comparison test. MPP, 1,3-Bis[4-hydroxyphenyl]-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; PHTPP, 4-[2-Phenyl-5,7-bistrifluoromethyl]pyrazolo[1,5-a]pyrimidin-3-yl]phenol; α-SMA, α-smooth muscle actin; SSc, systemic sclerosis; TGF-β, transforming growth factor-β.
Supplementary Figure S5. Estrogens interfere with the canonical Smad-dependent pathway through a proteasome-dependent degradation of pSmad2/3. (a, b) Estrogen inhibition of TGF-β response in the presence of the proteasome inhibitor MG132. Cell extracts from the cultured SSc fibroblasts, stimulated with TGF-β (10 ng/ml) and treated with 17-β-estradiol (10 µg/ml) for 24 hours (n = 5 for each) in the control dermal fibroblasts. (b) α-SMA mRNA levels upon TGF-β stimulation and treatment with 17-β-estradiol (10 µg/ml) for 24 hours (n = 5 for each) in the control dermal fibroblasts. (c) Representative images of immunofluorescence for α-SMA in control dermal fibroblasts upon TGF-β stimulation and treatment with 17-β-estradiol (10 µg/ml) for 24 hours. Bar = 20 µm. Nuclei are stained with DAPI (blue). (d) Amount of fluorescence quantified with the Image J software (NIH, Bethesda, MD) (n = 5 for each). (e–f) Protein expression of ER-α (e) and ER-β (f) assessed by western blotting in unstimulated SSc and control dermal fibroblasts (n = 5 for each). All the data are shown as the mean ± standard error of the mean of three independent experiments of each group. *P < 0.05, **P < 0.01, determined by Student’s t test. α-SMA, α-smooth muscle actin; ER, estrogen receptor; SSc, systemic sclerosis; TGF-β, transforming growth factor-β.

Supplementary Figure S4. Estrogens abrogate the profibrotic effects of TGF-β in control dermal fibroblasts. (a) Collagen release upon TGF-β stimulation (10 ng/ml) and treatment with 17-β-estradiol (10 µg/ml) for 24 hours (n = 5 for each) in the control dermal fibroblasts. (b) α-SMA mRNA levels upon TGF-β stimulation and treatment with 17-β-estradiol (10 µg/ml) for 24 hours (n = 5 for each) in the control dermal fibroblasts. (c) Representative images of immunofluorescence for α-SMA in control dermal fibroblasts upon TGF-β stimulation and treatment with 17-β-estradiol (10 µg/ml) for 24 hours. Bar = 20 µm. Nuclei are stained with DAPI (blue). (d) Amount of fluorescence quantified with the Image J software (NIH, Bethesda, MD) (n = 5 for each). (e–f) Protein expression of ER-α (e) and ER-β (f) assessed by western blotting in unstimulated SSc and control dermal fibroblasts (n = 5 for each). All the data are shown as the mean ± standard error of the mean of three independent experiments of each group. *P < 0.05, **P < 0.01, determined by Student’s t test. α-SMA, α-smooth muscle actin; ER, estrogen receptor; SSc, systemic sclerosis; TGF-β, transforming growth factor-β.
Supplementary Figure S7. Tamoxifen reverses the inhibitory effects of estrogens on TGF-β-induced collagen synthesis. (a–c) SSc dermal fibroblasts: mRNA levels of Col1a1 (a), Col1a2 (b), and collagen release (c) upon TGF-β stimulation (10 ng/ml) and treatment with 17-β-estradiol (5 µg/ml) for 24 hours (n = 5 for each). (d) α-SMA mRNA levels upon TGF-β stimulation (10 ng/ml) and treatment with tamoxifen (5 µg/ml) for 24 hours (n = 5 for each). (e) Representative images of immunofluorescence for α-SMA in control dermal fibroblasts upon TGF-β stimulation (10 ng/ml) and treatment tamoxifen (5 µg/ml) for 24 hours. Bar = 20 µm. Nuclei are stained with DAPI (blue). (f) Amount of fluorescence quantified with the Image J software (n = 5 for each). All the data are shown as the mean ± standard error of the mean of three independent experiments of each group. Statistical test: Student’s t test. α-SMA, α-smooth muscle actin; SSc, systemic sclerosis; TGF-β, transforming growth factor-β.
Supplementary Figure S8. Proposed model for the regulation of dermal fibrosis by controlling TGF-β signaling using estrogens and tamoxifen. In the absence of tamoxifen, estrogens inhibit the profibrotic effects of TGF-β in dermal fibroblasts by repressing the TGF-β-induced canonical cascade. A putative direct interaction between pSmad2/3 and ERα leads to the enhanced degradation of the Smad proteins via an ubiquitin-proteasome pathway. The estrogen-induced inhibition of TGF-β canonical signaling leads to decreased collagen synthesis, reduced α-SMA, and other TGF-β target gene expression. Tamoxifen induces a competitive inhibition of estrogens that prevents the putative interaction between estrogens and pSmad2/3, leading to enhanced collagen synthesis, increased α-SMA expression and transcription of other TGF-β target genes. Data that are still pending and not directly demonstrated in our study are represented by dotted arrows. Data emerging from our study are represented by plain arrows. αAMA; α-smooth muscle actin; ECM, extracellular matrix; ERα, estrogen receptor α; P, phosphorylation; RI/RII, receptor type I/II; SBE, Smad binding element; TGF-β, transforming growth factor-β.