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Interferon- γ Promotes Vascular Remodeling in Human Microvascular Endothelial Cells by Upregulating Endothelin (ET)-1 and Transforming Growth Factor (TGF) β 2

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Abstract

Systemic sclerosis (SSc) is a complex disease characterized by vascular alterations, activation of the immune system and tissue fibrosis. Previous studies have implicated activation of the interferon pathways in the pathogenesis of SSc. The goal of this study was to determine whether interferon type I and/or type II could play a pathogenic role in SSc vasculopathy. Human dermal microvascular endothelial cells (HDMVECs) and fibroblasts were obtained from foreskins of healthy newborns. The RT Profiler PCR Array System was utilized to screen for EndoMT genes. Treatment with IFN- α or IFN- γ downregulated Fli1 and VE-cadherin. In contrast, IFN- α and IFN- γ exerted opposite effects on the expression of α -SMA, CTGF, ET-1, and TGF β 2, with IFN- α downregulating and IFN- γ upregulating this set of genes. Blockade of TGF β signaling normalized IFN- γ -mediated changes in Fli1, VE-cadherin, CTGF, and ET-1 levels, whereas upregulation of α -SMA and TGF β 2 was not affected. Bosentan treatment was more effective than TGF β blockade in reversing the actions of IFN- γ , including downregulation of α -SMA and TGF β 2, suggesting that activation of the ET-1 pathway plays a main role in the IFN- γ responses in HDMVECs. IFN- γ induced expression of selected genes related to endothelial-to-mesenchymal transition (EndoMT), including Snail1, FN1, PAI1, TWIST1, STAT3, RGS2, and components of the WNT pathway. The effect of IFN- γ on EndoMT was mediated via TGF β 2 and ET-1 signaling pathways. This study demonstrates distinct effects of IFN- α and IFN- γ on the biology of vascular endothelial cells. IFN- γ may contribute to abnormal vascular remodeling and fibrogenesis in SSc, partially via induction of EndoMT.

Systemic sclerosis (SSc) or scleroderma, is a rare and progressive connective tissue autoimmune disease of unknown etiology affecting various organ systems. The disease is characterized by fibroproliferative vasculopathy, tissue fibrosis and activation of the immune system (Trojanowska, 2010). Vascular abnormalities also play a major role in organ dysfunction, including lung, heart, and kidney (Pattanaik et al., 2011). Various mediators have been implicated in the dysregulated vascular remodeling process in SSc (Trojanowska, 2010; Pattanaik et al., 2011; Piera-Velazquez et al., 2011), however, despite the significant

progress in this area, the mechanism underlying SSc vasculopathy remains poorly understood.

Endothelin (ET)-1, a potent vasoconstrictor and a profibrotic mediator, contributes to the vascular pathogenesis in patients with Raynaud's phenomenon and SSc (Sulli et al., 2009). Bosentan, a dual endothelin receptor antagonist, has been shown to be effective in preventing new digital and non-digital ulcers (Giordano et al., 2010; Taniguchi et al., 2012), as well as reducing the pro-inflammatory cytokine levels, including IFN γ , in SSc patients (Bellisai et al., 2011). Previous studies have reported various alterations in the dermal endothelial cells of SSc patients including a marked decrease in vascular endothelial VE-cadherin, a marker of endothelial barrier function (Fleming et al., 2008). Furthermore, expression of Friend leukemia integration (Fli) 1 transcription factor, an activator of the VE-cadherin gene, has been shown to be significantly reduced in endothelial cells in clinically uninvolved scleroderma skin (Kubo et al., 2003). We have observed that mice with a conditional deletion of Fli1 in endothelial cells (Fli1CKO) display vascular network disorganization and increased vascular permeability with a decrease in VE-cadherin and platelet endothelial cell adhesion molecule 1 (PECAM1), thus reproducing several aspects of scleroderma vasculopathy (Asano et al., 2010). Relevant to these findings, recent studies have shown that IFN- α signaling is activated concomitantly with the loss of VE-cadherin in the dermal endothelium of SSc patients (Fleming et al., 2008, 2009). Notably, similar to systemic lupus erythematosus, myositis, and rheumatoid arthritis, scleroderma patients show activation of the type I interferon pathway ("interferon signature") (Lafyatis and York, 2009; Higgs et al., 2011).

With respect to the effect of interferon on endothelial cells, previous studies have primarily focused on IFN- γ and its role in the pathogenesis of cardiac allograft vasculopathy, restenosis, and atherosclerosis (Tellides et al., 2000; Zohnhofer et al., 2001; Tellides and Pober, 2007). IFN- γ has been shown to promote immune cell recruitment (Choi et al., 2004), macrophage activation (Tellides and Pober, 2007), and smooth muscle cell (SMC) proliferation (Tellides et al., 2000). IFN- γ is produced predominantly by the natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (Schoenborn and Wilson, 2007). Scleroderma serum was shown to contain a higher level of the IFN- γ induced genes, CXCL10 and MIG/ CXCL9, which are known to have anti-angiogenic properties (Rabquer et al., 2011). It has been suggested that the initial stages of SSc are associated with the Th1 inflammatory response, while the later stages are characterized by the Th2 response (Antonelli et al., 2008, 2011). This suggests that IFN- γ may play an important role during the early stages of SSc. Notably, mice with a targeted knockout mutation in the IFN- γ gene were protected from bleomycin-induced pulmonary fibrosis, but the specific cell types involved in this protective response were not investigated (Segel et al., 2003). Furthermore, polymorphisms in the IFN- γ and IFN- γ receptor genes have been associated with SSc (Schrijver et al., 2004; Wastowski et al., 2009), supporting a pathogenic role of IFN- γ in SSc.

Given the potential pathogenic role of IFNs, particularly during the early, inflammatory stages of SSc characterized by endothelial cell injury, the goal of this study was to determine

the role of IFN- α and IFN- γ in SSc vasculopathy focusing on the effects of IFN- γ on the dermal microvascular endothelial cells. The results of this study show that both IFN- α and IFN- γ can contribute to the increased vessel permeability in SSc through downregulation of Fli1 and VE-cadherin. Furthermore, we show for the first time that IFN- γ upregulates expression of ET-1, TGF β and other genes related to the process of EndoMT, suggesting that IFN- γ could also contribute to pathological vascular remodeling in SSc. Together, this study suggests that in addition to its known antifibrotic role in fibroblasts, IFN- γ could also promote fibrosis through its action on endothelial cells.

Materials and Methods

Cell culture

Human dermal microvascular endothelial cells (HDMVECs) were isolated from human foreskins using previously described protocol (Richard et al., 1998). Upon informed consent and in compliance with the Institutional Review Board of Human studies, written approval was obtained from Perinatal Committee (IRB number H-29190) of Boston University Medical School. Briefly, primary cultures of human foreskins were established after the removal of epidermis. Such cultures consist of a mixture of HDMVECs, dermal fibroblasts, and some keratinocytes. Subconfluent cultures were treated with tumor necrosis factor- α for 6 h to selectively induce the expression of E-selectin in HDMVECs. HDMVECs were then purified using magnetic beads coupled to an anti-E-selectin monoclonal antibody. First passage cultures usually consist of >99% HDMVECs. A second immunomagnetic purification step ensures homogenous population of HDMVECs suitable for long term culturing. Purity of the HDMVEC cultures was evaluated using anti-CD31 and anti-von Willebrand factor antibodies. These cells were maintained on collagen-coated 6-well plate in EBM-2 medium (Lonza, Portsmouth, NH) supplemented with 10% Fetal bovine serum and Endothelial Cell growth supplement mix at 37°C under 5% CO₂ in air. Human dermal fibroblast cultures were obtained from foreskins of healthy newborns at Boston University Medical Center and were cultured in DMEM media with 10% Fetal bovine serum (FBS).

Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol reagent (MRC, Inc., Cincinnati, OH). Real-time PCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Briefly, 1 μ g of total RNA was reverse transcribed with random hexamers in total volume of 20 μ l using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Tuscon, CA) according to the manufacturer's protocol. The cDNA was diluted to 100 μ l. The real-time PCR was carried out using SYBR® Green PCR Master Mix (Applied Biosystems) with 2 μ l of diluted cDNA in duplicates with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control. Real-time PCR was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curve analysis of PCR products confirmed the absence of secondary product. The primer sequences used for real-time PCR are listed in Table 1.

Western blot analysis

Confluent HDMVECs were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride). Protein concentration was quantified using the BCA protein assay kit (Pierce, Rockford, IL). Proteins or collected media were separated using SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 4% milk in TBST (Collagen, VE-cadherin, SMA, TGF β 2, GAPDH) or 2% gelatin in TBST (CTGF) at room temperature for 1 h. The blots were probed with primary antibody overnight at 4°C (Collagen, VE-cadherin, SMA, TGF β 2, GAPDH) or at room temperature (CTGF) at the dilution of 1:1,000. Following washes with TBST, blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody and developed using the ECL kit (ThermoScientific, Rockford, IL). The following primary antibodies were used: collagen type I (Southern Biotechnology, Birmingham, AL), CTGF, VE-cadherin (Santa Cruz Biotechnology), SMA (Sigma, St. Louis, MO) at a 1:1,000 dilution, and β -actin (Sigma) loading control primary monoclonal antibody at a dilution of 1:5,000. Proteins levels were quantified using Image J densitometry software.

ELISA

The Endothelin-1 EIA kit (Enzo Life Sciences, Plymouth Meeting, PA) was used to assess the protein level of ET-1 in the media collected from stimulated and unstimulated HDMVECs. The assay was used according to manufacturer's protocol. Briefly, the conditional media from HDMVECs were diluted 1:2. Hundred microliter of the diluted samples along with the provided standards were pipetted to the bottom of the appropriate wells on the ET-1 ELISA plate and incubated for 1 h at room temperature. After washing the plate, 100 μ l of diluted α -ET1 antibody was added into each well except the blank and the plate was incubated for 30 min at room temperature. After washing, 100 μ l of substrate solution was added into each well and incubated for an additional 30 min at room temperature. After blocking the reaction by addition of 100 μ l of stop solution, the optical density (OD) was measured at 450 nm wavelength and background was eliminated by subtracting the average blank OD from the average OD for each standard and sample (Net OD). The Net OD for each standard was then plotted versus the ET-1 concentration for each standard. The concentration of the unknowns was calculated by interpolation and presented on the graph.

Immunofluorescence

Cultured HDMECs grown on collagen-coated cover slips were treated with IFN- α , IFN- γ , TGF β 1, or TGF β 2 for 48 h. Control and treated cells were fixed with 4% paraformaldehyde for 15 min followed by incubation with 0.15 M Glycine for 30 min. Non-specific protein binding was blocked with 3% BSA for 1 h. Next, cells were incubated at 4°C overnight with primary antibodies: goat anti-mouse VE-cadherin (1:300; Santa Cruz, CA). After washing, tissue sections were incubated with an Alexa fluor 488 donkey anti-goat and Rhodamine Phalloidin (Invitrogen, Grand Island, NY) for 1.5 h. Cells were mounted on slides using Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and examined using a

FluoView FV10i confocal microscope system (Olympus, Center Valley, PA) at 488 nm (green), 594 nm (red), and 405 nm (blue).

EMT real-time PCR array

The RT² ProfilerTM PCR Array System (SABiosciences, Frederick, MD) was used according to the manufacturer's manual to screen for the IFN- γ -regulated genes involved in the process of EMT/ EndoMT. Briefly, total RNA was isolated using TRIzol reagent (MRC, Inc.) followed by an additional step of DNase I treatment to remove DNA contamination from the samples. Then 1 μ g of total RNA was reverse transcribed with random hexamers in a total volume of 20 μ l using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. The cDNA was diluted to 102 μ l. Real-time PCR was carried out using the SYBR[®] Green PCR Master Mix (Applied Biosystems) and was performed using the StepOnePlusTM Real-Time PCR System (Applied Biosystems). Real-time PCR was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Analyses were performed using the Web-Based RT² ProfilerTM PCR Array Data Analysis version 3.4 software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). The obtained results were further confirmed by real-time PCR with the primers listed in the Table 2.

Statistical analysis

Results were compared using one-way ANOVA with Bonferoni posthoc or Student's paired *t*-test. *P* 0.05 was considered statistically significant.

Results

IFN- γ decreases Fli1 and VE-cadherin mRNA expression in HDMVECs

In the initial experiment HDMVECs were treated with increasing doses (50, 100, 500, 1,000, and 2,000 U/ml) of IFN- α or IFN- γ for 24 h. The expression levels of the typical IFN-response genes, tetratricopeptide repeats 1 (IFIT1) and C-X-C motif chemokine 10 (CXCL10), also known as interferon gamma-induced protein 10 (IP-10) were examined. Stimulation with either IFN- α or IFN- γ resulted in dosedependent increases in IFIT1 and CXCL10 mRNA (Fig. 1A, C) with maximal increases obtained at the highest concentration of 2,000 U/ml (134- and 5,736-fold) when compared to unstimulated cells. To determine time course of interferon stimulation, HDMVECs were stimulated with IFN- α or IFN- γ (100 U/ml) for 30 min, 1, 4, 8, 24, 48, 72, 96, and 120 h (Fig. 1B, D). The treatment for shorter time points (30min, 1, 4, 8 h) induced interferon-response genes, but did not reveal significant changes in the profibrotic genes (data not shown). IFIT1 and CXCL10 mRNA expression was significantly increased at 24 h (75- and 25,083-fold), but declined at a later time points, more rapidly in IFN- γ -stimulated cells. To determine whether IFN- α or IFN- γ induce HDMVEC apoptosis, we employed the caspase-3/CPP32 colorimetric assay. We have not observed any pro-apoptotic effects after IFN- α or IFN- γ treatment at the doses and time points used in this study (data not shown).

We next examined the effects of IFN- α and IFN- γ on expression of Fli1 and its target gene, VE-cadherin. Whereas both IFN- α and IFN- γ significantly downregulated Fli1 and VE-

cadherin in a dose- and time-dependent manner, IFN- α was more potent in reducing Fli1 mRNA levels, while IFN- γ was more potent in reducing VE-cadherin levels (Fig. 2A, B). No significant reduction of Fli1 and VE-cadherin at the shorter time points (30min to 8h) was observed (data not shown). Fli1 expression was significantly decreased at the lowest concentration of 50 U/ml IFN- α , while VE-cadherin expression was significantly decreased at 100 U/ml of IFN- α (Fig. 2A, B). A higher dose of IFN- γ (1,000 U/ml) was required for the consistent down-regulation of Fli1, while VE-cadherin was significantly downregulated across all doses of IFN- γ (Fig. 2C, D). These results suggest that IFN- α and IFN- γ have somewhat different effects on Fli1 and VE-cadherin gene expression. IFN- α transiently downregulates Fli1 and VE-cadherin genes at low, as well as high doses, whereas the effects of IFN- γ are more persistent, but require a higher dose.

Distinct effects of IFN- α and IFN- γ on the profibrotic gene expression in HDMVECs

Previous studies have shown that Fli1 negatively regulates expression of CTGF/CCN2 in fibroblasts and endothelial cells (Nakerakanti et al., 2006). Since Fli1 was downregulated in response to IFN treatments, we examined mRNA and protein expression of CTGF and other profibrotic mediators in IFN stimulated cells. The 24 h treatment with different dosage of IFN- α and IFN- γ were chosen for the further experiments based on the results of Fli1 and VE-cadherin mRNA expression shown in Figure 2. Interferon- α (100 U/ml) significantly downregulated expression of ET-1, CTGF, and α SMA mRNA without affecting TGF β isoforms mRNA levels (Fig. 3A). On the other hand, IFN- γ (1,000 U/ml) significantly increased ET-1, CTGF, α SMA, and TGF β 2 mRNA levels, but the levels of TGF β 1 and TGF β 3 remained unchanged (Fig. 3B). Similar effects were observed with a lower dose of IFN- γ , 100 U/ml (data not shown). The effects of IFN- γ were confirmed on the protein levels using immunoblotting for Fli1, VE-cadherin, CTGF, and SMA (Fig. 3C) and ELISA for ET-1 (Fig. 3D). HDMVECs stimulated with TGF β were used as positive controls for Western blot. These results suggest that IFN- α and IFN- γ have both overlapping and distinct effects on expression of genes related to vascular remodeling in HDMVECs. Unexpectedly, IFN- γ upregulated profibrotic genes, suggesting that it might have an unappreciated role in vascular pathogenesis.

TGF β and ET-1 mediate the effects of IFN- γ in HDMVECs

In order to determine whether autocrine TGF β 2 is involved in regulation of the IFN- γ -induced genes, TGF β signaling was blocked using a specific inhibitor of TGF β RI kinase (SB431542) and an anti-TGF β 1/2/3 neutralizing antibody. TGF β -stimulated HDMVECs were used as a control in this experiment. Pretreatment of cells with the inhibitor or the neutralizing antibody resulted in the inhibition of α SMA, CTGF, ET1, and TGF β 2, and a reversal of the Fli1 and VE-cadherin downregulation when compared to the TGF β 1 treated cells (Fig. 4A). Because of its well-established role in the process of EMT and fibrosis TGF β 1 was used as a positive control. In IFN- γ treated cells, blockade of TGF β signaling reversed the upregulation of CTGF and ET-1, whereas expression of Fli1, VE-cadherin, α SMA, and TGF β 2 were not affected by the inhibitor treatment (Fig. 4B, D, E). These data suggest that TGF β 2 mediates upregulation of ET-1 and CTGF in response to IFN- γ in HDMVECs, whereas Fli1, VE-cadherin, and α SMA appear to be regulated in a TGF β independent manner.

In order to examine the possible autocrine effects of ET-1 on the profibrotic gene expression in HDMVECs, the cells were pretreated with the dual endothelin receptor types A and B antagonist, bosentan, before IFN- γ treatment. The blockade of ET-1 signaling completely reversed the effects of IFN- γ on the HDMVEC gene expression (Fig. 4C–E). In addition, bosentan downregulated expression of ET-1 below the basal level, suggesting an autocrine ET-1 loops in HDMVECs. These results suggest that ET-1 is a key mediator of the effects of IFN- γ on HDMVEC gene expression, while TGF β 2 is involved in regulation of selected genes, including ET-1.

IFN- γ -activated HDMVECs induce fibroblast matrix production in a paracrine fashion

We reasoned that the IFN- γ -mediated upregulation of the profibrotic mediators, TGF β and ET-1, could be involved in a paracrine interaction between endothelial cells and adjacent fibroblasts. To investigate this possibility, HDMVECs were stimulated with IFN- α (100 U/ml), IFN- γ (1,000 U/ml) and as a positive control TGF β 1 (1 ng/ml) for 12 h, followed by replacement with fresh EGM-2 media without growth factors (0.1% BSA) and incubation for an additional 36 h. The media was collected and added to confluent, serum starved foreskin fibroblasts for 24 h. The RNA isolated from fibroblasts was used for real-time PCR analysis of the profibrotic genes. Fibroblasts treated with conditioned medium (CM) from the IFN- γ - or TGF β 1-stimulated HDMVECs displayed significant increase in ET-1, CTGF, and α SMA, as well as COL1A1 and PAI1 mRNAs (Fig. 5A). The conditioned medium from the IFN- α -treated endothelial cells not only failed to induce profibrotic genes, but resulted in a modest, but significant downregulation of these genes, suggesting that IFN- α may induce antifibrotic mediators in HDMVECs. We next assessed whether CM collected from the IFN- γ treated cells induce collagen secretion. Consistent with the mRNA data, collagen was increased in the fibroblast media after treatment with CM from the IFN- γ - and TGF β 1-stimulated, but not with CM from the IFN- α -stimulated HDMVEC cell lines (Fig. 5B, CM 1–3). In a control experiment, fibroblasts were directly stimulated with IFN- α , IFN- γ , or TGF β 1. In agreement with the previously published studies, IFN- α and IFN- γ reduced, while TGF β 1 stimulated collagen secretion (Fig. 5B, bottom part). These results suggest that IFN- γ is a potent activator of endothelial cells that functions both in an autocrine and a paracrine manner.

IFN- γ induces EndoMT related changes in HDMVECs

The observed changes in profibrotic gene expression in the HDMVECs after IFN- γ treatment raised the possibility that IFN- γ could facilitate phenotypic changes consistent with endothelial-to-mesenchymal transition (EndoMT). To investigate it further, the focused EMT RT² ProfilerTM PCR Array System was performed with the samples from the IFN- γ -treated HDMVECs. In addition to the upregulation of TGF β 2, changes in other genes were detected and further confirmed by a real-time PCR (Fig. 6A). The following genes were significantly increased: fibronectin (FN) 1 (3.0-fold), TGF β 2 (3.0-fold), Smad7 (4.1-fold), secreted frizzled-related protein (Sfrp) 1 (5.6-fold), wingless-type MMTV integration site family member (Wnt) 5b (4.5-fold), signal transducer and activator of transcription (STAT) 3 (2.4-fold), Snail1 (2.3-fold) and a regulator of G-protein signaling (RGS) 2 (4.4-fold), while Sfrp2 and Wnt11 were significantly downregulated. Additionally, 48 h treatment with IFN- γ induced morphological changes in HDMVECs similar to those induced by TGF β 1.

The morphological transformation correlated with downregulation of VE-cadherin, as well as major changes in the actin cytoskeleton as revealed by phalloidin staining. Untreated HDMVECs exhibited a cortical actin staining below the cell membranes, whereas the TGF β 1-, and to a lesser degree IFN- γ -treated cells displayed elongated F-actin stress fibers (Fig. 6B).

In order to determine whether autocrine TGF β and/or ET-1 mediate the IFN- γ -induced EndoMT, TGF β , and ET-1 signaling was blocked by the respective inhibitors, SB431542 or bosentan (Fig. 6C). Pretreatment of HDMVECs with either inhibitor resulted in a complete inhibition of IFN- γ -induced Snail1 expression. However, upregulation of FN1 and Smad7 was only reversed by pretreatment with SB431542, but was not affected by pretreatment with bosentan, suggesting that these genes are TGF β -dependent. Additionally, the effects of IFN- γ on morphological changes in HDMVECs were visualized by immunocytochemistry (Fig. 6E). Since TGF β 2 was upregulated by IFN- γ in HDMVECs, we used TGF β 2 treated cells as a positive control (Fig. 6D). TGF β 2 induced downregulation of VE-cadherin and cytoskeletal changes similar to those induced by TGF β 1 and pretreatment with either SB431542 or bosentan almost completely reversed these changes. On the other hand, in cells treated with IFN- γ bosentan was more effective than SB431542 in reversing downregulation of VE-cadherin, as well as the morphological rearrangements of the actin filaments.

Discussion

IFN- γ is one of the central endogenous regulators of immunity and inflammation (Billiau and Matthys, 2009; Hu and Ivashkiv, 2009). It plays an important role in macrophage activation, regulation of Th1 cell response, anti-infectious host defense, and autoimmunity (Billiau and Matthys, 2009). IFN- γ also induces the immunomodulatory function of endothelial cells by upregulating expression of MHC class I and II molecules, surface adhesion molecules, and chemokines (Tellides and Pober, 2007). In the present study we showed that IFN- γ induces expression of the two key profibrotic mediators, ET-1 and TGF β 2, and their target genes α SMA, CTGF, Fli1, and VE-cadherin. In addition, IFN- γ -mediated activation of endothelial cells resulted in a secretion of profibrotic mediators capable of stimulating collagen production in fibroblasts. Relevant to this study, it has been recently shown that pulmonary microvascular endothelial cells isolated from bleomycin-treated rats stimulate collagen production in fibroblasts in a CTGF and TGF β 1-dependent manner (Yin et al., 2011). The factors contributing to the activation of endothelial cells in vivo are currently unknown. Since ET-1 upregulates its own expression, it is conceivable that the initial exposure of endothelial cells to IFN- γ or other immune mediators may result in persistent upregulation of ET-1 leading to a prolonged profibrotic response. Consistent with this notion we observed that unlike CXCL10 that was only transiently increased by IFN- γ , upregulation of the profibrotic genes were sustained for up to 120 h (the longest time point investigated). Together, this study underscores the complexity of the IFN- γ function in fibrosis. In addition to its known antifibrotic effects on fibroblasts in vitro and in wound healing in vivo (Gillery et al., 1992; Grassegger et al., 1998; Laato et al., 2001; Kalra et al., 2003), IFN- γ has indirect profibrotic effects via endothelial cell activation as shown in this study.

The effects of IFN- γ on expression of α SMA, CTGF, TGF β 2 and other genes are mediated primarily through upregulation of ET-1. ET-1 is a potent vasoconstrictor and one of the key regulators of vascular homeostasis. Under physiological conditions, ET-1 is produced in small amounts mainly by endothelial cells (ECs). However in pathophysiological conditions, its production is stimulated in a large number of different cell types, including endothelial cells, vascular SMCs, fibroblasts, and inflammatory cells such as macrophages and leukocytes. In addition to its main role as a vasoconstrictor, ET-1 also contributes to inflammation, as well as fibrosis during various pathophysiological processes. SSc patients have higher circulating levels of ET-1 (Sulli et al., 2009). Bosentan, a dual ET receptor antagonist has beneficial effects on SSc vascular disease and is being used in the management of digital ulcers and pulmonary arterial hypertension (Steen et al., 2009; Hachulla and Denton, 2010). TGF β 2 was among the genes induced by IFN- γ through upregulation of ET-1 in endothelial cells and contributed to the regulation of a subset of genes, including CTGF, Fli1, and VE-cadherin. Notably, TGF β 2 mRNA has been shown to co-localize with the pro- α 1(I) collagen mRNA around blood vessels in patients in the inflammatory stage of SSc, but not in patients in the fibrotic stage (Kulozik et al., 1990). However, the effect of IFN- γ on ET1 expression by endothelial cells is dependent on the cellular origin; thus IFN- γ had no effect on ET expression in bovine aortic endothelial cells (Lamas et al., 1992). Our study suggests that IFN- γ activated endothelial cells may be a source of TGF β 2 in these early inflammatory lesions.

In addition to its immunomodulatory function, IFN- γ increases endothelial permeability through a RhoA-ROCK-dependent actin reorganization and increased endocytosis of tight junction components such as claudins and occludins (Capaldo and Nusrat, 2009). This process is enhanced during inflammatory conditions by a synergistic interaction of IFN- γ and TNF- α . We observed that IFN- γ downregulated expression of VE-cadherin, a key component of adherens junctions, which is consistent with the role of IFN- γ in vascular permeability. Interestingly, it has been reported that IFN- β blocked IFN- γ -induced downregulation of occludin and VE-cadherin in HUVECs (Minagar et al., 2003). In contrast, we have observed that IFN- α downregulated VE-cadherin in HDMVECs, possibly reflecting either differences between IFN- α and IFN- β or a different response between HUVECs and HDMVECs. Furthermore, IFN- α significantly decreased expression of Fli1 even at a very low concentration, suggesting that IFN- α may be responsible for the reduced expression of Fli1 in SSc microvessels in vivo (Kubo et al., 2003). IFN- γ indirectly contributes to Fli1 downregulation via the ET-1 and TGF β 2-dependent pathways.

The process of EndoMT has been gaining recognition as one of the mechanisms contributing to generation of activated fibroblasts/myofibroblasts in various fibrotic diseases (Piera-Velazquez et al., 2011). TGF β is one of the most widely studied inducers of EndoMT (Li and Jimenez, 2011), however other factors, especially ET-1 (Widyantoro et al., 2010), as well as some inflammatory factors have also been shown to induce this process in vitro and in animal models (Rieder et al., 2011). Interestingly, it has been shown that IFN- γ synergistically with TGF β increases the expression level of FN1 in human dermal fibroblasts, while inhibits the TGF β -induced expression of collagen (Varga et al., 1990). We have also observed induction of Snail1 and the morphological changes consistent with EndoMT in cells treated with IFN- γ , which were mediated by the autocrine ET-1 and

TGF β 2. Similarly, TGF β 2 has been shown to induce endothelial-to-mesenchymal transition and acquisition of a stem cell-like phenotype in vascular endothelial cells in an ALK2-dependent manner (Medici et al., 2010). Interestingly, IFN- γ also modulated the expression of the non-canonical Wnt ligands, Wnt11, and Wnt5b, and upregulated expression of the secreted frizzled-related protein 1 (Sfrp1), which sequesters both non-canonical and canonical Wnt ligands in the extracellular space. In comparison to canonical Wnt signaling, much less is known about non-canonical Wnt signaling, which has been studied primarily in development (Sugimura and Li, 2010). Together, these data suggest that IFN- γ may also contribute to the process of EndoMT, however more studies are needed to confirm this possibility.

Endothelial cell injury is an initiating event in the pathogenesis of SSc (Kahaleh, 2008). Endothelial cell apoptosis has been observed in the early stages of the disease, but this finding has not been consistent (Fleming et al., 2008). This study suggests that endothelial cells actively participate in the disease pathogenesis, not only as facilitators of the inflammatory process, but also as initiators of the fibrotic response. We show that exposure of endothelial cells to IFN- γ stimulates production of the well-known profibrotic mediators ET-1, CTGF and TGF β 2 that can induce transition of endothelial cells to myofibroblasts and activate adjacent pericytes/SMCs and fibroblasts. Interestingly, IFN- γ has been implicated in cardiac allograft vasculopathy, which is characterized by a concentric intra-luminal thickening of the intima of the coronary arteries, indistinguishable from the intimal thickening in patients with SSc (Tellides and Pober, 2007). In summary, this study suggests that IFN- γ may play an important role as a mediator of the crosstalk between immune and endothelial cells during pathological vascular remodeling in SSc and may represent an attractive therapeutic target.

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Fig. 1.

IFN- α and IFN- γ up-regulate the specific target genes in a dose- and time-dependent manner in HDMVECs. HDMVECs were stimulated with either IFN- α (A, B) or IFN- γ (C, D) at different dosage (50, 100, 500, 1, 000, and 2,000 U/ml) for 24 h (A, C) or at the concentration of 100 U/ml for different timepoints (24, 48, 72, 96, and 120 h) (B, D). mRNA expression was analyzed by real-time PCR. Values represent mean $\pm$ SEM from three independent cell lines. * $P < 0.05$

Fig. 2.

IFN- γ decreases Fli1 and VE-cadherin mRNA expression in HDMVECs. HDMVECs were stimulated with either IFN- α (A, B) or IFN- γ (C, D) at different dosage (50, 100, 500, 1,000, and 2,000U/ml) for 24 h (A, C) or at the concentration of 100 U/ml for different timepoints (24, 48, 72, 96, and 120 h) (B, D). Fli1 and VE-cadherin mRNA expression was analyzed by real-time PCR. Values are represented as mean $\pm$ SEM (n=3). * P < 0.05

Fig. 3.

Distinct effects of IFN- α and IFN- γ on the profibrotic gene expression in HDMVECs. HDMVECs were stimulated with 100 U/ml of IFN- α (A) or 1,000 U/ml of IFN- γ (B), or left untreated (UT) for 24 h. The mRNA expression was determined by real-time PCR. Values are represented as mean $\pm$ SEM ($n=5$). * $P < 0.05$. Cell lysates were analyzed for the indicated proteins by Western blotting with GAPDH used as a loading control (C). The Western blot images are representative of the three independent experiments. ET-1 was measured by ELISA (D).

Fig. 4.

TGFβ and ET-1 mediate the profibrotic effects of IFN-γ in HDMVECs. A, B: Human dermal microvascular endothelial cells were pretreated for 1 h with either the α-TGFβ1/2/3 neutralizing antibody (α-T) or the inhibitor of TGFβ type I receptor (ALK5) (SB431542) at the concentration of 2.5 μg/ml and 20 μM, respectively, followed by the stimulation with IFN-γ(1,000 U/ml) or TGFβ1 (1 ng/ml) for 24 h. Fli1, VE-cadherin, SMA, CTGF, ET-1, and TGFβ2 gene expression was analyzed by real-time PCR. C: HDMVECs were pretreated for 3 h with dual endothelin receptor antagonist (Bosentan) at the concentration of 10 μM, followed by the stimulation with IFN-γ(1,000 U/ml) for 24 h, or left untreated (UT). Fli1, VE-cadherin, SMA, CTGF, ET-1, and TGFβ2 gene expression was analyzed by real-time

PCR. Values represent mean $\pm$ SEM (n =6 or n =3). * P < 0.05. D: Western blot images are representative of the three independent experiments. E: Average protein was calculated using ImageJ densitometry software. Values represent mean $\pm$ SEM (n =3). # P < 0.05 when compared to UT. * P < 0.05 when compared SB +TGF β 1 to TGF β 1. ** P < 0.05 when compared SB +IFN- γ and Bos +IFN- γ to IFN- γ .

Fig. 5.

IFN- γ -activated HDMVECs induce matrix production in fibroblasts. HDMVECs were stimulated with IFN- α (100 U/ml), IFN- γ (1,000 U/ml), or TGF β 1 (1 ng/ml) for 12 h, followed by media change and incubation in the fresh starvation media (0.1% BSA) for 36 h. The conditioned media from three independent HDMVECs (CM 1–3) were collected and added to the confluent human dermal fibroblasts for 24 h. A: The mRNA expression of the indicated profibrotic genes was assessed in stimulated fibroblasts by real-time PCR. Values represent mean $\pm$ SEM. * P < 0.05 (n = 3). B: Collagen secretion in fibroblasts stimulated with CM 1–3 was examined by Western blotting. Bottom part (ctrl) shows collagen secretion in fibroblasts stimulated directly with IFN- α (100 U/ml), IFN- γ (1,000 U/ml), or TGF β 1 (1 ng/ml) for 24 h.

Fig. 6.

IFN- γ regulates selected genes involved in the process of EndoMT via upregulation of ET-1 and TGF β 2 signaling pathway (A) cDNA obtained from the IFN- γ -stimulated HDMVECs (1,000 U/ml) for 24 h was subjected to the EMT Profiler PCR Array System (SABiosciences). Gene validation was performed by real-time PCR. Values represent mean $\pm$ SEM from three experiments. * P 0.05. B: Immunofluorescence staining of VE-cadherin (green) and Phalloidin-F-actin (red) in HDMVECs treated for 48 h with 1,000 U/ml of IFN- γ , 1 ng/ml of TGF β 1 or left untreated (UT). Representative photographs are shown from three experiments. C: HDMVECs were pretreated for 1 h with either ALK5 kinase inhibitor (SB431542) or dual endothelin receptor antagonist (Bosentan) at the concentration of 2.5 μ M, 20 μ M, and 10 μ M, respectively, followed by the stimulation with IFN- γ (1,000 U/ml) or TGF β 1 (1 ng/ml) for 24 h. Selected EMT-specific genes were analyzed by real-time PCR. Values represent mean $\pm$ SEM (n=3–6). # P 0.05 when compared to UT. * P 0.05 when compared SB +TGF β 1 to TGF β 1. ** P 0.05 when compared SB +IFN- γ and Bos +IFN- γ to IFN- γ . D, E: Immunofluorescence staining of VE-cadherin (green) and Phalloidin-F-actin (red) in HDMVECs pretreated for 1 h with SB431542 or bosentan at the concentration of 20 μ M and 10 μ M, respectively, followed by the stimulation with IFN- γ (1,000 U/ml) or TGF β 2 (1 ng/ml) for 48 h or left untreated (UT).

TABLE 1

Human primers used for real-time PCR

	Forward 5' to 3'	Reverse 5' to 3'
IFIT1	gcctaatttacagcaacatga	tcatcaatggataactcccatgt
CXCL10	gaaagcagttagcaaggaaagg	gacataactccatgtagggaagtg
Fli1	ggatggcaaggaaactgtgtaa	ggttgtattataggccagcagtg
Ve-cadherin	aagcctctgattggcacagt	ctggccctgtcactggt
SMA	ccgaccgaatgcagaagga	acagagtatttcgcgccgaa
CTGF	ttgcgaagctgacctggaagagaa	agctcggtatgtcttcactggt
ET-1	gtcgtccctgatggataaaa	ccatacgaacaacgtgct
TGFβ1	gcagcacgtggagctgta	cagccggttgctgaggta
TGFβ2	ccttcttcccctccgaaac	agagcacctgggactgtctg
TGFβ3	aagaagcgggctttggac	cgcacacagcagttctcc
Col1a1	ccagaagaactgtacatcagca	cgccatactcgaactggaat
PAI1	gagaaaccagcagcagatt	tggtgctgatctcatccttg

TABLE 2

Human primers used to confirm the RT² ProfilerTM PCR Array by real-time PCR

	Forward 5' to 3'	Reverse 5' to 3'
Col1a2	gatgttgaactgttctgagg	tcttccccattcattgtctt
Col5a2	aggggagagaggtgaaact	tggcaccaggagtagccatc
Twist1	cggccaggtacatcgact	catcttgaggtccagctcgt
FN1	ctggccgaaaatacattgtaa	ccacagtcgggtcaggag
Smad7	agggggaacgaattatctgg	tagagcctccactctcg
FZD1	ctgtttatcggcacgtcctt	ccatgagcttctcagcttc
FZD7	gccagcttggtcctaataaga	agccgggagaaactcacag
Sfrp1	tcttctctgctcgtcttc	ctcggggaactgtcacact
Sfrp2	acggcatcgaataccagaac	gaagagcagcacagggaact
Wnt2	ctccctctgcttgcactg	gcacattatcgacatcacc
Wnt4	gctgtgacaggacagtgcac	gcctcattgtgtggagggt
Wnt9a	tgagaagaactgcgagagca	ctccacatagcagcaccaac
Wnt11	agctcgcccccaactatt	atacacgaaggccgactcc
Wnt5a	attgtactgcaggtgtacctaataac	ccccctataaatgcaactgttc
Wnt5b	ctgctgctgctgttcacg	caccgggtcaaagctaag
Stat3	cccttggttgagagtcaaga	aagcggctatactgctggtc
Snail1	gctgcaggactctaaccaga	atctccggaggtgggatg
RGS2	gaaaaggagctccaaagaga	ttctgggcagttgtaaagca
Ocln	aggaaccgagagccaggt	ggatgagcaatgcccttag
GAPDH	ggtctcctctgacttaaca	agccaaattcggtgtcctac