EXTENDED REPORT

Downregulation of miR-193b in systemic sclerosis regulates the proliferative vasculopathy by urokinase-type plasminogen activator expression

Naoki Iwamoto,1,2 Serena Vettori,1 Britta Maurer,1 Matthias Brock,1 Elena Pachera,1 Astrid Jüngel,1 Maurizio Calcagni,2 Renate E Gay,1 Michael L Whitfield,4 Jörg H W Distler,5 Steffen Gay,1 Oliver Distler1

Handling editor Tore K Kvien

Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2014-205326).

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterised by fibrosis of the skin and internal organs and by a widespread vasculopathy associated with high morbidity and mortality.1 In early stages of the disease, microvascular changes such as capillary dilatation with subsequent rarefaction occur.2 3 In addition to capillary rarefaction, another feature of the SSc vasculopathy is intimal hyperplasia of small arteries, which results from increased proliferation and decreased apoptosis of vascular smooth muscle cells (VSMCs).4 5 Together, these vascular changes reduce blood flow and contribute to life-threatening clinical manifestations such as pulmonary arterial hypertension.

Although the detailed pathogenesis of the SSc vasculopathy still remains unclear, several key pathways have been identified. One such pathway is the urokinase-type plasminogen activator (uPA) and the cellular receptor for uPA (uPAR) pathway. uPA is a serine protease that initiates the conversion of plasminogen to plasmin, and it is also involved in the activation of metalloproteinase and other proteins by enzymatic cleavage of precursors. uPA can trigger cellular responses such as cell migration, differentiation and proliferation on several cell types via uPA-uPAR dependent and uPAR-independent pathways.6–9 Moreover, there is evidence that uPA contributes to neointimal growth and vascular remodelling.9–11

Recent key studies have shown that the uPA–uPAR pathway is impaired in SSc. Matrix metalloproteinase-12, which is overproduced by SSc microvascular endothelial cells (MVEC) and SSc fibroblasts, cleaves uPAR on the surface of MVEC, and this modification contributes to the impaired angiogenesis of SSc.12 13 Accordingly, uPAR−/− mice develop increased apoptosis in endothelial cells in the dermis.14 MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression post-transcriptionally.15 16 miRNAs repress protein synthesis by destabilising target mRNAs or inhibiting mRNA translation through base-pairing with 3′ untranslated region of target mRNAs.17 There is growing evidence that the deregulation of miRNA is implicated in the pathogenesis of human diseases.18–20 and miRNAs are thought to represent novel disease biomarkers and potential therapeutic targets.21 Altered expression of miRNAs has also been reported in human autoimmune diseases including SSc.22–24 However, the role of miRNAs in the pathogenesis of vasculopathy in SSc has not been addressed.

In the present study, we found that microRNA-193b-3p (mIR-193b) was downregulated in cultured fibroblasts and skin biopsies of patients with SSc. Furthermore, we showed that the downregulation of miR-193b results in an overexpression of its target uPA. Increased levels of uPA...
contributed to the proliferative vasculopathy of SSc by inhibiting apoptosis and inducing proliferation of VSMCs in an uPAR-independent manner.

MATERIALS AND METHODS

More detailed information on methods is provided in the online data supplement.

Patients, biopsy specimens and cell culture

Skin biopsy and skin fibroblasts were obtained from patients with SSc (n=33, 5 diffuse SSc, 28 limited SSc) and normal healthy (NH) donors (n=25). All patients fulfilled the criteria for SSc as described by LeRoy et al.23 The study was approved by the local ethics committees. Primary human pulmonary artery smooth muscle cells (HPASMCs) were purchased from Invitrogen (Basel, Switzerland).

Reagents and stimulation assays

Dermal fibroblasts and/or HPASMCs were stimulated with transforming growth factor-β (TGF-β), platelet-derived growth factor B (PDGF-B), interleukin 1 β (IL-1 β) (all R&D Systems, Abingdon, UK), high molecular weight uPA (HMW-uPA) (Loxo, Dossenheim, Germany), low molecular weight uPA (LMW-uPA) (Loxo), tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (R&D Systems) or neutralising monoclonal antibodies against human uPAR (American Diagnostica, Pfungstadt, Germany). Carrier solution of stimulants was added at a similar volume for control experiments. Exposure to hypoxia was done by the local ethics committees. Primary human pulmonary artery smooth muscle cells (HPASMCs) were purchased from Invitrogen (Basel, Switzerland).

Transfection experiments

Dermal fibroblasts or HPASMCs were transfected with synthetic precursor miRNA (pre-miR), with inhibitors of miR-193b (anti-miR), or with scrambled controls (pre-miR/anti-miR negative control #1; Ambion/Applied Biosystems, Rotkreuz, Switzerland) with the use of Lipofectamine 2000 reagent (Invitrogen). Transfection efficiency was controlled by TaqMan real-time PCR.

Fluorescence activated cell sorting (FACS) analysis

For analysis of apoptosis, cells were detached with accutase and stained for annexin V and propidium iodide (PI) with the annexin V-FUOS staining kit (Roche, Mannheim, Germany). Cells were subsequently analysed on the FACSCalibur flow cytometer. Apoptotic cells were defined as PI-negative (indicating an intact plasma membrane) and annexin V-fluorescein isothiocyanate (FITC)-positive.27

RNA isolation and quantitative real-time PCR analysis

RNA was isolated from fibroblasts and fresh frozen skin tissue with the mirVana Isolation Kit (Ambion/Applied Biosystems) and from paraffin embedded sections with Recover All Total Nucleic Acid Isolation Kit (Ambion/Applied Biosystems). Reverse transcription and TaqMan real-time PCRs (for miRNAs) or SYBR green real-time PCR were performed as described.24

Western blot analysis, cell proliferation assay by WST-1 and immunohistochemistry

Western blots were performed using polyclonal antibodies against uPA (Abcam, Cambridge, UK), monoclonal antibodies against proliferating cell nuclear antigen (PCNA) (Imgenex, San Diego, California, USA) or monoclonal antibodies against α-tubulin (Abcam) according to standard protocols.29 Cell proliferation was measured using water-soluble tetrazolium salt (WST-1)/electro coupling solution (Abcam) as recommended by the manufacturer. Immunohistochemistry was performed using polyclonal rabbit anti-human uPA antibodies (Abcam), and monoclonal mouse antibodies against α-smooth muscle actin (α-SMA) (Sigma-Aldrich, Buchs, Switzerland). Irrelevant isotype-matched IgGs were used as controls. Staining was visualised with 3-amino-9-ethylcarbazole, diaminobenzidine or by fast blue using an alkaline phosphatase substrate kit (all from Vector, Burlingame, USA).

Statistical analysis

Data are expressed as mean and SEM. Normal distribution of data was confirmed using the Kolmogorov-Smirnov test. Statistical significance was evaluated by Student’s paired and unpaired t test. For non-parametric data, the Mann-Whitney U test and Wilcoxon matched-pairs signed rank test were used. p<0.05 was considered statistically significant.

RESULTS

Downregulation of miR-193b in SSc

In our preliminary experiments using the TaqMan Human MicroRNA Array (Ambion/Applied Biosystems), we found that miR-193b was one of the most significantly and most consistently downregulated miRs in dermal SSc fibroblasts compared with NH fibroblasts (not shown). To validate the microarray findings, quantitative real-time PCR with additional cultured fibroblasts was performed. In agreement with the array data, there was a significant difference in the expression of miR-193b between SSc fibroblasts and healthy control fibroblasts. As shown in figure 1A, the baseline levels of miR-193b were consistently downregulated in SSc fibroblasts to 31±9.6% (p<0.001).

Next, we investigated the expression of miR-193b in skin samples from patients with SSc. First, the expression of the miR-193b in tissue extracts isolated from paraffin-embedded skin sections was analysed, and similar to the findings in SSc fibroblasts, decreased levels of the miR-193b were observed. MiR-193b was downregulated in the dermis of patients with SSc as compared with healthy controls to 54±7% (p<0.01, figure 1B).

To exclude the possibility that paraffin processing influenced the content of miRNA in the tissue, we additionally investigated the expression of the miR-193b in fresh frozen skin biopsy samples. Consistent with the previous results, miR-193b was found to be downregulated to 62±20% (p=0.087) in patients with SSc as compared with healthy controls (figure 1C).

MiRs from the same cluster often show overlapping regulations and downstream effects. We therefore analysed the baseline levels of miR-365, which is the other member of the miR-193b-365 cluster.39 MiR-365 was also downregulated in cultured SSc fibroblasts, while no differences were detected in fresh frozen and paraffin embedded skin biopsies (not shown).

Downregulation of miR-193b is independent of profibrotic cytokines and hypoxia

To explore the factors that potentially downregulate the levels of miR-193b in SSc, we analysed the effects of major profibrotic cytokines and hypoxia. We first stimulated dermal SSc and NH fibroblasts with key profibrotic cytokines including IL-1β, TGF-β and PDGF-B for various time points (12 h, 24 h, 48 h and 5 days) and at different concentrations considered physiological in humans. None of these stimuli had any influence on the expression of miR-193b (see online supplementary figure S1). Similarly, exposure to hypoxia, which is thought to independently contribute to the progression of fibrosis,40 had no consistent effects on the expression of miR-193b compared with...
Expression of miR-193b in samples of patients with SSc was determined relative to healthy controls, which was defined as 100%. (A) miR-193b was markedly reduced in cultured SSc skin fibroblasts (n=12) as compared with healthy controls (n=6). (B) Similar to the in vitro findings, the expression of miR-193b in paraffin-embedded skin sections from patients with SSc (n=5) was downregulated as compared with healthy controls (n=5). (C) Expression of miR-193b in snap frozen skin biopsy samples from patients with SSc (n=9) was downregulated as compared with healthy controls (n=5). Values are the mean and SEM. *p<0.05 versus healthy controls.

Figure 1  Expression of microRNA-193b (miR-193b) in patients with systemic sclerosis (SSc) and healthy controls, as determined by TaqMan-based real-time PCR.

Overproduction of uPA in SSc
Next, we investigated whether uPA is upregulated in SSc. First, we compared the basal expression of uPA in SSc fibroblasts with healthy control fibroblasts. Baseline levels of uPA mRNA were not significantly changed compared with healthy control fibroblasts (3.58±1.58-fold; p=0.43). On the protein level, basal levels of uPA were slightly higher than in healthy controls without reaching statistical significance (n=5, 1.76±0.41-fold, p=0.14) (figure 3B). To simulate the profibrotic conditions present in SSc in vivo, fibroblasts were stimulated with TGF-β. Indeed, in TGF-β-stimulated fibroblasts, levels of uPA mRNA were strongly increased in SSc fibroblasts as compared with healthy control fibroblasts (8.5±3.91-fold, p<0.05, figure 3A).

We next analysed the expression of uPA using a recently published microarray data set derived from skin biopsies from distinct scleroderma subsets including 17 patients with diffuse SSc, 7 patients with limited SSc, 3 patients with morphea and 6 healthy controls.31 Analysis of this microarray revealed increased expression of uPA mRNA in the inflammatory subset of SSc, while it was expressed at low levels in normal-like subset that included all healthy controls (average fold change 2.59±0.47; p=0.000146 by t test). Taken together, these data show that there is an overexpression of uPA in SSc, which is related to TGF-β.

Localisation of uPA expression in human SSc skin sections
We next investigated the in situ expression of uPA in SSc skin biopsies by immunohistochemistry. Weak expression of uPA protein was seen in fibroblast-like cells, but pronounced expression of uPA protein was found in vascular structures (figure 4A). To confirm that uPA was preferentially expressed in vessels, we performed double staining with uPA and the VSMC marker α-SMA. Double staining showed that uPA-positive cells were double stained with α-SMA, suggesting that uPA is preferentially expressed by VSMCs in small arteries in SSc (figure 4B).
uPA induces proliferation and suppresses apoptosis on HPASMC

The localisation of uPA in SSc skin sections suggested that uPA might play a critical role in the vascular pathology of SSc. To assess whether uPA contributes to the proliferative vasculopathy of SSc, we first examined the proliferative effects of uPA on HPASMCs by WST-1 assay and PCNA protein expression. Cells were stimulated with various concentrations of HMW-uPA for 24 h and cell viability was assessed by using the WST-1 method, an indicator of active cell metabolism. HMW-uPA increased the proliferation of HPASMC by 21.75±7.5% (10 nM, p<0.004, figure 5A). This finding was confirmed by the observed expression of PCNA protein, another marker of proliferation. Consistent with the WST-1 assay, PCNA expression was increased by HMW-uPA (1.5±0.16-fold, p<0.03, figure 5B).

Because the proliferative vasculopathy of SSc is characterised by enhanced proliferation, and suppressed apoptosis of VSMCs, we next assessed the effect of uPA on apoptosis. To detect apoptosis, annexin V-PI double staining was performed and analysed by flow cytometry. As shown in figure 5C, HPASMC stimulated with 5 nM, 10 nM, 25 nM of HMW-uPA showed a decrease of 17%, 33%, 21% in the number of apoptotic cells, respectively (p<0.0001, 10 nM stimulation experiment). We also examined the effect of uPA on TRAIL-induced apoptosis. Similar to spontaneous apoptosis, stimulation with 5 nM, 10 nM, 25 nM of HMW-uPA in combination with TRAIL decreased the number of apoptotic cells by 17%, 48% and 18%, respectively (p<0.02, 10 nM stimulation experiment).

These results suggest that overproduction of uPA in SSc increases the number of VSMCs via suppression of apoptosis and increase of proliferation resulting in hyperplasia of VSMCs characteristic for the proliferative vasculopathy in SSc.

uPA suppressed apoptosis of HPASMC via uPA receptor-independent pathways

In SSc, receptor signalling via uPAR is impaired because of cleavage of uPAR by overproduction of matrix metalloproteinase-12. Thus, we next wanted to investigate whether the observed antiapoptotic effects of uPA on HPASMC are independent of uPAR. First, we performed blocking experiments for uPAR using uPAR neutralising antibodies. Indeed, uPA inhibited spontaneous and TRAIL-induced apoptosis even in the presence of uPAR neutralising antibodies (figure 6A). To confirm these results with an independent method, we stimulated HPASMC with LMW-uPA, which in contrast to HMW-uPA lacks the amino-terminal fragment and therefore does not bind to uPAR. LMW-uPA inhibited spontaneous apoptosis as well as TRAIL-induced apoptosis on HPASMC to a similar extent as
with HMW-uPA (figure 6B). These results suggest that uPA inhibits apoptosis in HPASMC via uPA-uPAR independent pathways.

**DISCUSSION**

Our study suggests a crucial role of miR-193b in the pathophysiology of SSc (see online supplementary figure S3). We have shown that the expression of miR-193b is decreased in SSc compared with NH controls, and that downregulation of miR-193b leads to increased production of uPA. Because (1) uPA was strongly expressed by VSMCs, (2) VSMCs are cellular key players in the pathophysiology of SSc-pulmonary arterial hypertension and (3) uPA has been described as a proliferative factor in several cell types, we next focused on the role of uPA on HPASMC. We could show that uPA is involved in the pathophysiology of proliferative vasculopathy via induction of cell proliferation and inhibition of apoptosis. These results suggest that uPA is an important player in the pathophysiology of the proliferative vasculopathy in SSc.

There is growing evidence that uPA is involved in the pathophysiology of SSc. Landmark studies by D’Alessio et al showed that the constitutive overproduction and secretion of matrix metalloproteinase-12 by SSc dermal MVECs and fibroblasts cause endothelial uPAR cleavage resulting in impaired angiogenesis. These findings suggest that the uPA-uPAR pathway is impaired in SSc. Thus, the increased levels of uPA mediated by decreased levels of miR-193b found in our study must exert their effects on the intimal hyperplasia mainly via uPAR-independent pathways. Indeed, our uPAR blocking experiments using uPAR neutralising antibodies, siRNA against uPAR as well as LMW-uPA demonstrated that uPAR-independent pathways play a major role in the ant apoptotic effects of uPA on HPASMCs. This creates a novel, complex dual role for uPA in the pathophysiology of SSc with (1) impaired angiogenesis and increased apoptosis due to the lack of functional uPAR on MVEC and (2) proliferation and decreased apoptosis of VSMCs leading to intimal hyperplasia via uPAR-independent pathways.

The increased expression of uPA in SSc, which we found in the present study, is also supported by other studies. Recently, increased secretion of uPA protein from limited

**Figure 3** Expression of urokinase-type plasminogen activator (uPA) in dermal fibroblasts from systemic sclerosis (SSc) skin and healthy controls. Expression of uPA in SSc fibroblasts was determined relative to the healthy controls, which was defined as 1. (A) On the mRNA level, expression of uPA was slightly, but not significantly increased in SSc skin fibroblasts (n=9) in comparison with healthy controls (n=6), as determined by SYBR green real-time PCR. However, after stimulation with transforming growth factor-β (TGF-β), the expression of uPA was markedly increased in SSc skin fibroblasts (n=10) as compared with healthy controls (n=6). (B) On the protein level, expression of uPA was slightly increased in SSc skin fibroblasts (n=5) as compared with healthy controls (n=4), as determined by western blotting (top), confirming the results from the mRNA analysis. Two representative experiments are shown. The semiquantitative analysis was performed by densitometry (bottom). Values are given as the means ±SEM. In the western blots, α-tubulin was used as loading control. *p<0.05 versus healthy controls.

**Figure 4** Urokinase-type plasminogen activator (uPA) expression in the skin of patients with systemic sclerosis (SSc). (A) Representative sections of SSc skin stained for uPA or IgG control. Positive staining of uPA appears as light brown colour. Expression is seen in vascular structures (arrows). Sections were counterstained with eosin. (B) SSc skin section double-stained for uPA (purple) and α-smooth muscle actin (α-SMA) (blue) or IgG control. Co-staining of uPA and α-SMA shows expression of uPA in vascular smooth muscle cells (arrows). One representative section is shown (n=5). Original magnification ×400.
Figure 5  Urokinase-type plasminogen activator (uPA) increased the proliferation and decreases apoptosis of human pulmonary artery smooth muscle cells (HPASMCs). (A) HPASMCs were stimulated with 5 nM, 10 nM, 25 nM high molecular weight uPA (HMW-uPA) for 24 h, and proliferation was assessed by water-soluble tetrazolium salt (WST)-1 assay. Values are reported in percentages of increase of cell viability as compared with unstimulated controls considered as 0%. Values are given as means±SEM. Results are from at least five independent experiments. (B) HPASMCs were stimulated with HMW-uPA at 10 nM for 24 h and proliferating cell nuclear antigen (PCNA) protein was determined by western blotting (top). Representative results are shown. The semiquantitative analysis was performed by densitometry (bottom). Values are given as means±SEM. In the western blots, α-tubulin was used as loading control. Expression of PCNA in stimulated samples was determined relative to the unstimulated controls, which were defined as 1. Results are from at least five independent experiments. *p<0.05 versus unstimulated controls. (C) HPASMCs were stimulated with several doses of HMW-uPA with or without TNF-related apoptosis-inducing ligand (TRAIL) (20 ng/mL) for 24 h. Flow cytometry was used to measure levels of propidium iodide (PI)-negative and annexin V-FITC-positive cells relative to controls. Apoptotic cells were defined as PI-negative (indicating an intact plasma membrane) and annexin V-FITC-positive. Values are given as relative changes in the number of PI-negative and annexin V-FITC-positive cells compared with controls. Values are given as means±SEM. Results are from at least five independent experiments. *p<0.05 versus respective controls.

Figure 6  Urokinase-type plasminogen activator (uPA) suppressed apoptosis in human pulmonary artery smooth muscle cells (HPASMCs) via uPA receptor (uPAR) independent pathways. (A) HPASMCs were stimulated with high molecular weight uPA (HMW-uPA) (10 nM), with or without TNF-related apoptosis-inducing ligand (TRAIL) (20 ng/mL) and neutralising monoclonal antibody against human uPAR (1.5 µg/mL) for 24 h. (B) HPASMCs were stimulated with or without TRAIL (20 ng/mL) and low molecular weight uPA (LMW-uPA) (10 nM) or HMW-uPA (10 nM) for 24 h. Flow cytometry was used to measure levels of propidium iodide (PI)-negative and annexin V-FITC-positive cells relative to control. Apoptotic cells were defined as PI-negative (indicating an intact plasma membrane) and annexin V-FITC-positive. Values are given as relative changes in the number of PI-negative and annexin V-FITC-positive cells compared with unstimulated control. Values are shown as means±SEM. Results are from at least five independent experiments. *p<0.05 versus respective controls.
cutaneous SSc fibroblasts compared with normal fibroblasts has been reported.36 We could also establish another link between TGF-β and vascular changes in SSc, as TGF-β strongly enhanced miRNAs and the proliferative vasculopathy in controls.

TGF-β and vascular changes in SSc, as TGF-β strongly enhanced miRNAs are often disease-specific and do not play major roles under normal physiological conditions in adult tissues or in quiescent cells.41 In fact, miRNA knockout mice often don’t display a specific phenotype, unless they are challenged with a stimulus causing cellular stress in target organs.42 Our main hypothesis derived from the present study is that increasing levels of miR-193b as a post-transcriptional regulator of uPA contributes to the proliferative vasculopathy characteristic for SSc. Therefore, strategies to maintain the expression or to prevent the repression of miR-193b have the potential to become a possible therapeutic strategy for the proliferative vasculopathy in SSc.

In conclusion, our study showed that downregulation of miR-193b in SSc leads to increased production of uPA, which is a key player in the pathogenesis of SSc. uPA increases the number of pulmonary artery smooth muscle cells and thereby contributes to the proliferative vasculopathy characteristic for SSc. Therefore, strategies to maintain the expression or to prevent the repression of miR-193b have the potential to become a possible therapeutic strategy for the proliferative vasculopathy in SSc.

Acknowledgements The authors thank Maria Comazzi for the excellent technical support.

Contributors NI: conception and design of the study, analysis and interpretation of data. JH: conception and design, microRNA microarray experiment. MLW: analysis gene expression microarray data. MC: supply clinical samples. SV, BM, MB, AJ, MC, REG, NI, OD: conception and design of the study, analysis and interpretation of data. All authors contributed to the writing of the manuscript. We declare that all authors listed contributed to the work.

Funding This study was supported by an Orphan Disease Program Grant from the European League Against Rheumatism (EULAR).

Competing interests OD has/had consultancy relationship and/or has received research funding in the area of systemic sclerosis and related conditions from Actelion, Pfizer, Ergonex, BMS, Sanoﬁ-Aventis, United BioSource Corporation, Roche/Genentech, Medac, Biovitrum, Boehringer Ingelheim Pharma, Novartis, 4D Science, Active Biotech, Bayer-Schering, Sinoxa, Serodapharm, Epipharm, GSK and Biogen, JHW has consultancy relationships and/or has received research funding from Actelion, Pfizer, Ergonex, BMS, Celgene, Bayer Pharma, Boehringer Ingelheim, JI Therapeutics, Sanoﬁ-Aventis, Novartis, Array Biopharma, KaroBio and Active Biotech in the area of potential treatments of scleroderma and is stock owner of 4D Science GmbH.

Ethics approval The local ethics committees.

Provenance and peer review Not commissioned; externally peer reviewed.

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Ann Rheum Dis 2016 75: 303-310 originally published online November 10, 2014
doi: 10.1136/annrheumdis-2014-205326

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