Under the aegis of:

BOSTON UNIVERSITY

Boston University School of Medicine
Scleroderma Program

UCL

Royal Free Hampstead NHS
NHS Trust

Centre for Rheumatology and Connective Tissue Disorders
University College London
(Royal Free Hospital Campus)

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Coordinators

Kate Brennan, Kim Fligelstone, Millie Williams, Pamela Yeomans

11th INTERNATIONAL WORKSHOP on Scleroderma Research

August 1st – 4th, 2010
Boston, Massachusetts

Understanding the pathogenesis of scleroderma, utilizing current therapies, identifying new targets and treatment strategies, leading to better management for patients with scleroderma

www.sclerodermaworkshop.org

Boston University Charles River Campus, Boston, Massachusetts

Acknowledgements

The organizers would like to thank the following for their support of the Workshop:

GOLD LEVEL SPONSERS

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Other Contributors

Conference Office (10)

10 Buick Street, Ground Floor, Room G15

Opening times:	Saturday	12.00pm -5.00pm
	Sunday to Tuesday	8.00am - 5.00pm
	Wednesday	8:00am – 2:00pm

Accommodations (10)

10 Buick Street

Workshop Sessions (9) & (7)

Dance Theatre @ Fit/Rec Center (9)
Burke Club Room @ Agganis Arena (7)

Scientific Sessions will be held in the Dance Theatre at the Fit/Rec Center (9), *with the exception of the Monday sessions*, which will be held in the Burke Club Room at Agganis Arena (7).

Poster Presentations (10)

10 Buick Street, 18th Floor Lounge

Access to the Lounge for set up will be from Saturday, July 31 at noon. Posters will remain up for the duration of the Workshop.

Top Ranking Abstract Awards (7)

Burke Club Room @ Agganis Arena (2nd Floor)

Presentations on the top 5 abstracts will take place in the Burke Room at Agganis Arena on Monday starting from 1pm.

Meals (4)

West Campus Dining Hall

Breakfast and lunch only. Please have your conference badge with you.

Welcome Reception (7)

Burke Club Room @ Agganis Arena (2nd Floor)

Sunday, 5:30-7pm

Dinner Banquet (39)

Metcalf Trustee Center Ballroom, One Silber Way, 9th Floor

Tuesday, August 3rd 6-9pm

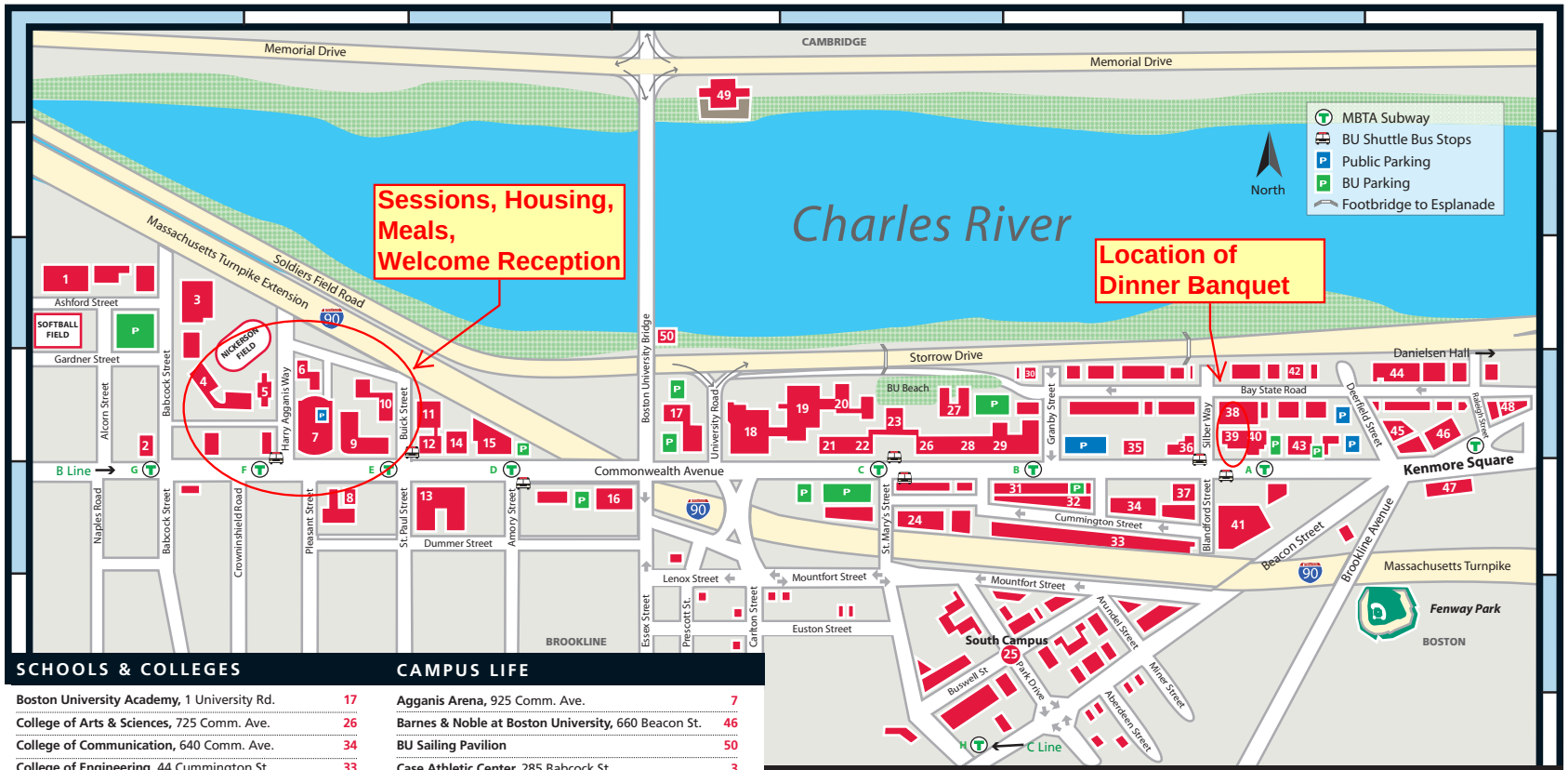
Getting to the Banquet:

Shuttle (Free): The Boston University Shuttle leaves the corner of Agganis Way and Commonwealth at 5:15, 5:30 and 6pm and arrives at the corner of Blandford Street and Commonwealth in about 6 minutes. Cross Commonwealth Ave and walk up Silber Way (Sherborn Street); the entrance to 1 Silber Way is on your right. The return shuttles leave from the corner of Commonwealth and Silber Way (Sherborn Street) at 8:14, 8:44, 9:14 & 9:44 and arrives back at the corner of Buick St. & Commonwealth in 7 minutes.

Trolley ($2.00 each way): Take the inbound Green Line trolley at the corner of Buick Street and Commonwealth (descend at Blandford Street stop) and the outbound trolley to return (descend at St. Paul Street on the return journey).

Walking: From Buick & Commonwealth to Sherborn & Commonwealth takes approximately 15 minutes.

Boston University Charles River Campus



SCHOOLS & COLLEGES

Boston University Academy, 1 University Rd.	17
College of Arts & Sciences, 725 Comm. Ave.	26
College of Communication, 640 Comm. Ave.	34
College of Engineering, 44 Cummington St.	33
College of Fine Arts, 855 Comm. Ave.	15
College of General Studies, 871 Comm. Ave.	14
Graduate School of Arts & Sciences, 705 Comm. Ave.	28
Metropolitan College & Extended Education, 755 Comm. Ave. (Summer Term)	21
College of Health & Rehabilitation Sciences: Sargent College, 635 Comm. Ave.	35
School of Education, 2 Silber Way	36
School of Hospitality Administration, 928 Comm. Ave.	8
School of Law, 765 Comm. Ave.	20
School of Management, 595 Comm. Ave.	40
School of Social Work, 264 Bay State Rd.	27
School of Theology, 745 Comm. Ave.	22
University Professors Program, 745 Comm. Ave.	22

The Henry M. Goldman School of Dental Medicine, School of Medicine, and School of Public Health are located at the Boston University Medical Campus in Boston's South End.

CAMPUS LIFE

Agganis Arena, 925 Comm. Ave.	7
Barnes & Noble at Boston University, 660 Beacon St.	46
BU Sailing Pavilion	50
Case Athletic Center, 285 Babcock St.	3
Fitness & Recreation Center, 915 Comm. Ave.	9
George Sherman Union, 775 Comm. Ave.	18
Marsh Chapel, 735 Comm. Ave.	23
Mugar Memorial Library, 771 Comm. Ave.	19
Student Activities Center, 1 University Rd.	17
Track & Tennis Center, 100 Ashford St.	1
Tsai Performance Center, 685 Comm. Ave.	29

MAJOR RESIDENCES

10 Buick Street	10
33 Harry Agganis Way	6
575 Commonwealth Avenue	43
1019 Commonwealth Avenue	2
Danielsen Hall, 512 Beacon St.	(not on map)
Myles Standish Hall, 610 Beacon St.	48
Shelton Hall, 91 Bay State Rd.	44
South Campus	25
The Towers, 140 Bay State Rd.	38
Warren Towers, 700 Comm. Ave.	31
West Campus, 273–277 Babcock St.	4

BUILDINGS & SERVICES

Admissions Reception Center, 121 Bay State Rd.	42
Boston University Police, 32 Harry Agganis Way	5
Center for English Language & Orientation Programs, 890 Comm. Ave.	13
Dean of Students, 775 Comm. Ave.	18
Disability Services, 19 Deerfield St.	45
Educational Resource Center, 775 Comm Ave.	18
Financial Assistance, 881 Comm. Ave.	12
Hotel Commonwealth, 500 Comm. Ave.	47
Housing Office, 25 Buick St.	11
Information Technology, 111 Cummington St.	32
International Students & Scholars Office, 888 Comm. Ave.	13
Metcalf Science Center, 590 Comm. Ave.	41
Photonics Center, 8 St. Mary's St.	24
President's Office, 1 Silber Way	39
Registrar, 881 Comm. Ave.	12
Student Accounting Services, 881 Comm. Ave.	12
Student Health Services, 881 Comm. Ave. (West)	12
University Service Center, 881 Comm. Ave.	12
808 Commonwealth Ave.	16

SPECIAL ATTRACTIONS

BU Art Gallery at the Stone Gallery, 855 Comm. Ave.	15
Boston University Experience, 602 Comm. Ave.	37
Boston University Theatre, 264 Huntington Ave.	(not on map)
The Castle, 225 Bay State Rd.	30
DeWolfe Boathouse, 619 Memorial Dr.	49
Gotlieb Archival Research Center, 771 Comm. Ave.	19

Stops

- A Blandford Street
- B Boston University East
- C Boston University Central
- D Boston University West
- E St. Paul Street
- F Pleasant Street
- G Babcock Street
- H St. Mary's Street

Campus Information

617-353-INFO (4636) | AskUs@bu.edu | www.bu.edu/infocenter

Visit Boston University Online Maps based on Google Maps for directions and more information at www.bu.edu/maps.

The distance from Kenmore Square to West Campus residences is approximately 1.3 miles.

SATURDAY, JULY 31

12-5pm Check-in and Registration
Conference Office, 10 Buick Street,
Ground Floor, Room G15

SUNDAY, AUGUST 1

8:45am Welcome Carol Black
Robert Lafyatis

Session 1 (9:00-10)
Challenges & Advances in Systemic Sclerosis
Chair – J Varga

9:00	Challenges facing the clinician	D Furst
9:30	Advances in scleroderma and the Workshop	C Denton
10:00	Coffee Break	

Session 2 (10:30-12)
Genetic Associations in Scleroderma: GWAS Studies
Chair – Josephine Hoh

10:30	Genetic associations in systemic sclerosis	M Mayes
10:50	Immune genetics in systemic sclerosis	T Radstake
11:10	Genetics & biomarkers in systemic sclerosis	P Pantelidis
11:30	Discussion	
12:00-1:30	Lunch	

Session 3 (1:30-3:00)
Genetic Regulation in Pulmonary Disease
Chair – F Arnett & B Kahaleh

	Keynote	
1:30	**Gene regulation in interstitial lung disease**	**K Gibson**
2:00	micro RNAs and TGFβ	A Hata
2:20	Lessons from gene expression in pulmonary hypertension	T Bull
2:40	Discussion	
3:00	Tea	

Session 4 (3:30-4:30)
Genetic Markers of Systemic Sclerosis
Chair – C Denton & B White

3:30	Subclassification of scleroderma patients using microarray gene expression	M Whitfield
3:50	Interferon-regulated gene expression in systemic sclerosis	S Assassi
4:10	Discussion	

4:30-5:30 **Poster Session (Authors stand by posters)**
18th Floor Lounge, 10 Buick Street

5:30-7:00 **Welcome Reception** **Agganis Arena Burke Club Room**

Free Evening

MONDAY, AUGUST 2 *(Please note: All Monday sessions are in the Burke Club Room, 2nd floor of Agganis Arena)*

Session 5 (9:00-10:30) Agganis Arena Burke Club Room
Pulmonary Fibrosis – Clinical Update & Pathogenesis
Chair – F Wollheim & T Wynn

	Keynote	
9:00	**Oxidative stress, hemoxygenase, cell apoptosis and lung injury**	**A Choi**
9:30	Clinical – radiological correlations in pulmonary fibrosis	A Wells
9:50	Inflammasome and innate immunity in pulmonary fibrosis	F Sutterwala
10:10	Discussion	
10:30	Coffee Break	

Session 6 (11:00-12:00) Agganis Arena Burke Club Room
Mediators of Fibrotic Lung Disease
Chair – A Choi & A Wells

	Keynote	
11:00	**Matrix and proteases in fibrosis**	**G Laurent**
11:30	IGFBP5 and gene regulation in scleroderma-associated pulmonary fibrosis	C Feghali-Bostwick
11:50	Discussion	

12:00 Lunch

Abstract Presentations (1-2:40)
Agganis Arena - Burke Club Room
Chair – L Mouthon

1:00	Inhibition of thrombin as a novel strategy in the treatment of scleroderma-associated ILD	Bogatkevich
1:20	Genetic deletion or pharmacologic antagonism of LPA_1 ameliorates dermal fibrosis in a mouse model of systemic sclerosis	Castelino
1:40	Post-natal fibroblast-specific deletion of the *Pten* gene in fibroblasts induces a scleroderma phenotype	Parapuram
2:00	Semaphorin 7a regulates fibrocyte accumulation in TGF- ß1 induced pulmonary fibrosis	Herzog
2:20	The early growth response gene Egr-2 (Krox20) is a novel transcriptional target of transforming growth factor-β that is up-regulated in systemic sclerosis and mediates profibrotic responses	Feng

3:00 Tea

Session 7 (3:20-5:00) Agganis Arena Burke Club Room
Emerging Therapeutics I
Chair – R Simms & M Baron

3:20	Targeting B cells and IFN in scleroderma	B White
3:40	SAP in fibrosis	M Lupher
4:00	Actemra (Tocilizumab)	J Pope
4:20	Tyrosine kinase inhibitors in SSc	P Gergely
4:40	Discussion	

Session 8 (5:20-6:40) Agganis Arena Burke Club Room
Emerging Therapeutics II
Chair – M Matucci Cerinic & T Medsger

5:20	Anti- αvβ6	S Violette
5:40	Anti-TGFβ	J Mannick
6:00	miRNA therapeutics	TBA
6:20	Discussion	

Free Evening

TUESDAY, AUGUST 3

Session 9 (8:30-10)
Mechano-sensing in Fibrosis
Chair – K Rubin & T Krieg

	Keynote	
8:30	**Mechano-sensing in fibrosis**	**D Ingber**
9:00	Myofibroblasts & fibrosis	D Tschumperlin
9:20	Integrin regulation of fibroblast phenotype	B Eckes
9:40	Discussion	
10:00	Coffee Break	

Session 10 (10:40-12)
Inflammation & Fibrosis
Chairs- R Lafyatis & A Postlethwaite

10:40	T cells in graft versus host disease	S Katz
11:00	Immune activation & fibrosis	A Coyle
11:20	IL-13 & alternative macrophage activation	T Wynn
11:40	Discussion	
12-1:30	Lunch	

Session 11 (1:30-3)
Progenitors, Stem Cells & Regenerative Medicine
Chair - D Abraham & S Jiménez

	Keynote	
1:30	**Wnt regulation of fibrosis**	**T Rando**
2:00	IPS cells & lung regeneration	D Kotton
2:20	PPARγ in mesenchymal cell differentiation	J Varga
2:40	Discussion	
3:00	Tea	

Session 12 (3:30-5)
Pulmonary Arterial Hypertension
Chair – H Farber & L Mouthon

	Keynote	
3:30	**Pathogenic mechanisms in pulmonary hypertension**	**R Tuder**
4:00	IL-6 in pulmonary hypertension	A Waxman
4:20	Altered endothelial cells in scleroderma vasculopathy	M Trojanowska
4:40	Discussion	

6-9	**Dinner Banquet**	**Metcalf Trustee Center Ballroom, 1 Silber Way**

Speaker: Robert F. Meenan, MD, MPH, MBA
Dean, Boston University
School of Public Health

WEDNESDAY, AUGUST 4

Session 13 (8:30-10)
Vascular Inflammation & Injury
Chair – A Gabrielli & R Tuder

	Keynote	
8:30	**Pulmonary arterial hypertension -Lessons from new models**	**M Rabinovitch**
9:00	The immune response in PAH	G Grunig
9:20	Anti-endothelial cell antibodies & oxidative stress in scleroderma	F Batteux
9:40	Discussion	
10:00	Coffee Break	

Session 14 (10:30-12)
Regulation of Vascular Phenotype
Chair – M Rabinovitch & M Trojanowska

	Keynote	
10:30	**Vascular inflammation**	**J Loscalzo**
11:00	Endothelial mesenchymal transition	S Jiménez
11:20	EPCs and manufacturing vascular networks in vivo	J Bischoff
11:40	Discussion	
12-1:20	Lunch	

Session 15 (1:20-4)
Scleroderma Clinical Trials Consortium Session
Chairs – J Seibold & R Simms

1:20	Development of outcome measures in CTD-ILD	L Saketkoo
2:00	Trial design for SSc-ILD	A Wells
2:20	Lung & blood biomarkers in SSc-ILD	C Feghali-Bostwick
3:00	Lessons learned from recent trials	J Seibold
3:20	Utilization of skin biomarkers in SSc clinical trials	R Lafyatis
3:40	Discussion	

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Five Highest Ranking Abstracts (Travel Award Winners)

Inhibition of thrombin as a novel strategy in the treatment of scleroderma-assoicated interstitial lung disease

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Background/Aims
Activation of the coagulation cascade leading to generation of thrombin has been extensively documented in various forms of lung injury including systemic sclerosis-associated interstitial lung disease (SSc-ILD). We previously demonstrated that the direct thrombin inhibitor (DTI) dabigatran inhibits profibrotic signaling in lung fibroblasts isolated from scleroderma patients. The aim of this study is to provide a molecular basis for therapeutic interventions in SSc-ILD by inhibition of thrombin.

Materials and Methods
Thrombin activity was measured by fluorometric assay in bronchoalveolar lavage fluid (BALF) and lung tissue from SSc-ILD patients and from mice with bleomycin-induced pulmonary fibrosis. Lung injury was induced in 6-8 week old female C57BL/6 mice by a single intratracheal instillation of bleomycin. DTI dabigatran etexilate was given as supplemented chow beginning on day 1 (early treatment, anti-inflammatory effect) or on day 8 (late treatment, anti-fibrotic effect) following bleomycin instillation. Two and three weeks after bleomycin instillation mice were euthanized; lung tissue, bronchoalveolar lavage fluid (BALF), and plasma were investigated. For in vitro studies, lung fibroblasts and alveolar epithelial cells (AEC) were stimulated with or without thrombin (1U/ml) and dabigatran (100ng/ml). Apoptosis was measured by propidium iodine exclusion, cell death detection ELISA, and in situ cell death detection assay. Caspase-3, Akt, and α-smooth muscle actin (SMA) were studied by immunoblotting and immunofluorescent staining.

Results
We observed significantly higher thrombin activity in BALF and lung tissue from SSc-ILD patients and bleomycin-treated mice compared with normal controls ($p<0.001$).Thrombin induces apoptosis in AEC by cleaving caspase-3 and increasing DNA fragments by 3.7-fold compared with control. Thrombin differentiates lung fibroblasts to a profibrotic myofibroblast phenotype resistant to apoptosis by inducing α-SMA expression 6-fold and by rapid phosphorylation of Akt (within 10 min of thrombin treatment). Both early and late treatment with dabigatran etexilate attenuated the development of bleomycin-induced pulmonary fibrosis in mice. Dabigatran etexilate significantly reduced thrombin activity and levels of TGF-β1 and PDGF-AA in BALF, while simultaneously decreasing inflammatory cells and protein concentrations. Histological lung inflammation and fibrosis were significantly decreased in dabigatran etexilate-treated mice. Additionally, dabigatran etexilate reduced collagen, CTGF, and α-SMA expression in mice with bleomycin-induced lung fibrosis, whereas it had no effect on basal levels of these proteins. Dabigatran strongly reduced thrombin-induced apoptosis of AEC and activation of fibroblasts in vitro and in vivo.

Conclusions
We conclude that activation of thrombin following lung injury leads to apoptosis of AEC while increasing survival of myofibroblasts resulting in persistent pulmonary fibrosis. Inhibition of thrombin using the oral DTI dabigatran etexilate has marked anti-inflammatory and anti-fibrotic effects in a bleomycin model of pulmonary fibrosis. Therefore, inhibition of thrombin by DTI dabigatran etexilate may serve as a potential novel therapeutic avenue for the treatment of SSc-ILD.

Genetic deletion or pharmacologic antagonism of LPA_1 ameliorates dermal fibrosis in a mouse model of systemic sclerosis

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Rationale: Systemic sclerosis (SSc) is a potentially fatal autoimmune disease of unknown etiology, characterized by progressive multi-organ fibrosis that is largely refractory to currently available pharmacological therapies. We have previously implicated the lipid mediator lysophosphatidic acid (LPA) in the pathogenesis of pulmonary fibrosis. Here, we studied the roles of LPA and two of its G protein-coupled receptors, LPA_1 and LPA_2, in the development of dermal fibrosis in a bleomycin-induced mouse model of SSc, by using mice deficient in these receptors. Additionally, we investigated the therapeutic potential of targeting LPA_1 pharmacologically, by using the novel selective LPA_1 antagonist AM095 in this model.

Methods: Wild type (WT) and LPA_1- and LPA_2-deficient (LPA_1 KO and LPA_2 KO) mice were injected subcutaneously with bleomycin (10μg/ml) or phosphate buffered saline (PBS) once per day. After 28 injections, full thickness 6mm punch biopsies were obtained from the injection sites. Dermal thickness was determined by measuring the distance between the epidermal-dermal and dermal-fat junctions using hematoxylin and eosin-stained skin sections. Collagen in skin sections was visualized by Masson's trichrome staining, and quantified by hydroxyproline measurement. Myofibroblasts and cells responding to TGF-β, were identified in sections of lesional skin by immunohistochemical staining with anti-α-smooth muscle actin (α-SMA) and anti-phosphoSmad2 antibodies, respectively. The selective LPA_1 antagonist, AM095 or vehicle control was administered to bleomycin- or PBS-challenged C57Bl/6 mice by oral gavage twice daily on weekdays and once daily on weekends. AM095 or vehicle treatment was either administered concurrently with bleomycin or PBS, or initiated at 7 or 14 days after bleomycin challenge, for total treatment durations of 28, 21 or 14 days, respectively. At the conclusion of these AM095 and vehicle treatment schedules, dermal thickness and collagen content were assessed as above.

Results: LPA_1 KO mice were markedly protected from dermal fibrosis as assessed by both dermal thickness and hydroxyproline content. Comparing LPA_1 KO and WT mice, genetic deletion of LPA_1 attenuated bleomycin-induced increase in dermal thickness by 91% and hydroxyproline content by 90%. Bleomycin-induced increases in dermal α-SMA^+ and $phosphoSmad2^+$ cells were also markedly attenuated in LPA_1 KO mice. Bleomycin challenge increased the number of α-SMA^+ myofibroblasts by 70% in WT mice, but only by 5% in LPA_1 KO mice. Similarly, bleomycin challenge increased $phosphoSmad2^+$ cells by 81% in WT mice, with no increase in LPA_1 KO mice. In contrast, LPA_2 KO mice were not protected from bleomycin-induced increases in dermal thickness and collagen content when compared to WTs. Pharmacologic antagonism of LPA_1 with AM095 also significantly attenuated bleomycin-induced dermal fibrosis. Although the greatest reductions in dermal thickness and collagen content were seen with 28-day administration of AM095, both delayed treatment regimens significantly reduced dermal fibrosis as well.

Conclusions: These results suggest that LPA signaling through LPA_1, but not LPA_2, is critically required for bleomycin-induced dermal fibrosis. Both genetic deletion and pharmacological inhibition of LPA_1 markedly attenuated dermal fibrosis in this model. LPA acting through LPA_1 appears to importantly contribute to both myofibroblast accumulation and activation of the TGF-β signaling pathway. Targeting LPA_1 therefore has the potential to be an effective new therapeutic strategy for systemic sclerosis.

Post-natal fibroblast-specific deletion of the *Pten* gene in fibroblasts induces a scleroderma phenotype

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Skin fibrosis, characterised by increased collagen deposition and matrix contraction by resident connective tissue fibroblasts is a principal feature of scleroderma and is believed to result from a dysregulated tissue repair program. The protein phosphatase and tensin homolog (PTEN) suppresses specific functional activities of cells including migration, contractility, survival and collagen production and thus could be a key modulator of tissue repair. We hypothesized that altered expression of PTEN in fibroblasts could result in the activation of a tissue repair program and fibrogenesis. To test the hypothesis, we first used Western blot and immunohistochemical analyses to assess the PTEN protein expression in fibroblasts of scleroderma skin. PTEN expression was significantly

decreased in fibroblasts of scleroderma skin (N = 6; $p < 0.05$) compared to controls. To evaluate whether this defect was sufficient for fibrogenesis *in vivo*, we studied the role of PTEN by selectively deleting *Pten* gene in fibroblasts. Mice with *Pten* gene flanked by *loxP* sites were mated with mice expressing tamoxifen-dependent Cre recombinase under the control of fibroblast-specific type I collagen promoter/enhancer (Zheng B et al., 2002 Am J Pathol, 160 (5):1609-17). Mice homozygous for *loxP Pten* and hemizygous for Cre were either treated with tamoxifen (to delete *Pten* specifically in adult fibroblasts) or corn oil (vehicle; to generate control mice). Compared to controls (*Pten*+/+), conditional deletion of the *Pten* gene (*Pten*-/-) in the fibroblasts resulted in increased thickness of the dermis (*Pten*+/+: 168 ± 57µm, *Pten*-/- : 372 ± 112µm, $p < 0.05$) and elevated collagen deposition (hydroxyproline content: *Pten*+/+: 3.7 ± 1.0, *Pten*-/-: 4.9 ± 0.8 g/100g tissue $p < 0.05$). Dermal collagen fibrils showed a 35% increase in diameter in *Pten*-/- mice when examined by transmission electron microscopy (*Pten*+/+: 67.5 ± 9.8nm, *Pten*-/- : 91.9 ± 9.2nm, $p < 0.001$). There was increased expression of pAKT in *Pten*-/- dermis. The excessive collagen production by cultured *Pten*-/- dermal fibroblasts was significantly suppressed by selective inhibitors of the PI3K/Akt signaling pathway (LY294002 and wortmannin). Our findings indicate that decreased expression of PTEN is sufficient for fibrogenesis *in vivo* and hence could be major factor in the development of fibrosis in scleroderma. PTEN appears to be a master regulator of tissue repair, and PTEN agonists could represent important novel anti-fibrotic treatments for the skin fibrosis in scleroderma.

Semaphorin 7a regulates fibrocyte accumulation in TGF- ß1 induced pulmonary fibrosis

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Introduction: Profibrotic cells derived from monocytes include collagen-producing cells called fibrocytes (1). Semaphorin 7a (Sema 7a) is a GPI anchored membrane protein with important immunologic effects that signals through two known receptors, ß1 integrin and Plexin C1. (2) Our murine work demonstrates Sema 7a also critically regulates TGF-ß1 induced pulmonary fibrosis. (3) However, a role for Sema 7a in regulation of fibrocytes has not been proposed. In addition, the relationship of Sema 7a to Scleroderma-related ILD (SSc-ILD), a fibrosing lung disorder associated with TGF-ß1 over-activation (4) and accumulation of circulating fibrocytes (5) has not been assessed. We hypothesized that TGF-ß1 mediates its pulmonary effects in part through the stimulation of fibrocyte accumulation and that Sema 7a- ß1 integrin interactions play a critical role in the pathogenesis of these responses

Methods: We characterized fibrocytes in lung-targeted TGF-ß1 transgenic mice, determined if the ability of transgenic TGF- ß1 to induce fibrocyte accumulation was altered in mice that had null mutations of Sema7a or were treated with antibodies against ß1-integrins, and used bone marrow chimeras to determine the tissue compartment (circulation or stroma) in which these effects were initiated. In addition, a cohort of treatment naïve patients with SSc-ILD were recruited and characterized, and peripheral blood mononuclear cells (PBMCs) were obtained and analyzed for fibrocytes by flow cytometry, expression of Sema 7a and its receptors (q-RT-PCR), and Sema 7a expressing monocytes and lymphocytes. The role of Sema 7a in fibrocyte outgrowth was assessed using blocking antibodies to Sema 7a and ß1 integrin.

Results: TGF-ß1 overexpression induced robust accumulation of fibrocytes (characterized by the co-expression of CD45/Col-1) ($p<0.05$). When repeated in TGF-ß1 x Sema 7a-/- mice, fibrosis and fibrocyte accumulation was abolished ($p<0.05$). Antibody mediated blockade of ß1 integrin similarly decreased in fibrosis, inflammation and fibrocytes ($p<0.05$ for all comparisons). The chimera studies indicated that Semaphorin 7a on circulating cells was sufficient to restore fibrosis, inflammation, and fibrocytes in the Sema 7a null mice ($p<0.05$). Our human studies showed that fibrocytes are increased in the blood of patients with SSc-ILD when compared to normal controls. Expression of Sema 7a, ß1 integrin, and Plexin C1 were all increased in SSc-ILD, as were absolute numbers of PBMCs expressing Sema 7a ($p<0.05$ all comparisons). Sema 7a was found to localize to lymphocytes and not monocytes or fibrocytes in increased numbers. In vitro studies demonstrate that blockade of Sema 7a or ß1 integrin decrease fibrocyte outgrowth in patients with SSc-ILD.

Conclusions: These data demonstrate that Semaphorin 7a on circulating cells regulates TGF-ß1 induced lung fibrosis and fibrocyte accumulation, and that Sema 7a regulates fibrocyte biology in patients with SSc-ILD.

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The early growth response gene Egr-2 (Krox20) is a novel transcriptional target of transforming growth factor-β that is up-regulated in systemic sclerosis and mediates profibrotic responses

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Introduction: Fibrosis, the hallmark of scleroderma, is caused by the deposition of extracellular matrix (ECM) with activated fibroblasts. Transforming growth factor β (TGF-β) has been implicated as an essential mediator of fibrosis. Egr-2 is a member of the early growth response gene family that regulates cell proliferation and differentiation. While Egr-2 is known to play essential roles in nerve myelination, the regulation of Egr-2 expression and its role in the fibrosis are poorly understood. To explore this notion, we characterized the role of Egr-2 in fibrosis pathogenesis.

Methods: The regulation of Egr-2 expression was examined *in vitro* using skin fibroblasts and *in vivo* using biopsies. Cellular Egr-2 and regulation of collagen gene expression were examined by real-time PCR, Western blot, immunocytochemistry and transient transfection assays.

Results: We show here that in normal fibroblasts transforming growth factor-β (TGF-β) induced a Smad3-dependent sustained stimulation of Egr-2 gene expression. Ectopic expression of Egr-2 was sufficient to induce profibrotic responses such as collagen gene expression and myofibroblast differentiation, whereas these responses showed attenuated stimulation by TGF-ß in Egr-2-depleted fibroblasts. Genome-wide expression profiling revealed that many genes involved in tissue remodeling and wound healing were up-regulated by Egr-2 in fibroblasts, but the pattern of Egr-2-induced gene expression only partially overlapped with Egr-1-induced gene expression. Mice with bleomycin-induced scleroderma showed elevated Egr-2 expression. Moreover, elevated Egr-2 expression was noted in skin and lung biopsies from patients with the prototypic fibrosing disorder systemic sclerosis (SSc). These results provide the first evidence that Egr-2 is a functionally distinct TGF-β-regulated transcription factor that is both necessary and sufficient for profibrotic responses, and is aberrantly expressed in the skin and lungs in SSc and in a model of scleroderma.

Summary: Egr-2 is a novel transcriptional target induced by transforming growth factor-β. It is up-regulated in systemic sclerosis and mediates profibrotic responses.

Conclusion: These results provide the first evidence that Egr-2 is a TGF-β-inducible transcription factor that induces collagen gene expression and might play an important role in the pathogenesis of fibrosis. Targeting Egr-2-mediated TGF-β2 signaling pathway may have potential pharmaceutical application for the control of fibrosis.

Submitted Abstracts/Posters (Alphabetical by Author)

1. Cadherin-11 in Systemic Sclerosis and its Role in Dermal Fibrosis

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Background: Cadherin-11 (Cad-11) is a mesenchymal cadherin initially identified in osteoblast cell lines but subsequently found to be expressed on other cells including synovial fibroblasts where it imparts a mesenchymal phenotype and promotes cellular invasion. Recently, 2 independent microarray studies demonstrated that cadherin-11 was amongst a large list of upregulated genes in scleroderma skin
Objective: Given the expression of Cad-11 on fibroblasts and the increased Cad-11 levels in scleroderma skin, we hypothesized that Cad-11 is a critical mediator of dermal fibrosis.
Methods: Scleroderma and normal skin biopsies were used for qRTPCR to quantitate Cad-11 levels and for immunohistological (IHC) analyses to determine expression pattern of Cad-11. Dermal fibroblasts were stimulated with TGFb and qRTPCR was used to determine if TGFb increased Cad-11 expression. To determine if Cad-11 is a mediator of dermal fibrosis, Cad-11 deficient (def) and wild type (WT) mice were compared in the bleomycin (bleo)-induced dermal fibrosis model.
Results: Quantitative RTPCR analyses of scleroderma skin biopsies (n=6) demonstrated an increase in Cad-11 mRNA relative to healthy control skin biopsies (n=9). IHC analyses of skin biopsies demonstrated Cad-11 reactivity in the dermis of scleroderma biopsies, but not control biopsies, that was predominantly expressed on fibroblast-like cells. Additional in vitro studies were performed on cultured dermal fibroblasts demonstrating that TGFb stimulation induces an upregulation of Cad-11 on control and scleroderma dermal fibroblasts. Using the bleo-induced dermal fibrosis model, Cad-11 def. mice had markedly attenuated dermal fibrosis as quantitated by skin thickness, collagen levels, and myofibroblasts accumulation in the lesional skin, relative to WT mice. Lastly, Col1a1 and CTGF mRNA levels but not IL-6 levels were significantly decreased in lesional skin of Cad-11 def. compared to WT mice.
Conclusions: These data demonstrate that Cad-11 levels are increased in SSc skin. Furthermore, Cad-11 def. mice have decrease dermal fibrosis in the bleomycin induced dermal fibrosis model, demonstrating that Cad-11 is a critical mediator of dermal fibrosis, suggesting that Cad-11 is a potential therapeutic target in SSc.

2. Assessing the activity of agonistic autoantibodies in systemic sclerosis and their effects on vascular smooth muscle cells

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Background: Systemic sclerosis (SSc) is characterized by fibrosis, vascular disease and the presence of specific autoantibodies. It is suggested, although not without controversy, that SSc autoantibodies may stimulate the platelet-derived growth factor receptor (PDGFR) on fibroblasts, leading to intracellular signalling events, the production of reactive oxygen species, and collagen gene expression, thereby forming a link between the autoimmune and fibrotic disease components. Vascular complications are significant in SSc. If anti-PDGFR autoantibodies are present in the circulation, they might also encounter vascular smooth muscle cells (VSMCs), which also express the PDGFR. Thus, the present study aims to characterize the effects of SSc IgG on VSMC and to determine if stimulatory autoantibodies directed to the PDGFR of VSMCs can be detected.
Methods: IgG was purified from female diffuse cutaneous SSc (dcSSc) patient serum or from control (age- and sex-matched osteoarthritis patient) serum, using Protein A/G chromatography. The purified IgG (200ug/mL) was then used to stimulate quiescent rat VSMCs for different periods of time. Cellular extracts were prepared and proteins were analyzed by immunoblot analysis to verify the state of ERK and Akt activation. Total RNA was also isolated to analyze *Col1a1, Col2a1, Tgfb1, Tgfb2 and Tgfb3* mRNA expression using real-time RT-PCR. Cellular proliferation was measured through [^{3}H]-thymidine incorporation and the capacity of SSc IgG to selectively bind the PDGFR was assessed in immunoprecipitation

experiments. VSMCs exposed to purified IgG were also treated with PDGFR inhibitors before IgG exposure.

Results: IgG from both control and SSc patients immunoprecipitated the PDGFR. However, only faint PDGFR bands were detected in control treatments, while 75% of the SSc IgG samples tested immunoprecipitated the PDGFR much more strongly. Similarly, in terms of ERK activation, phosphorylation above basal levels occurred in response to both SSc and control IgG, although not in all samples. The phopho-ERK signal in response to most (68%, 19/28) SSc-IgG samples was markedly stronger than in controls, of which only 27% (3/11) gave an above-basal signal. Akt signalling was fainter, and only detectable above basal levels in SSc samples. However, the use of AG1296, a specific inhibitor of the PDGFR did not block these IgG-induced phosphorylations, despite effectively blocking these signals in PDGF-stimulated cells. PDGFR knockdown experiments are underway in order to conclusively verify the involvement of the PDGFR in this response to SSc IgG. We detected no changes in *Col1a1* or *Col2a1* expression in response to SSc or control IgG. On the other hand, mRNA expression of *Tgfb1*, a pro-fibrotic cytokine, was upregulated 2-fold ($p<0.001$) in VSMC treated with SSc IgG, as compared to those treated with control IgG. Interestingly, mRNA of *Tgfb2*, which has been shown to have an anti-fibrotic role in the avian SSc model, was down-regulated by 20 to 40% ($p<0.001$) in SSc-IgG treated VSMCs. Gene expression of *Tgfb3*, whose role in fibrosis is less well-defined, appears to be upregulated in response to treatment with SSc IgG. Cellular proliferation assays showed no significant difference between SSc or control IgG-treated cells, all having very weak to no mitogenic effects on VSMC.

Conclusions: Our results suggest that SSc IgG has effects on VSMCs. These effects include upregulation of the pro-fibrotic *Tgfb1* gene, and down-regulation of the anti-fibrotic *Tgfb2* gene, thus suggesting a role in fibrosis in VSMCs. Although SSc IgG does bind to the PDGFR of VSMCs, and although ERK and Akt phosphorylation is upregulated by SSc IgG, we have so far been unable to prove that these effects are mediated by the PDGFR. Our future investigations will clarify the involvement of the PDGFR. Finally, these autoantibodies seem to be present in most, but not all, SSc patients.

3. β-arrestin 1 is a novel regulator of profibrotic TGFβ/S1P$_1$ signaling.

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RATIONALE: TGFβ is an important regulator of physiological connective tissue biosynthesis and plays a central role in pathological tissue fibrosis. The complexity, diversity, and flexibility of this pathway stems from the cross-talk with other cellular pathways that modulate TGFβ signaling. One of such a pathway is sphingosine 1-phosphate (S1P) pathway. β-arrestins (ARRBs) are multifunctional adaptor proteins best known for their role in G protein-coupled receptor desensitization. In addition, ARRBs have emerged as important regulators of other signaling pathways through their ability to scaffold signaling complexes. This study was undertaken to assess the role of ARRBs in profibrotic TGFβ/S1P$_1$ signaling.

METHODS: Human dermal fibroblast culture was obtained from foreskins of healthy newborns. The spontaneously immortalized clonal murine embryo fibroblasts (MEFs) were obtained from ARRB1/2 knockout mice. Using siRNA technology the depletion of ARRB1/2 and S1P1 receptor was performed. Using ARRB1/2 adenoviruses the overexpression of ARRB1/2 was performed.

RESULTS: Using siRNA mediated gene silencing we show that in human fibroblasts ARRB1 and S1P$_1$ receptor are required for the TGFβ-induced expression of CCN2 and collagen mRNA and proteins. Specifically, ARRB1 and S1P$_1$ receptor were required for the TGFβ-induced Smad1 and ERK1/2 phosphorylation, but were not involved in activation of the Smad3 pathway. The Smad1-induced target gene, Id1 was upregulated upon TGFβ stimulation, but was not present in the cells with depleted ARRB1 and S1P$_1$ receptor. Depletion of ARRB2 did not change the TGFβ-induced level of Id1. Upon TGFβ stimulation, TGFβRI formed complexes with ARRB1 and S1P$_1$ receptor. Additional experiments using MEFs from ARRB1/2 knockout mice confirmed the profibrotic function of ARRB1 via the TGFβ/S1P$_1$/Smad1/Id1 axis. We also show that TGFβ induces phosphorylation of ARRB1 on Ser 412 and that this phosphorylation event is required for the profibrotic function of ARRB1. In contrast, ARRB2 was not involved in these TGFβ effects.

CONCLUSIONS: This study establishes a novel role for ARRB1 in non-canonical TGFβ signaling and suggests that ARRB1 may represent an attractive therapeutic target to treat fibrosis.

4. The role of PTEN down-regulation on PPM1A expression in scleroderma fibroblasts

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Recent in vivo and in vitro studies suggest that PTEN, a lipid and protein phosphatase, is a negative regulator of fibrosis and there is increasing evidence that PTEN deficiency is associated with fibrosis in different organs. Previous studies provided evidence that PTEN protein levels are reduced in both cultured scleroderma fibroblasts and lesional scleroderma skin in vivo. A novel function of nuclear PTEN as a co-factor of the Smad2/3 phophatase, PPM1A, has been recently demonstrated. In the presence of PTEN, PPM1A is protected from degradation induced by the TGF-β signaling, resulting in rapid dephoshorylation of Smad2/3.

The aim of this study was to examine the expression levels of PPM1A and to test the role of PTEN down-regulation on PPM1A gene expression in scleroderma fibroblasts in culture. We hypothesized that in the presence of low levels of PTEN, PPM1A is also down-regulated, leading to constitutive phosphorylation of Smad2/3 and up-regulation of fibrosis. Additionally, we hypothesized that restoration of PTEN levels in scleroderma fibroblasts will lead to a decrease in the levels of phosphorylated Smad3 and up regulation of PPM1A.

We first analyzed the protein levels of PPM1A by Western blot in 6 scleroderma dermal fibroblasts and 6 normal (control) dermal fibroblasts. PPM1A levels were significantly reduced (2.7 fold down-regulation) in scleroderma fibroblasts as compared with normal controls. Furthermore, the decrease in PPM1A correlated with the increased total protein levels of connective tissue growth factor (CTGF/CCN2). We next examined 3 scleroderma and 3 normal control fibroblasts and, consistent with previous reports, found that PTEN protein levels were significantly decreased in all scleroderma cell lines as compared with healthy controls and these levels correlated with significantly decreased PPM1A protein levels. These results suggest that the decrease in PPM1A protein levels in scleroderma cells could be due to reduced endogenous PTEN levels. To further investigate our hypothesis, we restored the PTEN levels using adenovirus-mediated overexpression of PTEN in scleroderma fibroblasts and observed a significant increase in PPM1A levels in these cells. Similar increase in PPM1A protein levels was observed in healthy control fibroblasts after adenovirus mediated PTEN overexpression.

Our findings suggest that PPM1A is down-regulated in scleroderma fibroblasts, possibly due to reduced levels of PTEN phopshatase. Additionally, PTEN down-regulation could play a role in Smad2/3 phosphorylation and up-regulation of collagen synthesis in scleroderma.

5. Caveolin-1 overexpression in a subset of SSc dermal fibroblasts promotes TGFβ/Smad1 signaling and contributes to MMP1 down-regulation

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Published evidence suggests that caveolin-1 (cav-1), the principal component of caveolae, has antifibrotic effects by virtue of its capacity to down-regulate the canonical TGFβ/Smad3 signaling. Scleroderma fibroblasts secrete exaggerated amounts of ECM proteins, including collagen and CCN2, and have decreased levels of MMP1. Our recent studies showed that the profibrotic TGFβ/Smad1 pathway is constitutively activated in a subset of SSc fibroblasts and is required for the TGFβ induced CCN2 up-regulation. **Cav-1 has been documented to promote Alk1/Smad1 signaling in endothelial cells (1).** Currently, very little is known about how Smad1 is constitutively activated or how MMP1 is down-regulated in SSc.

The aim of this study was to examine the role of cav-1 on TGFβ/Smad1 signaling and profibrotic gene expression in cultured SSc and normal dermal fibroblasts.

In contrast to published observations that showed down-regulation of cav-1 in SSc skin, our preliminary data shows that a significant subset of SSc patients (9 out of 12 SSc/NS pairs) have up-regulated cav-1 expression in their dermal fibroblasts. Additionally, we found that constitutive Smad1

phosphorylation correlates with cav-1 overexpression in these cells. Confirming its role as a positive regulator of Smad1 signaling in endothelial and vascular smooth muscle cells, cav-1 interacted with Alk1 receptor in human dermal fibroblasts. Additionally, depletion of cav-1 using siRNA resulted in inhibition of TGFβ/Smad1 signaling in SSc and normal dermal fibroblasts. Furthermore, adenovirus mediated cav-1 overexpression in normal dermal fibroblasts led to up-regulation of TGFβ/Smad1 signaling and to a dose dependent increase in CCN2 protein.

In order to investigate the molecular mechanisms that might contribute to reduced MMP1 expression by SSc fibroblasts we examined the effects of siRNA mediated cav-1 depletion or adenovirus mediated cav-1 overexpression on the MMP1 mRNA and protein levels using real time RT-PCR and western blot analysis. siRNA treatment almost completely depleted the mRNA levels of cav-1 (up to 93%) and resulted in a potent and significant up-regulation of MMP1 mRNA (up to 5x) and protein levels, while cav-1 overexpression to the levels seen in SSc resulted in a two fold decrease in MMP1 mRNA levels, suggesting that cav-1 is a negative regulator of MMP1 gene expression. The up-regulation of MMP1 protein following cav-1 blockade correlated with an increase in Erk1/2 activation, suggesting that cav-1 controls MMP1 gene expression via an Erk1/2 dependent mechanism.

Taken together these results suggest that cav-1 is up-regulated in the skin of a significant subset of SSc patients and that this could contribute to the profibrotic SSc phenotype by enhancing TGFβ/Smad1 signaling and down-regulating MMP1 expression. By bringing additional insights into the role of cav-1 in SSc fibrosis our study suggests that we need to carefully select the patients that might benefit of treatment with cav-1 peptide.

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6. Identification of new autoantibody specificities in different subsets of systemic sclerosis patients

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Abstract

Objectives. Identify new target auto-antigens in patients with systemic sclerosis (SSc) and antinuclear antibodies (ANA) without identified specificity.

Methods. We have used two-dimensional electrophoresis and immunoblotting with HEp-2 cell total and nuclear protein extracts as sources of autoantigens. Sera from 45 SSc patients were tested in 15 pools of 3 phenotypically identical patients. Sera pool of 12 healthy blood donors was used as control.

Results. Serum IgG in 15 pools of SSc patients recognized 142±44 and 175±77 protein spots in total and nuclear protein extracts, respectively. Twenty-three spots were specifically recognized by IgG from at least 4/11 pools of patients with ANA without identified specificity. Fourteen and 12 proteins were recognized by IgG from at least 75% of the 15 pools of patients in total and nuclear protein extracts, respectively. A number of these antigens were recognized with a higher intensity by IgG from patients with ANA without identified specificity and not by IgG from other patients and healthy blood donors, including Triosephosphate isomerase and Superoxide dismutase [Mn], mitochondrial precursor in total protein extract, Heterogeneous nuclear ribonucleoprotein L in nuclear protein extract and Lamin A/C in both protein extracts. In addition, target antigens specifically recognized by IgG from subsets of phenotypically identical patients with ANA without identified specificity were identified, including Cofilin 1, Peroxiredoxin-2 and Calreticulin.

Conclusions. In SSc patients with ANA without identified specificity, we have identified a number of new target antigens either shared among these patients or specific for a given phenotype.

7. Antibodies to vascular smooth muscle cells in idiopathic and systemic sclerosis-associated pulmonary arterial hypertension

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Rationale. The pathophysiology of pulmonary arterial hypertension (PAH) is characterized by remodeling of the pulmonary arteries due to smooth muscle cell (SMC) and endothelial cell hyperproliferation and fibroblast activation. We have previously reported the presence of antibodies to endothelial cell and to fibroblasts in patients with idiopathic or systemic sclerosis (SSc) associated PAH. **Objectives**. To detect antibodies directed toward vascular SMC in idiopathic PAH (iPAH) and SSc-associated PAH (SSc-PAH), characterize their target antigens and investigate their function. **Methods**. Sera from 15 patients with iPAH, 15 with SSc-PAH, 15 with SSc without PAH and 12 healthy individuals were tested for the presence of IgG antibodies to vascular SMC by using one and two dimensions immunoblot with protein extract of immortalized human mammary artery SMC. The effect of whole serum or purified IgG on aortic vascular SMC was investigated by using an *in vitro* assay of collagen matrix contraction. **Results.** In one dimension immunoblot, serum IgG from SSc patients, patients with iPAH and healthy individuals tested individually reacted with 7-10, 4-8 and 2-5 protein bands respectively, with qualitative and quantitative differences between groups. In 2 dimensions immunoblot, IgG of pools of patients with iPAH, IgG of pools of patients with SSc with or without PAH, and IgG of a pool of healthy individuals recognized 145±48, 127±26, 130±25 and 150 protein spots respectively. Twenty-one protein spots were recognized by more than 80 % of IgG of pools of sera in each group of patients and not by IgG of healthy individuals. The great majority of IgG of pools of patients recognized 27 protein spots with a higher intensity than IgG of pools of healthy individuals. Identified proteins were components of cytoskeleton, proteins involved in oxidative stress such as stress-induced phosphoprotein 1 and peroxyredoxin 6 and proteins involved in regulation of cell energy metabolism such as triosephosphate isomerase. Furthermore, whole serum and purified IgG of patients with iPAH, SSc-PAH or SSc without PAH induced significantly increased contraction of a collagen matrix than did whole serum or purified IgG from healthy individuals. **Conclusion**. We have identified antibodies to vascular SMC in the serum of patients with idiopathic and SSc-associated PAH. These antibodies bind to cytoskeleton, oxidative stress and cell cycle antigens and can induce the contraction of vascular SMC.

8. CD39/CD73 double knockout mice are protected against bleomycin-induced dermal fibrosis.

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BACKGROUND: Adenosine may be formed intracellularly from adenine nucleotides or extracellularly through sequential dephosphorylation by nucleoside triphosphate diphosphohydrolase (CD39) and ecto-5'nucleotidase (CD73). Deficiency in either or both results in vastly decreased adenosine levels in tissues. The resultant decrease in tissue adenosine protects against bleomycin-induced pulmonary fibrosis. To further characterize the contribution of endogenous adenosine to skin fibrosis, we determined whether changes in nucleoside levels regulate dermal fibrosis.

METHODS: Male CD39/CD73-double deficient mice (CD39/73 KO) were injected with the fibrosing agent bleomycin (0.1U sc qod) for 18 days and compared to wild-type littermates. After sacrifice, dermal morphometric measurements were assessed on freshly excised skin and 6mm skin punch biopsies (4 biopsies from the dorsum of each animal). Dermal collagen content was measured by hydroxyproline determination and skin adenosine levels were assessed by HPLC.

RESULTS: CD39/CD73 KO mice showed lower dermal thickness (0.29±0.05 vs. 0.36±0.10mm), skin-fold thickness (0.79±0.16 vs. 0.97±0.37mm), tensile strength (198.2±7.3 vs. 248.7±7.0g) and hydroxyproline content (21.7±1.2 vs. 26.5±1.1μg/mg tissue) ($n=6$, $p<0.01$ for each) compared to control mice after bleomycin treatment; correlating with a two-third decrease in adenosine levels compared to wild-type controls.

CONCLUSIONS: The skin is relatively resistant to the fibrosing effects of the well-known sclerosant, bleomycin, in an animal model of low tissue adenosine. Adenosine is necessary for the full sclerosing effects to occur in response to a toxic insult such as bleomycin. Blockade of adenosine or its downstream effectors may be useful in the treatment of diseases such as scleroderma where dermal fibrosis is a prominent manifestation.

9. Role of n-terminal pro-bnp in detecting heart involvement in systemic sclerosis patients

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Background: Cardiac involvement is common in Systemic Sclerosis (SSc) and is often clinically occult. However, it is recognised as a poor prognostic factor contributing significantly to mortality among these patients. Early detection of cardiac disease with non-invasive tools is therefore critical. In recent years, non-invasive approaches with biological markers including B-type natriuretic peptides has been demonstrated to be potentially valuable in the assessment of pulmonary arterial hypertension in SSc.
Aim: To assess the role of N-TproBNP in SSc related cardiac involvement in a retrospective cohort of patients.
Methods: 19 SSc patients (13 dcSSc and 6 lcSSc, 13 female) patients with cardiac involvement were enrolled in this study. Cardiac involvement was defined as haemodynamically significant arrhythmias, pericardial effusion or congestive heart failure, requiring specific treatment. All patients had normal pulmonary artery systolic pressure and none had serum creatinine over 140 mol/l This group of patients was compared with 19 age- and sex-matched SSc patients without evidence of cardiac involvement or pulmonary hypertension. Serum N-TproBNP levels were measured with the Roche Modular Analytics E-170 (Eleccys module) immunoassay. Normal N-TproBNP levels were less than 20 pmol/l. Associations between N-TproBNP and left ventricular ejection fraction, Troponin-I, systolic and diastolic blood pressure, lung function (FVC and DLCO) and mRSS were determined by Pearson's correlation coefficient. Univariate mortality analysis was performed with Kaplan-Meier method, the level of significance of the differences among survival curves was assessed by Log-rank test. Unpaired t-test was used to compare N-TproBNP values between subgroups based upon disease subset, gender, presence of cardiac involvement or requirement for intracardiac defibrillator. N-TproBNP levels were compared at presentation of cardiac involvement and at six month follow up using a paired t-test. Within the group with cardiac involvement, high levels of N-TproBNP levels were defined as above the median value of 219 pmol/l. Statistical analysis was performed using Minitab 15.

Results: Compared with those without cardiac involvement, N-TproBNP was significantly increased in SSc patients with cardiac involvement (mean±SD 14.9±14.5 pmol/l versus 1043±2053 pmol/l respectively, p=0.037; 95%CI 67,1989). Among those with cardiac involvement, there were no significant differences in N-TproBNP between genders (mean±SD 11829±1921 pmol/l in males versus 1021±2259 pmol/l in females, p=0.876; 95%CI -2048,2370) or disease subsets (mean±SD 1262±2500 pmol/l for diffuse versus 651±909 pmol/l in limited, p=0.459; 95%CI -1102,2324). There was a progressive reduction in N-TproBNP after the acute phase of cardiac involvement (mean±SD 301±330 pmol/l) during 6 months follow-up (mean±SD 87±113 pmol/l; p=0.048, 95%CI 2.8,425). Serum N-TproBNP levels correlated negatively with left ventricular ejection fraction (with logarithmic transformation of N-TproBNP values, R^2=0.52, p=0.001). However, there was no correlation between N-TproBNP and systolic BP (R^2=0.32, p=0.018), diastolic BP (R^2=0.04, p=0.434), troponin-I (R^2=0.27, p=0.047), mRSS (with logarithmic transformation of N-TproBNP values, R^2=0.07, p=0.282), FVC (with logarithmic transformation of N-TproBNP values, R^2=0.10, p=0.242), and DLCO (with logarithmic transformation of N-tproBNP R^2=0.024, p=0.563). Moreover, there was no significant difference in the serum N-TproBNP levels between those that required an intracardiac defibrillator and those that did not (mean±SD 783±768 pmol/l versus 1205±2519 pmol/l respectively, p=0.59, 95%CI -1210,2054). In addition, higher levels of N-TproBNP did not predict survival within the two groups of patients with cardiac involvement (p=0.959, at Log rank analysis).

Conclusions: These data suggest that N-TproBNP peptide may be a surrogate marker for cardiac involvement in SSc. It may selectively identify those patients with severe impairment of cardiac function. Further studies are required to evaluate the utility of N-TproBNP levels for cardiac assessment in SSc patients.

10.Alternative Activation of macrophage/monocytes (AAM) is involved in Pulmonary Arterial Hypertension in Scleroderma disease

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Objective: to explore the alternative activation of macrophage/monocytes (AAM) and possible biomarkers in SSc patients.

Methods: Blood samples (PBMC) were collected from healthy controls (HC, n=8) and SSc patients (n=51; ACR criteria) that were classified as diffuse SSc (dSSc; n=16), limited SSc without PAH (lSSc-noPAH; n=19) and limited SSc with PAH (lSSc-PAH; n=11) by heart catheterization. Microarray analysis was performed and expression of mRNA of relevant genes were analyzed on PBMCs and in CD14- and CD14+ populations (Siglec1, MX1, IL13Ra1, CCR1 and JAK2). MRC1 expression, an AAM marker, was also analyzed and after 18 hours of PBMCs stimulation with IL-13. IL-13 and IL-4 were measured in plasma (ELISA).

Results: An increased PBMC expression of IFN-regulated and biomarkers genes distinguished SSc patients from HC (microarray analysis). The biomarker cluster differentiated lSSc-PAH from lSSc-noPAH and the mRNA expression of IFN-regulated genes Siglec1 (p= 0.02) and MX1 (p= 0.03) were increased in all SSc subsets. CCR1 (p<0.001) and JAK2 (p<0.001) were highly expressed in lSSc-PAH patients and in CD14+ population. MRC1 had an increased expression exclusively in lSSc-PAH patients (p< 0.0001), strongly related to PAP (r= 0.52, p= 0.03) and correlated with higher mortality (p= 0.02). A higher MRC1 expression was found in CD14+ population and, IL-13 stimulation increased its expression on PBMCs. IL-13 in plasma was also exclusively higher on LSSc-PAH patients (p< 0.0001).

Conclusion: Alternative activation of macrophage/monocytes may be an important key for the development of PAH in scleroderma disease. Furthermore, MRC1 was found as a biomarker for this severe complication and was related to mortality.

11.Dyspnea in Scleroderma Patients from the PHAROS Registry: A Major Contributor to Disability

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Purpose: Patients with systemic sclerosis (SSc) experience substantial disability related to systemic manifestations of their disease. We sought to determine the impact of dyspnea, as measured by the University of

California San Diego (UCSD) Shortness-of-Breath Questionnaire (SOBQ), on disability in SSc patients with incident pulmonary hypertension (PH) and those at high risk for developing PH.

Methods: We used data from patients enrolled in the Pulmonary Hypertension Assessment and Recognition of Outcomes in Scleroderma (PHAROS) study. Criteria for enrollment include age >18, clinical diagnosis of SSc, and either PH diagnosed by right heart catheterization (mean pulmonary artery pressure ≥25mmHg) within 6 months of enrollment (definite PH) OR evidence of "early" PH defined as: either right ventricular systolic pressure (RVSP) of ≥40mmHg on echocardiogram OR either forced vital capacity (FVC) >70% and diffusing capacity of carbon monoxide (DLCO) <55% of predicted OR an FVC/DLCO ratio >1.6. For this analysis, we included subjects with complete data for self-reported measures at baseline. Early and definite PH groups were compared using Student's t-test and chi-squared tests. Univariate and multivariate linear regression were used to determine the extent to which individual factors predict disability as measured by the Health Assessment Questionnaire (HAQ).

Results: 353 patients (223 early PH, 130 definite PH) with complete baseline data had a mean age of 56.9±11.6 years and disease duration from first non-Raynaud's symptom of 10.6±14.2 years. The majority of patients was female (86%), Caucasian (73%), and had limited cutaneous disease (69%). Patients with definite PH had higher mean dyspnea scores on the SOBQ (1.7±1.1 vs. 1.2±1.0, scale 0-5, $p<0.0001$) and a trend toward greater disability as measured by the HAQ (0.99±0.75 vs. 0.85±0.77, scale 0-3, $p=0.09$) than patients with early PH. Univariate analyses identified the SOBQ ($r=0.69$, $p<0.0001$), 6 minute walk distance (6MWD) ($r=0.32$, $p<0.0001$), DLCO ($r=0.12$, $p=0.03$), RVSP ($r=0.16$, $p=0.004$), and New York Heart Association functional class (FC) ($r=0.33$, $p<0.0001$) as significant predictors of the HAQ. After accounting for age, gender, race, 6MWD, FVC, DLCO, RVSP, FC, and early vs. definite PH group, the SOBQ explained an additional 34% of the observed variance in disability (increase in adjusted R^2 from 0.14 to 0.48, $p<0.0001$).

Conclusions: Dyspnea in SSc patients with incident PH and at high risk for developing PH is a major contributor to disability. The UCSD SOBQ may be a useful tool in assessing dyspnea in this patient population.

12. Evaluation of an Imatinib Response Gene Signature in Patients with Systemic Sclerosis

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Purpose: We previously defined a set of imatinib-regulated genes from lesional skin of two patients with systemic sclerosis (SSc), termed the "imatinib response signature." We sought to further evaluate the clinical effects and gene expression changes induced by imatinib in patients with SSc.

Methods: Patients were treated with imatinib for 24 weeks at an initial dose of 100 mg daily, and titrated up to a maximum of 400 mg daily as tolerated. The primary endpoints of the study were safety and change in cutaneous sclerosis as measured by the modified Rodnan Skin Score (mRSS, scale 0-51) at 24 weeks compared with baseline. Lesional skin biopsies of the upper extremities were obtained at baseline and 4 and 24 weeks after therapy. Total RNA was extracted from skin biopsies using Qiagen RNeasy fibrous tissue kit. RNA was amplified using Agilent's Quick Amp Labeling Kit, no dye. Amplified skin RNA (labeled with NEN brand Cyanine 3-CTP, 100 nmol) and amplified Stratagene Human Universal Reference RNA (labeled with NEN brand Cyanine 5-CTP, 100 nmol) were competitively hybridized to Agilent Whole Human Genome 4x44K oligo microarrays.

Results: Seven patients with diffuse cutaneous SSc (dcSSc) and progressive skin disease (median disease duration 1year, range 0.5-13) and 2 patients with limited cutaneous SSc (lcSSc) and interstitial lung disease (ILD) were initially enrolled in the study. Baseline mRSS in the dcSSc patients ranged from 19-49 (median 36). Seven patients (6 dcSSc, 1 lcSSc) completed 24 weeks of imatinib therapy at a median dose of 300 mg daily. One patient withdrew from the study at 4 weeks due to a keratopathy related to underlying SSc, and one patient with lcSSc and severe ILD died after 8 weeks of therapy due to pneumonia and subsequent sepsis. Common adverse events affecting two-thirds of patients included gastrointestinal complaints, edema, and infections. The mean mRSS improved by 32% at week 24 ($p=0.005$), with an average improvement of 11 points in the dcSSc patients, but one patient experiencing minimal improvement (49 to 46).

Skin biopsies pre- and post-treatment were available from four additional patients, all of whom showed gene expression changes that were significantly enriched for the originally defined "imatinib response" gene set ($p=0.025$ to 2.27×10^{-11}, hypergeometric distribution). One patient with lcSSc showed imatinib-induced changes in gene expression that were very similar to our original 2 patients ($p=4.6 \times 10^{-6}$). This patient experienced clinical improvement at week 24 with mRSS decreasing from 6 to 2, decreased ground glass opacities on high resolution CT of the chest, and decreased right ventricular systolic pressure from 69 to 54 mmHg on echocardiogram. The remaining 3 patients demonstrated significant repression of the genes induced by imatinib in our initial patients ($p=0.029$ to 4.9×10^{-9}). These 3 patients had variable clinical responses to imatinib therapy: one patient with dcSSc had improvement in cutaneous sclerosis but worsening ILD; the second was the lcSSc patient who died; and the third was the dcSSc patient who experienced minimal improvement in skin score.

Conclusions: Imatinib therapy at low to moderate doses is tolerated by patients with SSc and may result in clinical improvement in a subset of patients. Further analyses are necessary to confirm the presence of an imatinib response gene signature and its correlation with clinical response in a larger number of patients.

13. Characterization of ECM gene expression in a Fli1 knockdown mouse model of fibrosis.

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Background

Fli1 is in the Ets family of transcription factors and is important for hematopoiesis, embryogenesis and angiogenesis. Due to its important role in vessel formation, $Fli1^{-/-}$ mice are embryonic lethal at E11.5 with a hemorrhagic phenotype. Extensive in vitro studies have also implicated Fli1 as an important anti-fibrotic transcription factor. Fli1 can bind to promoters of genes involved in fibrosis such as Col1a2, CCN2, TN-C and MMP1 and act as either an activator or repressor of gene transcription. Fli1 levels are significantly diminished in the skin of Scleroderma (SSc) patients. This suggests that Fli1 downregulation may be involved in the excessive extracellular matrix deposition seen in SSc. Scleroderma patients suffer from severe skin fibrosis but the complication that causes the highest mortality is pulmonary fibrosis. We hypothesized that a similar gene program which causes skin fibrosis may also lead to fibrosis in the lung. To test this hypothesis we used lungs from $Fli1^{+/-}$ mice which had previously been used to study skin fibrosis in our lab.

Methods

Fli1+/- and control male and female mice age 8-12 weeks were sacrificed and skin was harvested for experiments. Whole collagen was extracted using a pepsin/acetic acid digest of an 8mm skin punch. Dermal fibroblasts were cultured and protein and mRNA was collected for western blot analysis and qPCR, respectively.

Fli1+/- and WT control male mice were sacrificed at 8 months of age and lungs were taken for analysis. qPCR data was analyzed using the $\Delta\Delta$Ct method.

Results

qPCR from skin showed approximately 50% reduction in Fli1 mRNA levels of $Fli1^{+/-}$ mice. An increase of approximately 1.5 fold gene expression in collagen mRNA levels was observed in skin of $Fli1^{+/-}$ mice. However, levels of total collagen protein were shown to be significantly increased in skin by pepsin/acetic acid extraction, this was further confirmed by western blot of collagen type 1. Preliminary data from lung samples show an increase in expression of pro-fibrotic genes in $Fli1^{+/-}$ mice. A significant increase in collagen I was observed at the mRNA level in lungs, Col1a1 (1.4 fold, $p=0.04$), Col1a2 (1.3 fold, $p=0.04$). The largest increase in mRNA levels observed in the lung was Tenascin-C (TN-C, 3.4 fold, $p=0.02$), an important matrix modifying gene. Interestingly, there was also a significant increase of TIMP3 (1.5 fold, $p=0.03$) in lungs of $Fli1^{+/-}$ mice, this suggests an inability to breakdown collagen efficiently.

Conclusion

Our data shows activation of the profibrotic genes Col1a1, Col1a2, TN-C and TIMP3 in the lung of $Fli1^{+/-}$ mice and thus reproduces the fibrotic phenotype shown in the skin of these mice. This suggests that these mice could be a valuable mouse model of SSc.

14. Perturbed VEGF signalling within the pulmonary vasculature of a TGFβ dependent mouse model of systemic sclerosis

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Purpose: Vascular complications of systemic sclerosis (SSc) are a major cause of mortality and morbidity. A role for altered VEGF signaling in SSc-PAH is supported by our recent data that correlate circulating VEGF with mPAP at diagnosis. There is a trend for VEGF levels to fall after initiation of endothelin receptor antagonist therapy. High circulating VEGF levels may be a marker of repair in response to vascular injury. We have therefore examined VEGF signaling in a TGFβ-dependent mouse model of SSc with evidence of a constitutive pulmonary vasculopathy.

Methods: The transgenic mouse strain TβRIIΔk-fib expresses a kinase-deficient type II TGFβ receptor linked to a fibroblast-specific promoter leading to balanced ligand-dependent upregulation of TGFβ signalling. Pulmonary vasculopathy was confirmed by histological assessment of vessel architecture, isolated organ bath and *in vivo* haemodynamic studies performed on adult male transgenic and littermate wildtype animals (up to n=8 in each group). Biochemical analysis of VEGF and endothelin signalling axes were performed assessing RNA by quantitative PCR and protein by Western blotting of cultured using pulmonary artery smooth muscle cells, and by immunostaining of tissue sections. Results were then compared to the same cells cultured under hypoxic conditions.
Results: Within the pulmonary arterial circulation, transgenic intimal diameter was increased, particularly in the smaller 30-60μm vessels due to an increase in smooth muscle ($p<0.05$). Pulmonary arterial ring responses to direct and receptor-mediated contractile stimuli were reduced in the transgenic animals and right ventricular pressures were higher in transgenic animals (wildtype mean 29mmHg±4, transgenic mean 37 mmHg±3, $p=0.14$). Transgenic vascular smooth muscle cells showed upregulation of TGFβ responsive genes including VEGF and VEGFRA which were further upregulated in the pulmonary arterial circulation when compared to aortic cells from the same animals (for example, PA wildtype VEGFRA mRNA 3400±500, PA transgenic 6300±800, $p<0.05$, student's t-test). Endothelin receptor A gene expression was reduced in transgenic animals. Hypoxic culture resulted in upregulation of VEGF and VEGFRA in cells from both wildtype and transgenic animals, again more marked in the pulmonary arterial cells.
Conclusions: The histological, biochemical and functional phenotype of this transgenic mouse model offers insight into the altered vascular dynamics reported in human SSc, and supports a potential role for perturbed TGFβ, endothelin and VEGF activity in this process.

15. Internal Organs are Affected Very Early in Diffuse Scleroderma: Implications for Clinical Trials

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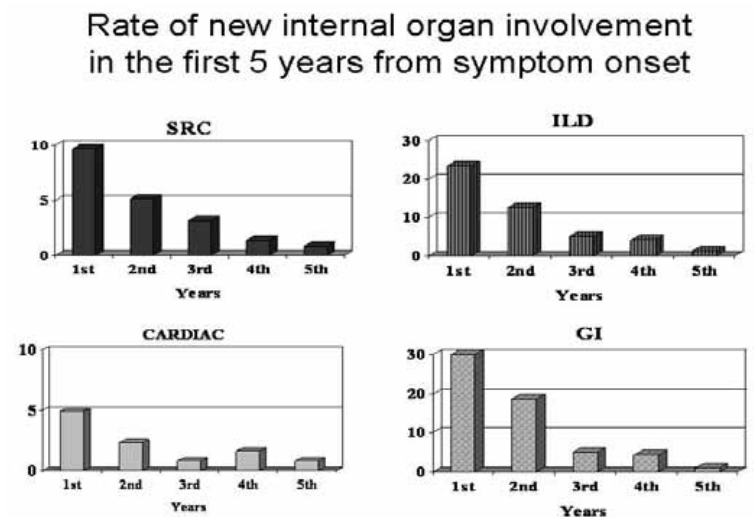
Objective:
To determine the frequency of new internal organ involvement during the first five years of disease in a population of diffuse systemic sclerosis (dcSSc) patients.

Patients and Methods:
We included SSc patients from the prospectively enrolled University of Pittsburgh Scleroderma Databank who were seen for an initial visit between 1980 and 2007. A minimum of two separate evaluations within five years after the first symptom attributable to SSc was required. We considered both patient-reported symptom onset attributable to a specific disease manifestation, as well as objective criteria. We determined the rate of new organ involvement for each year of time from symptom onset by dividing the incident cases into those at risk for each year (x 100). We examined scleroderma renal crisis (SRC), interstitial lung disease (ILD), cardiac involvement, gastrointestinal (GI) involvement and pulmonary arterial hypertension (PAH).



Results:

A total of 695 SSc patients met inclusion criteria. The mean age at the first symptom was 45.9 years with a standard deviation (SD) of 14.5 years. The median time from first symptom to initial visit was 1.11 years with an interquartile range of 0.73 – 2.02 years. 76% of patients were female, 90% Caucasian and 6% African-American. Mean skin score at first visit was 23.7 (SD 11.8). A total of 21% developed SRC, 42% ILD, 18% cardiac involvement, 55% GI involvement and 4% pulmonary hypertension not attributable to ILD.

The rate of new organ involvement by each year after symptom onset is shown to the left in Figure 1. For PAH the risk was 0.7% in year 1, 0.1% in year 2, 0.3% in year 3, and 0.4% in years 4 and 5. For all other organ systems the rate was highest in the first year after symptom onset and rapidly diminished over the next 4 years. The timing of organ involvement was the same when objective criteria were satisfied.

Conclusions:

For each major internal organ involvement, early dcSSc patients were at greatest risk in the first and second years after symptom onset. For each organ system, the occurrence of new internal organ involvement was less than 6% in years 3 – 5. These data strongly suggest that treatment with a goal of preventing new organ involvement in dcSSc should be initiated during the first year of disease. Similarly, clinical trials in dcSSc should enroll only patients with disease duration less than 2 years after the first symptom of disease if new organ system involvement is considered an important endpoint.

16. Selective targeting the Platelet-derived growth factor ligand-receptor pathway: relevance to the pathogenesis of Systemic Sclerosis

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Background. Platelet derived growth factors (PDGFs) are released during the inflammatory process. They are potent activators of migration and proliferation in mesenchymal cells such as fibroblasts. PDGFs have previously been implicated in the pathogenesis of scarring and fibrosis. Recent reports of PDGF receptor (PDGFR) autoantibodies in Systemic Sclerosis (SSc), coupled with on-going trials using selective PDGFR kinase inhibitors (such as Imatinib), has lead to more focused studies on PDGFs and their receptors in SSc. There are five different PDGF ligands: PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and PDGF-AB, which bind three dimeric receptors: PDFGRαα, PDGFRββ and PDGFRαβ, with varying specificities and affinities. However, it is not known which ligand/receptor combinations are expressed in SSc and contribute to excess scar formation.

Objectives. To analyse the differences in distribution of each PDGF ligands and receptors in SSc and normal skin. To assess changes in gene and protein expression and cell function associated with loss of either PDGFRα or PDGFRβ *in vitro* and *in vivo* using global and conditional gene-deletion strategies. To delineate PDGFR-specific signal transduction pathways.

Methods. Immunohistochemistry was used to analyse expression and distribution of PDGFRs in sections of early diffuse SSc and normal skin. As depletion of PDGFRs is embryonic lethal, fibroblasts were harvested from transgenic mice harbouring either a conventional knockout of PDGFRβ or an inducible tissue-specific deletion of PDGFRα under the control of a fibroblast-specific promoter. In the latter case, efficient gene deletion was achieved utilising adenoviral expressed Cre technology. PCR and Western blotting with receptor-selective antibodies was used to verify the deletion of the individual receptors. Phospho-protein profiling of normal and Imatinib-treated fibroblasts was used to examine the phosphorylation of PDGFR and downstream targets.

Results. PDGFRs and in particular PDGFRβ, were widely distributed within the dermis of SSc patients, notably within inflammatory infiltrates,

fibroblasts and pericytes. Three approaches were employed to delineate receptor-specific effects. As the PDGFR knockouts are embryonic lethal, we harvested embryonic fibroblasts from conventional PDGFRβ knockout mice and we utilised adenoviral Cre to knockout a loxP-flanked PDGFRα *in vitro*. PCR and Western blot confirmed efficient deletion of the respective cell types. Changes in profibrotic endpoint in receptor-deficient cells are being assessed to delineate receptor specific effects. In parallel, the effect of knockdown of the individual receptors in normal and SSc fibroblasts is being assessed using siRNA in conjunction with pharmacological inhibitors. Selective pharmacological inhibition of PDGFRα and PDGFRβ in normal fibroblasts (using Imatinib) blocks PDGF-BB-dependent phosphorylation of PDGFR, suppressed activation of mitogen-activated protein kinase ERK and induced changes in the phosphor-protein profile.

Conclusions. The signalling pathways medicated by PDGFRα and PDGFRβ are important regulators of SSc. Receptor specific targeting may provide more focused therapies to reduce fibrosis in the intractable disease.

17. Effects of the Green Tea Antioxidant Epigallocatechin-3-Gallate on Collagen Production and Fibroblast Activity in Systemic Sclerosis.

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Background: Systemic sclerosis (SSc) is characterized by increased deposition of extracellular matrix (ECM) proteins such as collagen and fibronectin, as well as oxidative stress. Previously we have shown enhanced oxidation of lipoproteins and also abnormal nitric oxide metabolism in the plasma of patients with SSc and in the tight skin mouse model of fibrosis. The current study investigates the anti-fibrotic potential of the antioxidant epigallocatechin-3-gallate (EGCG) on activated dermal fibroblasts from SSc patients.

Methods: Dermal fibroblasts from a cell line (AG), healthy individuals (CON), and SSc patients were treated with EGCG, TGF- , PDGF-BB or other antioxidants (SOD, catalase, NAC, DPI). Collagen type I, fibronectin, CTGF, -SMA, and MAP kinases were measured by ELISA and/or Western blot. Fibroblast contractile forces were measured by collagen gel contraction. Reactive oxygen species (ROS) was assessed by DCF-DA fluorescence, and NF B activity by DNA binding assay.
Results: EGCG (1-100 M) dose-dependently decreased collagen type I secretion in culture medium after 24 h in AG fibroblasts. Collagen type I protein expression in cell lysates was also significantly reduced by 40% in EGCG (40 M) treated cells. Furthermore, EGCG also down-regulated TGF- -induced collagen type I, fibronectin, and CTGF. Similarly, in CON fibroblasts EGCG decreased basal and stimulated collagen type I, fibronectin, and CTGF after 24 h, while in SSc the effects of the antioxidant were apparent after 48 h. Fibroblast-mediated contraction of collagen gels was inhibited by EGCG as early as 1 h in AG fibroblasts, and in CON and SSc. Additionally, EGCG also inhibited TGF- -stimulated gel contraction similar to other antioxidants DPI and NAC, but not SOD or catalase. EGCG suppressed TGF- -induced ROS production in all fibroblasts. Furthermore, EGCG inhibited TGF- or PDGF-BB-induced phospho-ERK1/2 MAP kinase and NF B activity in SSc fibroblasts.
Conclusion: The results suggest that the antioxidant, EGCG, can reduce ECM production, the fibrotic marker CTGF, and inhibit contraction of dermal fibroblasts from SSc patients. Furthermore, EGCG was able to suppress intracellular ROS, ERK1/2 kinase signalling, and NF B activity. Taken together, EGCG may be a possible candidate for therapeutic treatment aimed at reducing both oxidant stress and the fibrotic effects associated with SSc.

18. Evaluation of the durometer to access skin thickness in healthy children

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Background: The evaluation of the skin thickness manually for the Modified Rodnan Skin Score has a low correlation coefficient. In several studies with adult patients the Durometer was applied to access the skin thickness. The measured arbitory score from 0 to 100 does changes during treatment trials. The are currently no norm values for children.
Objectives: to establish norm values for skin thickness for different body parts in children.
Methods: Consecutive patients of the paediatric rheumatology clinic, without a skin disease, or a skin involvement of the rheumatic disease, were prospectively evaluated for the Durometer score to establish norm values for the different anatomic areas. The points of the assessment of the anatomic area was standardized. Certain anatomic areas with an underlying bone structure were excluded.
Results: In 244 consecutive patients the skin thicknes with the Durometer was evaluated. The mean age of the patients was 11.9 years. The mean tanner score was 2.1. Most of the patients had the diagnosis of juvenile idiopatich arthritis,exluding psoriatc arthritis. The mean values were for the upperarm 24.4, for the lower arm 29.8, for the hand 15.9, for the upper leg 30.5, for the lower leg 28.8, for the back of the feet 24.5, for the abdomen 19.8, for the subclavicular region 14.5. The mean values for the region over the finges were 50, because of the bony underlayer.
Conclusion: We established the norm values of skin thickness for certain anatomic areas with the durometer for healthy controls. The durometer can not be applied in regions , where there is bony structure directly under the skin surface, like fingers. The values for the upper arm are in the similar range as established in healthy adults. The application of the durometer will help to have a more objective way to access the MRSS, as one of the primary outcome measures in therapeutic trials for systemic sclerosis.

19.Preliminary results for 6 minute walk values in healthy German children.

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Introduction
6 minute walk is a primary outcome measure in therapeutic studies for patients with pulmonary hypertension. Currently we have a two of sets of data (Lammers[1] et al and Li [2]et al)regarding test results in the 6 minute walk test (6MWT) in healthy children with a large span in the norm values in the different age groups.

Aim of the study
To establish norm values for healthy German children for the 6 Minute Walk Test.
Method
The team of an occupational therapist and a study nurse is visiting schools, were previously the parents agreed on the participation of the students on the test. Always just students from one class are invited to participate in the test. The students are performing the test according the international guidelines. The demographic data of the students are collected and the parents fill out a short survey regarding the physical activity and the health condition. Children with chronic diseases, which decrease the stamina are excluded.
Results
Up till now 208 students participated from the age 7 to 10 years. 90 of the 208 were female. 30 in the age group of 7; 50 in the age group of 8 years; 67 in the age group of 9 years and 61 in the age group of 10 years. The mean 6 minute walk distance was 463.73 m in the age group of 7; 479.09 m in the age group of 8 ; 492,72 m in the age group of 9 and 488.32 m in the age group of 10.
Conclusion
Our results are in the range of the patients from the UK published by Lammers et al [1] and are in significantly lower range than in the Chinese population collected data by Li et al.[2]. This reflects the importance of this study to gain norm values for our patient population.
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Background: Juvenile systemic sclerosis (jSSc) is a rare autoimmune disease. Currently just retrospective data exist without a standardized assessment of the organ involvement. Our project is the first projects, where prospectively and with a standardized assessment data of early jSSc patients are collected.
Objectives: to learn about the evolvement of juvenile systemic sclerosis
Methods: Using the proposed standardized patient assessment protocol patients with early jSSc ,entry into the cohort within the first 24 months of disease, are prospectively assessed. All participating centres approved the protocol over the own IRB.
Results: 42 centres from 20 countries applied to participate on the project. The assent and consent forms were translated into the local native languages. Up till now 14 patients were enrolled, the mean follow up of the patients in the cohort are 1.6 years. Twelve of the 14 patients were female. The mean age at the onset of the non-Raynaud symptomatic were 12.4 years. Seven of the 14 have diffuse subtype, one of them have overlap features and 7 of the 14 have a limited subtype and 3 of these have overlap features. At the time of the inclusion the mean modified Rodnan Skin Score was 16.5 (range, 2 to 46). 12 were ANA positive, and 5 of them were anti-Scl 70 positive. None of them was anticentromere positive. Twelve of the 14 have Raynaud´s, 9 of them have capillary changes and 4 of them already ulcerations. 6 of them have cardiopulmonary involvement , 4 of them have interstitial lung disease. One has renal involvement associated with hypertension, but not renal crisis. Seven of them have gastrointestinal involvement, and 5 of them oesophageal involvement. Twelve of ten have musculoskeletal involvement, 9 of them with joint contractures.
Conclusion: We present the data on the first 14 prospectively assessed patients with jSSc. The current recruitment data confirms that pediatric patients are different from the adult patients. We are only at the beginning of this project and hope to recruit up to 50 patients and follow them prospectively over the next 2 to 4 years at least.

21. Repression of collagen type I by TGF-ß induced CUX1.

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TGF-β is a major inducer of transcriptional activation of fibrillar collagens, which in pathological conditions leads to excessive extracellular matrix deposition and fibrosis. We hypothesized that identifying a mechanism to switch off collagen type I would prove beneficial in preventing and treating established fibrosis. In this study we report that the homeobox transcription factor CUX1 is a negative regulator of fibroblast activation, a crucial event in tissue remodeling often leading to scarring and fibrosis. We show for the first time that CUX1, a CCAAT binding factor displacement protein, is associated with reduced expression of collagen type I. This effect is generated following enhanced expression of CUX1 or the addition of TGF-β. We present evidence to show that CUX1 is responsible for suppressing collagen type I via binding to with two CCAAT boxes located within the promoter of collagen, thus interfering with normal occupancy of the *cis*-acting sequence by CBF/NF-Y. We established an in vivo model of progressive interstitial fibrosis and observed that CUX1 expression was significantly in fibrotic tissue compared with tissue from sham-treated animals. Conversely, CUX1 knockdown in kidney fibroblasts increased the

production of collagen type I. These studies demonstrate that modifications of CUX1 expression lead to down-regulation of collagen type I, which may provide a molecular basis for fibrogenesis. Taken together, we postulate that CUX1 represents an important regulatory factor in fibrosis and a novel therapeutic target.

22. The Role of GATA-6 in the pathogenesis of Pulmonary Arterial Hypertension in Scleroderma

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Background Pulmonary arterial hypertension (PAH) is an increase in blood pressure in the pulmonary artery or lung vasculature and in severe cases can lead to right ventricular heart failure and death. PAH can be idiopathic (cause unknown), familial (genetic), or associated with a variety of other conditions, such as connective tissue diseases like systemic sclerosis (SSc). The key features of this disease are vasoconstriction, followed by intimal proliferation and fibrosis, in-situ thrombosis, and plexogenic changes. Several factors are known to be perturbed in endothelial cells (ECs) during PAH: TGF-β/Bone Morphogenetic Protein (BMP) components, nitric oxide (NO), and endothelin-1 (ET-1). GATA-6 is a zinc finger transcription factor that is indispensable for embryogenesis and subsequent tissue-specific gene regulation. Recent studies have demonstrated that GATA-6 is downregulated in both intramyocardial arteries from spontaneously hypertensive rats and rat carotid arteries following balloon-mediated injury. The goal of this work was to evaluate the potential role of GATA-6 as a novel regulator of gene programs altered in PAH.

Methods The protein levels of GATA-6 were assessed by immunostaining of lung specimens from 9 patients with SSc-PAH and 4 healthy controls. To identify genes regulated by GATA-6 in microvascular ECs, we performed a microarray analysis after AdsiRNA-mediated GATA-6 knockdown, and subsequently validated putative target genes using commercial siRNA oligos followed qRT-PCR and western blotting. To investigate if GATA-6 is a direct transcriptional regulator of these genes, we performed chromatin immunoprecipitation (ChIP) analysis. The mRNA and protein levels of GATA-6 and its potential targets were also examined in the lungs of the monocrotaline (MCT) rat model of PAH by using qRT-PCR and western blot.

Results GATA-6 protein levels are dramatically reduced in ECs (15% stained positive in SSc-PAH versus 85% in controls) within occluded and non-occluded vessels of patients. Genes altered in endothelial cells after suppression of GATA-6 included components of the BMP pathway, endothelial cell markers and matrix remodeling proteins. Silencing of GATA-6 with siRNA oligos in ECs confirmed that GATA-6 might be a negative regulator of MMP1 and MMP10 and a positive regulator of eNOS, VE-cadherin and BAMBI gene expression. ChIP analysis demonstrated that GATA-6 is a direct transcriptional regulator of BAMBI, eNOS, MMP1 and MMP10. We also found significant reductions in GATA-6 expression as well as decreased mRNA levels of its putative targets, BAMBI, eNOS and VE-cadherin at both early and late stages of disease in the MCT rat model.

Conclusion These findings suggest that GATA-6 reduction occurs before vessel occlusion and may reflect an initial phase of EC activation and/or dysfunction and, therefore, may play a critical role in development of PAH by regulating genes associated with vascular remodeling.

23. Gene-Gene Interaction between *IL1A* Promoter Polymorphism (-889C/T) and Major histocompatibility complex (MHC) class II alleles in Systemic Sclerosis

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Introduction: In the pathogenesis of systemic sclerosis (SSc), one candidate for an autocrine factor that could regulate the phenotype of SSc

fibroblasts is the proinflammatory cytokine IL-1α, which has been found to be constitutively expressed in epidermal cells and fibroblasts cultured from the lesional skin of SSc patients. A single-nucleotide polymorphism (SNP) within the promoter region, at position -889 (-889C/T), of the *IL1a* gene has been suggested to influence gene transcription and IL-1α production. We have previously shown association of this polymorphism with SSc and its centromere and limited subsets. This polymorphism also has been reported to confer a poor functional response to cyclophosphamide in fibrosing alveolitits. MHC class II allelic associations with SSc have been reported with *DRB1*, *DQB1*, *DQA1* alleles and their haplotypes. The purpose of this work was to investigate any possible gene-gene interactions between the most common MHC class II alleles/haplotypes (on chr 6) and the *IL1a* (on chr 2) SNP.

Methods: SNP genotyping for *IL1a* polymorphism was performed using the Taqman Assay (Applied Biosystems) in 800 Caucasian patients along with 492 race-matched controls. HLA-*DQA1*, -*DQB1* alleles were oligotyped and *DRB1* alleles were directly sequenced. All SSc patients fulfilled ACR criteria or had at least 3 of the 5 CREST features. Chi-square (χ^2), Fishers exact test, linkage disequilibrium, mutual information (MI) and logistic regression (LR) were used for statistical analyses.

Results: Only the major MHC class II alleles showing association with SSc from previous studies were tested for their interaction with *IL1a* polymorphism. There was an extremely significant interaction in SSc group between -889C/T and *DQB1**0301 with χ^2 $p=1.75\times10^{-12}$ and MI $p= 2.31\times10^{-5}$ and OR (95%CI)= 4.97(2.5-9.9) for risk allele in both the genes. The risk conferred by IL1a alone was 1.82(1.2-2.8) and with *DQB1**0301 alone 1.50(1.2-1.9). In centromere antibody + subset besides *DQB1**0301, there was a significant interaction with -889C/T and *DRB1**01, and -889C/T and *DQB1**0501 with both χ^2 p and MI $p <10^{-8}$. For a double hit in -889C/T and *DRB1**01 the OR (95%CI) was 6.19(2-19) and in in -889C/T and *DQB1**0501 the OR (95%CI) was 8.42(2.7-26). In Limited SSc subset, there were significant interactions between -889C/T and *DRB1**0404, *DRB1**1104, *DRB1**1104 with OR (95%CI) of 3.14(1.2-8), 5.2(1.7-16.4), 5.44(2.2-13.5) respectively and both χ^2 p and MI $p <10^{-4}$. All these interactions were also significant by the logistic regressional analysis and remained significant after bonferroni's correction.

Conclusion: We demonstrate an interaction in SSc between two non-linked loci- *IL1a* and *DRB1* and *IL1a* and *DQB1* genes using 3 different statistical tools- chisquare, mutual information and logistic regression. The risk for SSc susceptibility increases when there are polymorphisms on both the genes as compared to each of them alone. This interaction is also observed in centromere + and limited subsets of SSc. These results open up the door to investigate the interactions amongst other SSc susceptibility linked loci. The exact role of these interactions and their function in SSc susceptibility needs to be elucidated experimentally.

24. Differential expression of junctional adhesion molecules (JAMs) in systemic sclerosis (SSc) skin

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Objective: Junctional adhesion molecule (JAM)-A and JAM-C are immunoglobulin superfamily members which regulate leukocyte-endothelial cell (EC) interactions by virtue of their ability to undergo heterophilic binding with the leukocyte integrins LFA-1 and Mac-1, respectively. In addition, their propensity to interact homophilically at endothelial and epithelial junction sites suggests that JAMs may participate in the regulation of paracellular permeability. JAMs have been implicated in a variety of physiological and pathological processes involving leukocyte transmigration, tight junction assembly, vascular endothelial permeability and angiogenesis. SSc is characterized by early perivascular inflammatory infiltrates, vascular damage and defective angiogenesis in the skin and internal organs. Our aim was to investigate the expression of JAM-A and JAM-C in SSc skin and in dermal microvascular endothelial cells (MVECs) challenged with sera from SSc patients.

Patients and Methods: Skin biopsy samples were obtained from the clinically involved skin of 16 SSc patients (9 lcSSc, 7 dcSSc, 10 early and 6 late disease phase) and from 10 healthy control subjects. Peripheral blood samples were also obtained. Serial skin sections were immunostained with rabbit polyclonal antibodies against human JAM-A or JAM-C, and immune reactions were revealed using fluorochrome-conjugated secondary antibodies. A monoclonal antibody against podoplanin (D2-40, a lymphatic EC specific marker) was used to differentiate blood (D2-40-negative) and lymphatic (D2-40-positive) microvessels. Primary human dermal MVECs were grown to confluence and then stimulated with human recombinant VEGF or with early SSc (n=5) and healthy control (n=4) sera for different

time points (1, 6, 24 hours). MVECs were double-immunolabeled for JAM-C and the human tight junction protein zonula occludens-1 (ZO-1), as well as for JAM-A and the pro-angiogenic $\alpha_V\beta_3$ integrin which form a complex on EC surface. Immunostained tissue sections and cells were examined by confocal laser scanning microscopy. Densitometric analysis of immunofluorescent staining intensity was performed on digitized images using ImageJ software.

Results: In control skin, constitutive expression of JAM-A was observed in dermal blood and lymphatic ECs, fibroblasts and keratinocytes. In early SSc skin, JAM-A expression was increased in blood and lymphatic vessels and fibroblasts. Moreover, perivascular inflammatory cells showed strong JAM-A immunopositivity. In late SSc, JAM-A expression was weaker than in controls. JAM-C was weakly expressed in control skin. In early SSc skin, JAM-C expression was markedly observed in blood and lymphatic vessels and fibroblasts, as well as in perivascular inflammatory infiltrates. Instead, in late SSc skin JAM-C expression was similar to controls. The stimulation of MVECs with early SSc sera increased the expression of JAM-A and $\alpha_V\beta_3$ integrin on the cell surface. JAM-C expression was found in the cytoplasm of MVECs at basal conditions and under stimulation with healthy control sera. Upon challenging with early SSc sera, JAM-C was rapidly recruited from the cytoplasm to the tight junctions where it was colocalized with ZO-1, showing the maximun effect after 1 hour. Similar effects were observed after 1 hour stimulation with VEGF.

Conclusions: JAM-A and JAM-C are abnormally expressed in SSc skin and differentially expressed in early and late phases. Early SSc sera affect the expression and subcellular localization of JAMs in dermal MVECs. Our data suggest that, in SSc, JAMs may be key players in the early inflammatory process, EC activation and impaired angiogenesis.

Keywords: systemic sclerosis, junctional adhesion molecules, skin, microvascular endothelial cells

25. Identification of target antigens of endothelial cells antibodies in systemic sclerosis and idiopathic pulmonary arterial hypertension

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Purpose: to identify target antigens of anti-endothelial cells antibodies (AECA) in systemic sclerosis (SSc) and in idiopathic pulmonary arterial hypertension (iPAH).

Material and methods: we have used a two-dimensional electrophoresis and immunoblotting technique with protein extracts of human umbilical vein endothelial cells (HUVEC). We have tested sera from 18 SSc patients with (n=9) or without PAH (n=9), 9 sera from iPAH patients (3 sporadic, 3 familial and 3 dexfenfluramin-associated PAH) and 12 sera from healthy blood donors as negative controls. These sera were tested at a dilution of 1/100 by pools of 3 for patients and in a pool of 12 for healthy controls. Targeted spots were identified by mass spectrometry.

Results: Serum IgG from SSc patients with or without PAH recognized 42±4, 37±7 protein spots respectively. IgG from patients with sporadic, familial or dexfenfluramin-associated PAH recognized 46, 30 and 30 protein spots respectively, whereas 53 protein spots were recognized by IgG from healthy donors. 64 protein spots were recognized specifically by IgG from SSc patients including 17 spots which were recognised specifically by PAH patients. Only six protein spots were recognized by IgG from more than 75% of patients' pools of sera and not by the IgG from healthy donors. Antigens targeted by IgG AECA from SSc and/or PAH patients have been identified by mass spectrometry (MALDI-toff) including triosephosphate isomerase, peroxiredoxin, cathepsin D, prohibitin and phosphoglycerate mutase 1.

Conclusions: We identified target antigens of AECA in SSc and in idiopathic PAH patients. Additional experiments are needed to characterize their potential pathogenetic role.

26. Major differences among proteomes of macro-vascular and micro-vascular endothelial cells: 2D-DIGE approach

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Rationale. Progress on the isolation and culture of various endothelial cells (EC) has allowed comparison of their biochemical and physiologic properties. However, very few studies compared the proteomes of EC from different sources.**Objective.** To compare proteomes of macro-vascular and micro-vascular EC. **Methods.** Proteomes of human umbilical vein EC (HUVEC) and 2 sources of micro-vascular EC, human pulmonary (HMVEC-P) and dermal micro-vascular EC (HMVEC-D) from healthy caucasian donors (4 in each group) were compared using two-dimension differential in gel electrophoresis (2D-DIGE) at pH ranges of 3-11 and 4-7 and mass spectrometry. **Results.** Among the 2167+/-50 protein spots detected in pH 4-7 gels, 100 were differentially expressed between HUVEC and micro-vascular EC with a ratio ≥ 2 and a T-test ≤ 0.01. Sixty-three proteins were identified including fatty acid binding protein 4 and retinal dehydrogenase 1 that were over-expressed in micro-vascular EC at 235.7 and 5.8 average ratio, respectively. Ingenuity software analysis interestingly showed that numerous proteins over-expressed in micro-vascular EC are implicated in the retinoic acid pathway. Sixteen protein spots were differentially expressed between HMVEC-D and HMVEC-P with a ratio ≥ 2 and a T-test ≤ 0.01 in pH 4-7 gels and 9 were identified. In pH 3-11 gels, 41 protein spots were differentially expressed between HUVEC and HMVEC-D and HMVEC-P with a ratio ≥ 2 and for a T-test ≤ 0.01. Among these protein spots, 33 were identified. Four protein spots were differentially expressed between HMVEC-D and HMVEC-P with an average ratio ≥ 2 and for a T-test ≤ 0.01 and were identified, corresponding to cytoskeleton proteins or enzymes implicated in glycolysis. **Conclusion.** Major differences were observed between proteomes of macro-vascular and micro-vascular EC. Some of the differentially expressed proteins might be of great importance in the homeostasis and pathophysiology of EC.

27. Differential Expression of the Chitinase 3-like protein Chi3L1 Identifies Subgroups of Scleroderma (SSc) Patients

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Scleroderma (SSc) is characterized by connective tissue fibrosis occurring in the skin and, in its diffuse form, also in many organs of the body. Dermal fibroblasts are one of the main effector cells involved in the development of fibrotic lesions, and their biological activity responds to a variety of inflammatory cytokines and growth factors, many of which have been implicated in the progressive development of these lesions in SSc patients.

Chitinase 3-like protein 1 (Chi3L1 also known as YKL-40 and gp39) is a component of the innate stress response of connective tissue cells, and patients with SSc have elevated serum levels of this protein. Therefore we investigated the capacity of fibroblasts from SSc patients and from healthy individuals to synthesize Chi3L1 and the regulation of this process by growth factors and inflammatory cytokines. Fibroblasts were isolated by outgrowth from skin biopsies taken from the abdomen and the arm and maintained in monolayer cell culture. To evaluate Chi3L1 production, cells were exposed to IL1, IL6, oncostatin M (OSM), TGF-ß or PDGF in the absence of serum. Chi3L1 in culture media was analyzed by SDS-PAGE and immunoblotting. Cells actively producing Chi3L1 were identified by immunolocalization.

Our results show that Chi3L1 expression is not detectable in unstimulated normal skin fibroblasts, and its production was not induced by any of the cytokines or growth factors used in this study. In contrast, fibroblasts from some patients showed endogenous production of Chi3L1. Exposure to IL-1 or OSM resulted in a strong upregulation of this process in most patient samples investigated, including those where no endogenous production was observed.

Based on their capacity for endogenous production of Chi3L1 and its regulation by OSM, three response patterns were observed. Group 1 SSc fibroblasts constitutively express Chi3L1 and its expression is up-regulated upon stimulation with OSM. Group 2 SSc fibroblasts do not endogenously

express Chi3L1 but its expression is induced with OSM. Group 3 SSc fibroblasts behave essentially like cells from healthy individuals, in that they do not endogenously express Chi3L1 and its expression is not inducible with OSM.

Fibroblasts derived from the lesion sites on the arm of patients always responded to OSM treatment with increased production of Chi3L1, although endogenous production was not observed in all samples. Fibroblasts derived from abdominal biopsy sites from some patients behaved like their counterparts from the arm, while others behaved like normal skin fibroblasts with no Chi3L1 production under any condition.

Identification of Chi3L1-producing cells in these different fibroblast isolates indicated that only a fraction of the total cell population produced Chi3L1 spontaneously and/or responded to OSM treatment. These cells did not stain positively for α-smooth muscle actin, which identifies myofibroblasts, suggesting that the cells producing Chi3L1 and responding to OSM represent a distinct cell population in fibroblast isolates from SSc patients. The Chi3L1 profiles observed in SSc skin fibroblasts might characterize the different pathological events associated with lesion development. Therefore Chi3L1production could be used for characterizing disease severity and progression of SSc.

28. Non-invasive imaging for the assessment of dermal fibrosis

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Introduction:
Scleroderma (Systemic Sclerosis, Ssc) is a heterogeneous chronic multisystem rheumatological disorder which can affect the immune system, vasculature, skin, and major internal organs. A hallmark of the disease is excessive dermal and organ scarring, thus SSc is generally considered as the prototypic fibrotic disease. The complexity within the disease spectrum includes the extent and degree of cutaneous involvement, the nature and extent of internal organ involvement, serological features, and outcome. Dermal biopsies have been extensively used to evaluate the degree of scarring and development of fibrosis using global gene expression profiling, fibroblast cell culture and detailed histological analysis. This invasive technique only allows a 'snapshot' of one region of the skin. Here we used a pre-clinical model of scleroderma to assess the potential for applying non-invasive magnetic resonance imaging for the analysis of dermal fibrosis. We analysed structural changes in the skin of mice harbouring mutations which resulted in modulation of the TGFβ signalling pathways ($T\beta RII\Delta k^{fib}$ mice) by histological staining and high resolution magnetic resonance imaging (MRI) scans to detect and compare changes in skin architecture and composition.

Methods:
Full-thickness dermal biopsies were taken from the dorsal region of adult mice and fixed in formalin. Samples were placed within a customised holder containing Fomblin Perfluorosolv PFS-1 (Solvay Solexis, Milan, Italy), positioned within a quadrature volume coil (ID, 39mm; Rapid Biomedical GmbH, Rimpar, Germany), and MRI performed on a 7 Tesla VMRIS scanner (Varian Inc, Palo Alto, USA) using a gradient echo MRI sequence with the following parameters: repetition time, 250ms; echo time, 2.5ms; field of view, 10 x 10mm; matrix size, 128 x 128; 40^{o} flip angle and 14 consecutive transverse, 1mm thick slices (in plane resolution in 78μm). Thickness of the dermis and panniculus adiposus were measured using Image J with 7 measurements, distributed across the image, for each sample. After scanning, samples were embedded in paraffin and serial sections (3 microns) were stained with H&E for routine histology and specific extracellular matrix stains for the degree of fibrosis (Picrosirius red). Images were captured at 10x magnification using an Axioskop Mot2plus microscope. Thickness of the dermis and panniculus adiposus were measured using Axioskop software with at least 30 measurements, distributed across the length, for each sample.

Results:
Histological staining allows easy identification of the dermal and hypodermal compartments, namely the dermis, panniculus adiposus, panniculus carnosus, subcutaneous loose connective tissue, and subcutaneous muscle. Comparison with the MRI images showed that high resolution MRI can also distinguish between dermis, panniculus adiposus, panniculus carnosus, subcutaneous loose connective tissue, and subcutaneous muscle. By gradient echo MRI dermis, panniculus carnosus,

and muscle have higher signal intensities than panniculus adiposus and loose connective tissue.
Thickness measurements of dermis and panniculus adiposus are consistent between histological and MRI images, however a better correspondence is observed for dermis than panniculus adiposus. TβRIIΔk^{fib} mutant mice were found to have thicker dermis than wildtype mice, which is consistent with the known increase in collagen in the skin of the mutant mice. Interestingly, thickening of the dermis is also accompanied by a thickening of the underlying panniculus adiposus.

Conclusion:
High resolution MRI scanning is a promising non-invasive technique for assessing dermal fibrosis that warrants further optimisation in pre-clinical models prior to translation into a clinical setting.

29. Mice lacking the receptor-like protein tyrosine phosphatase CD148 are protected from bleomycin-induced pulmonary fibrosis

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PURPOSE: The molecular mechanisms underlying pulmonary fibrosis, one of the most morbid complications of scleroderma, remain incompletely characterized. Protein tyrosine phosphatases and kinases regulate the equilibrium of tyrosine phosphorylation signaling pathways important in cell growth and differentiation. Tyrosine kinases have been implicated in fibrosis, and studies testing the anti-fibrotic activity of tyrosine kinase inhibitors such as imatinib in scleroderma patients are underway. The receptor-like protein tyrosine phosphatase (RPTP) CD148 is widely expressed on various hematopoietic and non-hematopoietic lineages, including lung epithelial cells, endothelial cells, and fibroblasts. Given the importance of tyrosine phosphorylation pathways in fibrosis, we explored the role of CD148 in the bleomycin mouse model of pulmonary fibrosis.

METHODS: Mice with a targeted deletion of the CD148 transmembrane domain (CD148KO) have been generated by our lab and are used in all studies described. Acute lung injury was measured using extravasation of radioactive iodine-labeled albumin and wet-to-dry ratios. Fibrosis was evaluated by both Masson Trichrome staining of lung sections as well as by the Sircol Collagen Assay (Biocolor).

RESULTS: Following intratracheal instillation of bleomycin at a dose of 3 units/kg, WT mice showed significantly impaired survival, with 4 of 6 WT mice (67%) dying between 10-16 days, whereas none of the 8 CD148KO mice (0%) died (p=0.007). Masson Trichrome staining of lungs demonstrated markedly increased fibrosis in WT mice, whereas fibrosis was significantly attenuated in the CD148KO mice. At day #13 following a lower dose of bleomycin (2.5 U/kg), WT bleomycin-treated mice had a 4.2-fold increase in lung collagen content whereas CD148KO mice showed only a 1.7-fold increase in collagen (p=0.0009). Lung collagen levels in WT bleomycin mice (66.1+/-6.1 ug/ml) were significantly higher than in WT saline mice (15.8+/-0.8 ug/ml) (p=0.0008), whereas lung collagen levels in CD148KO bleomycin mice (46.1+/-8.2 ug/ml) vs. CD148KO saline mice (26.6+/-7.2 ug/ml) were not significantly different (p=0.157). The acute lung injury response at day #5 post bleomycin (2.5 U/kg) was equivalent between genotypes. Endothelial permeability was 1.7% +/-0.33% in WT saline mice vs. 1.5% +/-0.39% in CD148KO saline mice. Following bleomycin, the increase in endothelial permeability in WT mice to 3.9% +/-0.67% and in CD148KO mice to 3.9% +/-0.39% was equivalent between genotypes (n=4 mice per genotype).

CONCLUSION: Mice lacking CD148 phosphatase activity show improved survival. Attenuation of bleomycin-induced fibrosis does not appear to be the consequence of a diminished early acute lung injury response to bleomycin. Future studies will interrogate the specific cell types mediating this response, as well as elucidating the pathways regulated by CD148 underlying this phenotype. These data suggest that inhibition of the RPTP CD148 may present an attractive anti-fibrotic therapeutic strategy.

30. Arsenic trioxide abrogates the development of HOCl-induced systemic sclerosis in mice.

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Background: HOCl-induced systemic sclerosis (SSc) is a newly described murine model that mimics the main features of the human disease, i.e. fibrosis of skin and visceral organs, endothelial hyperreactivity and immunological dysregulations. On the other hand, arsenic trioxide (As_2O_3) is a safe and efficient drug used in the treatment of haematologic malignancies. It can trigger apoptosis and abrogate cellular proliferation through various pathways including protein kinase and reactive oxygen species signalling, caspase activation. As_2O_3 can also act on the immune response by modulating the Th1/Th2 cytokine profile. Here, we have evaluated the therapeutic effects of As_2O_3 on the fibrotic, vascular, and immunological disorders that characterize HOCl-induced SSc.

Materials and Methods: SSc was induced in BALB/c mice by daily subcutaneous injections of hypochlorous acid daily for 6 weeks. As_2O_3 was concomitantly administered intraperitoneally. Dermal thickness, skin and lung collagen contents, anti-topoisomerase-1 Abs and total IgG and IgM levels were determined to assess the development of the disease. Glutathione levels in dermal fibroblasts, serum concentrations of advanced oxidation protein products (AOPP) and cytokine production were measured to investigate the mechanisms of action of As_2O_3.

Results: As_2O_3 significantly reduced skin thickness and collagen accumulation in both skin and lung of HOCl-mice ($p=0.0232$ and $p=0.0311$ for skin and lung collagen concentration respectively). The skins of diseased mice treated with As_2O_3 showed lower levels of α-smooth muscle actin (α-SMA) than skins of untreated mice. Levels of anti-topoisomerase-1 antibodies and total IgG and IgM Abs were decreased in mice with SSc treated with As_2O_3, compared to non-treated mice. The splenocytes of SSc mice treated with As_2O_3 produced lower levels of IL-4 than splenocytes of untreated mice, moreover, As_2O_3 abrogated the local production of IL-13 in the skin of HOCl-injected mice. Those changes in the cytokine profile allowed the reduction of auto-antibody production and the limitation of skin fibrosis. On the other hand, As_2O_3 restored intracellular glutathione levels in skin fibroblasts isolated from SSc mice, thus limiting the toxic effects of the oxidative stress. This effect resulted in the decrease in serum AOPP levels, a marker of systemic oxidative stress, in diseased mice ($p=0.029$).

Conclusions: Arsenic trioxide reduces skin and lung fibrosis, abrogates the production of auto-antibodies and the oxidative stress observed in mice with SSc induced by HOCl. These therapeutic effects are exerted through several mechanisms. On the local level, As_2O_3 abrogates IL-13 production and restores fibroblasts glutathione levels; on the systemic level, the molecule limits the systemic oxidative stress and acts on IL-4 production by splenocytes, thus limiting the production of auto-antibodies. Therefore, arsenic trioxide is a novel potential therapeutic agent in the treatment of SSc.

31. Fibrocyte recruitment and myofibroblast diffrentiation after acute lung injury is blocked by selective inhibition of tgfbeta signalling in resident interstitial fibroblasts

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Purpose

TGFbeta overactivity is implicated in the development of lung fibrosis and may be a potential therapeutic target in fibrotic disease such as scleroderma (SSc). We have used post-natal deletion of the high affinity type II TGFbeta receptor (TbetaRII) in fibroblasts to test the role of TGFbeta signalling in resident lung fibroblasts in the fibrotic response to lung injury.

Methods

TbetaRII was deleted using a compound Cre-Lox genetic strategy with post-natal administration of tamoxifen over 5 days to activate Cre-recombinase in mice harbouring a conditional allele of TbetaRII to delete the receptor from fibroblasts exclusively. Illumina microarray gene profiling was used to confirm anergy to TGFbeta (2ng/ml) in explanted lung fibroblasts. A bleomycin lung injury model was used to induce lung fibrosis. Multichannel immunofluorescence was used to define the cell populations after lung injury at 7 and 14 days on tissue sections and fibrocytes were defined by co-expression of CD34, Col1 (Collagen I) and alphaSMA. Myofibroblasts were identified by co-expression of Col1 and alphaSMA.

Results

There was almost complete attenuation of lung fibrosis in mice treated with intratracheal bleomycin (Null-B) after deletion of TbetaRII in resident fibroblasts. At 7 days after injury there was evidence of epithelial mesenchymal transdifferentiation (EMT) but the number of fibrocytes and myofibroblasts was substantially reduced. Using high power field counts (hpf) the number of fibrocytes in Null-B lungs at 7 days was decreased compared wildtype littermate controls (WT-B;3.6±2.22 cells/hpf, 26.6±4.96 cells/hpf p=0.007 respectively). At 14 days this reduction was sustained (8.6±2.06 cells/hpf compared with 46.6±4.947 cells /hpf p=0.0007). Furthermore, myofibroblast expression was reduced in 7 day Null-B lungs compared with WT-B (134.2±28.54 cells/hpf compared to 11.4±3.25 cells/hpf, p=0.01). Again, this was maintained at day 14 (72.2±20.73 cells/hpf, 7.6±1.46 cells/hpf p=0.03). Analysis of gene expression defined a cohort of TGFbeta responsive genes that were not upregulated in fibroblasts after deletion of TbetaRII. This included the key profibrotic mediators CTGF and ET-1, raising the possibility that defective induction of these mediators may underlie the altered fibrocyte recruitment and myofibroblast differentiation that we observe (see Table).

Mean (±SEM) Normalised Gene Expression in Lung Fibroblasts						
	Wildtype (n=6)			Null (n=3)		
	Basal	**TGFb**	**P value**	**Basal**	**TGFb**	**P value**
CTGF	2282 ±1781	9926 ±2115	0.031	1753 ±951	2027 ±1190	NS
ET-1	401 ±22	838 ±197	0.035	541 ±49	504 ±65	NS

Conclusion

Intact TGFbeta signalling in resident lung fibroblasts is essential for lung fibrosis to develop and our results support a key regulatory role of these cells in determining fibrocyte recruitment and myofibroblast differentiation.

32. Circulating Adiponectin Levels Are Correlated with Skin Involvement in Systemic Sclerosis: role for peroxisome proliferator-activated receptor gamma (PPAR .

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Background: Adiponectin is a hormonal adipokine with insulin sensitizing and anti-inflammatory properties produced by white fat. In healthy individuals, serum adiponectin levels are inversely correlated with body mass, and are elevated in lean subjects. Adiponectin production from tissue-resident and perivascular adipocytes is mediated through the ligand-inducible nuclear orphan receptor peroxisome proliferator-activated receptor gamma (PPAR ..and levels of circulating adiponectin reflect the activity of PPAR . in patients with type 2 diabetes. We have shown that PPAR . disrupts Smad2/3-dependent TGF-ß signaling in normal fibroblasts resulting in abrogation of TGF-ß-mediated fibrotic responses in vitro, and ameliorates bleomycin-induced fibrosis in the mouse in vivo. The levels of PPAR expression, and PPAR -dependent transcriptional activity, were found to be markedly reduced in lesional tissue from a subset of patients with diffuse cutaneous SSc, suggesting a role for impaired PPAR . in the pathogenesis of fibrosis in SSc. To evaluate PPAR . activity in SSc in vivo, we determined serum levels of adiponectin in a cohort of well-characterized SSc patients.

Materials and Methods: A cross-sectional study was conducted. Serum samples from 129 patient with dcSSc or lcSSc, and 86 healthy controls were analyzed. Adiponectin levels were measured by multiplex assays. Demographic, clinical and laboratory data were obtained from the electronic medical record.

Results: Serum adiponectin levels were reduced in female [but not male] SSc patients compared to sex-matched controls (SSc females 17.1±10.1 g/ml versus control females 19.7±10.5 g/ml; SSc males 16.0±11.2 g/ml versus controls 12.8±6.5 g/ml), particularly in patients with diffuse cutaneous SSc (dcSSc) (13.2±8.7 µg/ml in dcSSc versus 19.3±10.5 µg/ml in lcSSc; p<0.01). African-Americans had lower adiponectin levels that other ethnic groups. Levels of adiponectin were lowest in patients with early

disease (<18 months). There was no correlation between adiponectin levels and BMI, autoantibody status (Scl-70, ACA, RNA pol III), lung function tests or chest HRCT findings. However, a significant inverse correlation between adiponectin levels and Rodnan skin score was found (Spearman; N=124; p<0,01).

Conclusions: In SSc, diffuse cutaneous involvement and early-stage disease are associated with reduced adiponectin levels in the srum. Since adiponectin is a sensitive indicator of PPAR activity, these results are consistent with impaired PPAR expression or function in dcSSc, and suggest that determination of serum adiponectin might be useful as a biomarker. On-going longitudinal studies will evaluate if adiponectin levels are sensitive to change over time, and whether they reflect changes in skin score or progression of fibrosis in patients with SSc.

33. Functional interaction between ER stress/UPR and TLR ligands promotes inflammation in endothelial cells

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PURPOSE. The presence of the HLA-B35 allele has emerged as an important risk factor for the development of Pulmonary Hypertension (PHT) in patients with Scleroderma (SSc). Our recently published data (1) showed that the presence of HLA-B35 could contribute to endothelial cell dysfunction via ER (Endoplasmic Reticulum) stress and UPR (Unfolded Protein Response) mediated induction of ET-1 in patients with PHT, supporting a pathogenic role of HLA-B35 in PHT. Toll-Like Receptors (TLRs), the key mediators of the innate immunity, have recently been implicated in Scleroderma pathogenesis (2). There is evidence of interactions between ER stress/UPR pathways and inflammation from other experimental models. The goal of our study was to determine the effect of TLR ligands on endothelial cells in the presence of ER stress.
METHODS. Human dermal microvascular endothelial cells (HDMECs) were transduced for 48 hours with Adenovirus expressing HLA-B35 or control virus or treated 24 hours with Tunicamycin (TM) (10μg/ml), a known ER stress inducer. The cells were treated with TLR ligands (Pam3CSK4 1μg/ml (TLR2), Poly I:C 2,5μg/ml (TLR3) and LPS 10μg/ml (TLR4) for 24 hours. Total RNA from cell cultures was extracted and the quantitative real-time polymerase chain reaction (qPCR) was performed to check upregulation of ER stress/UPR and inflammation genes. The Mann-Whitney U-test was used to assess significant changes in mRNA expression.
RESULTS. Two UPR mediators, transcription factor ATF4 and ATF6, were examined in ECs after overexpression of HLA-B35 or treatment with TM. HLA-B35 modestly up-regulated both ATF4 and ATF6 mRNA levels, while TM alone markedly up-regulated ATF4 only (12 fold increase). We next tested the effects of TLR ligands on expression of these UPR genes. ATF4 was stimulated by TLR3 ligand (5 fold increase) or TLR4 ligand (3 fold increase), but expression levels of ATF6 was not responsive to these treatments. However, when TLR ligands were used in combination with HLA-B35, there was a synergistic enhancement of ATF4, as well as ATF6 expression levels. When TLR ligands were used in combination with TM, only ATF4 expression was synergistically up-regulated. We also investigated the effects of TLR ligands alone or in combination with HLA-B35 or TM on expression level of IL-6, a major pro-inflammatory cytokine. IL-6 was up-regulated by either HLA-B35 (5 fold) or TM (2 fold), but TLR ligands had only minimal effect on expression of IL-6. However, a combination of the ER stress/UPR inducer and the TLR ligand synergistically enhanced IL-6 gene expression; in particular, in the presence of HLA-B35 IL-6 was induced by TLR2 ligand 16 folds, by TLR3 ligand 19 folds and by TLR4 ligand 12 folds.
CONCLUSION. Our data suggest that in endothelial cells ER stress/UPR and TLR activation are linked to regulation of inflammatory gene transcription. This regulatory loop may contribute to the enhanced inflammatory process in patients with PHT.

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34. Global gene expression profiling of scleroderma lung fibroblasts identifies a heterogeneous expression pattern of activated genes and consistent repression of interferon related pathways

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Background: Lung fibroblasts are the main cell type responsible for the excessive extracellular matrix synthesis observed in scleroderma-associated interstitial lung disease (SSc-ILD). The study of lung fibroblast global gene expression profiles is likely to allow insights into their role in fibrotic lung disease and to elucidate key signalling and transcriptional mechanisms underlying their profibrotic phenotype.

Methods: The global gene expression profile of lung fibroblasts isolated from eight SSc-ILD and ten control lungs was assessed by using Affymetrix oligonucleotide U133A2 microarrays. Cluster analyses were performed using dChip, and *in silico* promoter analysis using MatInspector (Genomatix). Transcription factor (TF) DNA binding activity in nuclear extracts from SSc-ILD and control fibroblasts was determined using Panomics oligonucleotide array technology.

Results: Significant differences in the gene expression patterns between SSc-ILD and control fibroblasts were observed in a wide range of genes, both up and downregulated. Significant heterogeneity was observed among upregulated genes; this was used to identify distinct clusters of co-expressed genes, likely to contain functionally related transcripts with shared regulatory mechanisms. Clusters included expected TGF- response/myofibroblast associated genes, as well as many novel to SSc. Further *in silico* analysis of two members of one such group, CTGF and PAI-1, revealed striking co-expression across samples, and functionally conserved regulatory promoter motifs, including TF binding sites already implicated in SSc fibroblast gene regulation (e.g. Sp1, Ets), as well as several novel to SSc. Preliminary screening of TF/DNA binding activity in nuclear extracts from SSc-ILD and control fibroblasts, confirmed differential binding at a number of consensus binding sites, including those identified in the CTGF and PAI-1 genes. Perhaps most relevant to the disease, a core group of genes was observed to be consistently downregulated in all of the analysed scleroderma lung fibroblasts. These related to a number of interferon induced pathways, including genes involved in host defence response, negative regulation of proliferation, and apoptosis/cell death.

Conclusions: The gene expression profile of SSc-ILD lung fibroblasts differs significantly from that of control lung fibroblasts. We have identified distinct co-expression groups suggesting tight co-regulation of groups of genes, providing the opportunity to identify underlying transcriptional and other molecular mechanisms. Furthermore, the most consistent change across all SSc-ILD fibroblasts was a downregulation of interferon related pathways, suggesting that inhibition of a cluster of constitutively active genes is needed to develop a pro-fibrotic phenotype in SSc-ILD.

35. CCN2-expressing pericytes are required for skin fibrosis

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Fibrosis is characterized by excessive production of collagens and their contraction by fibroblasts. It is the major cause of internal organ failure. There is no effective therapy for fibrotic disease and the origin of fibrotic cells within lesions is unclear. The matricellular protein connective tissue growth factor (CTGF/CCN2) is a marker of fibrotic cells and is considered playing an important role in fibrogenesis. However, the specific role of CCN2 in connective tissue biology in general and in fibrogenesis in particular is unclear.

To investigate the role of CCN2 in fibrogenesis, mice harboring a floxed CCN2 allele were generated. Fibroblast specific CCN2-Knockout mice were created by crossing mice carrying floxed CCN2 with mice carrying a tamoxifen-inducible Cre-recombinase under the control of a fibroblast-specific regulatory sequence from the proα2(I) collagen gene. Cutaneous sclerosis was induced by subcutaneous injection of bleomycin. Dermal thickness, collagen production, and the number of α-smooth muscle actin (αSMA) and NG2 (pericyte marker)–positive cells were determined.

Loss of CCN2 results in resistance to bleomycin-induced skin fibrosis. In response to bleomycin, wild-type mice possess, but CCN2-deficient mice lack, abundant NG2/α-SMA-expressing myofibroblasts within fibrotic lesions.

Our results indicate that CCN2 is required for the formation of pericytes and that CCN2-expressing pericytes are essential for bleomycin-induced skin fibrosis. These data suggest that therapeutic strategies blocking CCN2/pericyte differentiation in vivo may be of benefic in combating fibrotic skin disease.

36. CCN2-expressing pericytes are required for bleomycin-induced skin scleroderma

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There is no therapy for fibrotic disease. Moreover, the origin of fibrotic cells within lesions is unclear. The pro-adhesive matricellular protein connective tissue growth factor (CTGF/CCN2) is a marker of fibrotic cells and is significantly overexpressed in scleroderma. CCN2 has been proposed to be a specific target for anti-fibrotic drug therapy in scleroderma. However, the specific role of CCN2 in connective tissue biology in general and fibrogenesis in particular is unclear. Using adult mice bearing a smooth muscle cell/fibroblast-specific deletion of CCN2, we show that loss of CCN2 results in resistance to bleomycin-induced skin scleroderma in that, in response to bleomycin, CCN2-deficient mice show resistance to bleomycin-induced skin thickness and collagen production (N=6, $p<0.05$). In response to bleomycin, wild-type mice possess, but CCN2-deficient mice lack, abundant ˜SMA-expressing myofibroblasts within fibrotic lesions (N=6, $p<0.05$). Essentially all (~95%) of the myofibroblasts induced in response to bleomycin not only express CCN2 but also express the pericyte-specific marker NG2, suggesting that the myofibroblasts induced in bleomycin-exposed skin are derived from pericytes. Collectively, these results indicate that CCN2-expressing pericytes are essential for bleomycin-induced skin scleroderma. These data indicate that therapeutic strategies blocking CCN2/pericyte differentiation in vivo may be of benefit in combating the skin fibrosis in scleroderma.

37. Preliminary results of studies on susceptibility genes to systemic sclerosis in Chinese population

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- **Purpose:** To verify the association of candidate genes and their SNPs from previous studies to systemic sclerosis (SSc) in Chinese population, and do in-depth analysis of relationship between these SNPs and different phenotypes of SSc.
- **Methods:** In this study, Sequenom Massarray SNP analysis platform was used to genotype 32 SNPs of different gene loci in 265 systemic sclerosis patients and 122 controls. Statistical analysis of both genotype and allele frequencies was done combined with subgroup analysis of serum antibodies spectrum and subtypes of SSc
- **Results:** MHC rs7763822 and rs7764491 ($p=0.004$ and 0.0006 respectively) and STAT4 rs7574865 ($p=0.027$) are associated with SSc; MHC rs3128930, rs7763822 and rs7764491 are associated with anti-Scl-70 autoantibodies (p values as 0.001, 0.00001 and 0.00001 respectively); STAT4 rs7574865 is related to ACA ($p=0.04$).
- **Conclusion:** STAT4 is a susceptibility gene to SSc in Chinese population; MHC genes are in the strongest association with SSc in Chinese population; multiple gene loci are associated with SSc subtypes, anti-Scl-70 antibodies and ACA.
- **Key words:** systemic sclerosis, Chinese population, single nucleotide polymorphism

38. A genetic variant located in the promoter of the uPAR (CD87) gene is associated with the vascular complications of systemic sclerosis

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Objective: The urokinase-type plasminogen activator receptor (uPAR, or CD87) gene encodes a pleiotropic cell surface receptor involved in fibrosis, immunity, angiogenesis and vascular remodeling. Previous studies have implicated uPAR in systemic sclerosis (SSc) vasculopathy and impaired angiogenesis. We investigated whether uPAR gene promoter polymorphisms may be associated with SSc susceptibility and clinical phenotypes in the European Caucasian population.

Patients and Methods: A total population of 1,339 individuals was studied. The Italian discovery cohort comprised 388 SSc patients and 391 healthy controls. The French replication cohort consisted of 344 SSc patients and 216 healthy controls. Cutaneous subsets, anticentromere (ACA) and anti-topo I antibodies, interstitial lung disease, pulmonary arterial hypertension (PAH, defined by right heart catheterization), past and/or present occurrence of digital ulcers (DU) were assessed. Genomic DNA was purified from peripheral blood mononuclear cells. The uPAR rs344781 and rs4251805 single-nucleotide polymorphisms (SNPs) were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.

Results: Genotype frequencies were in Hardy-Weinberg equilibrium in the control population for the 2 SNPs investigated. In the Italian cohort, the rs344781 G allele was associated with SSc-related DU (OR 1.39, p=0.03), SSc-PAH (OR 1.81, p=0.003), ACA-positivity (OR 1.45, p=0.02) and limited cutaneous SSc (lcSSc) (OR 1.37, p=0.02). The rs344781 GG genotype was associated with SSc-PAH (OR 3.79, p=0.0003), ACA-positive SSc (OR 2.17, p=0.02) and lcSSc (OR 1.96, p=0.02). No association was observed for rs4251805. Allelic and genotypic associations of rs344781 with SSc-DU and ACA-positive SSc were replicated in the French sample (for SSc-DU: G allele OR 1.49, p=0.04; GG genotype OR 3.20, p=0.01; for ACA-positive SSc: G allele OR 1.57, p=0.03; GG genotype OR 3.09, p=0.02). A pooled analysis comprising the 2 cohorts revealed a strong association of rs344781 G allele and GG genotype with SSc-DU (allele OR 1.41, p=0.005; genotype OR 2.15, p=0.005), SSc-PAH (allele OR 1.65, p=0.004; genotype OR 3.16, p=0.0006), ACA-positive SSc (allele OR 1.47, p=0.002; genotype OR 2.40, p=0.001) and lcSSc (allele OR 1.34, p=0.004; genotype OR 1.77, p=0.02). In a multivariate logistic regression analysis model including the above associated subgroups of SSc patients, the rs344781 GG genotype remained an independent risk factor for SSc-DU (OR 1.96, p=0.04) and SSc-PAH (OR 2.68, p=0.01).

Conclusions: Our data provide the first genetic evidence for a defined vascular phenotype of SSc patients tagged by the uPAR rs344781 gene variant that may be used as biomarker in the future. This study provides new insight into the pathogenesis of SSc, including clues to the mechanisms leading to the development of severe vascular complications, such as DU and PAH, and opens new therapeutic perspectives.

Keywords: systemic sclerosis, uPAR, gene polymorphism, pulmonary arterial hypertension, digital ulcers.

39. Association of a functional polymorphism in the MMP-12 promoter region with diffuse cutaneous systemic sclerosis (SSc) and SSc-related pulmonary fibrosis in the Italian population

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Objective: Systemic sclerosis (SSc) is a life-threatening connective tissue disease characterized by progressive fibrosis of the skin and internal organs. Pulmonary fibrosis is a major cause of morbidity and mortality in SSc patients. Matrix metalloproteinase-12 (MMP-12, or macrophage metalloelastase) is believed to play an important role in chronic pulmonary inflammation and fibrosis. We have recently shown that dermal fibroblasts and microvascular endothelial cells isolated from diffuse cutaneous SSc (dcSSc) patients constitutively overexpress and release MMP-12. Interestingly, MMP-12 overproduction by SSc cells has been shown to be a permanent alteration over multiple generations in vitro. The human MMP-12 gene, on chromosome 11q22.3, contains a common A/G functional single-nucleotide polymorphism in the promoter region (rs2276109) which modulates transcriptional activity in an allele-specific manner. The A allele has a greater affinity to the activator protein-1 (AP-1) transcription factors, thus resulting in increased promoter activity and enhanced MMP-12 expression. We investigated the possible implication of the MMP-12 gene in the genetic predisposition to SSc susceptibility and clinical phenotype.
Patients and Methods: The MMP-12 rs2276109 polymorphism was selected as genetic marker and genotyped by PCR-RFLP assay in 513 unrelated subjects of Italian Caucasian ancestry: 250 SSc patients (146 with limited cutaneous SSc (lcSSc), 104 with dcSSc) and 263 healthy individuals. Genetic association was assessed by using univariate and multivariate logistic regression analyses.
Results: No deviation from the expected population genotype proportions predicted by Hardy-Weinberg equilibrium was detected, both in SSc patients and controls, at the MMP-12 rs2276109 polymorphic site. A significant difference in MMP-12 rs2276109 genotype distribution between SSc patients and controls (p=0.0003), and between lcSSc and dcSSc (p=0.003) was observed. The A allele frequency was significantly higher in SSc patients than in controls (p=0.0002), and in dcSSc than in lcSSc (p=0.003). After adjustment for age and sex the homozygosity for the A allele significantly influenced the predisposition to SSc, and to dcSSc (OR 2.44, 95%CI 1.61-3.71, p<0.0001; OR 4.69, 95%CI 2.36-9.33, p<0.0001, respectively). A trend towards an association between the A allele and lcSSc was observed (p=0.06). The homozygosity for the A allele was also significantly and independently associated with anti-topoisomerase I antibody-positive SSc (OR 6.39, 95%CI 2.18-18.76, p=0.001) and SSc-related pulmonary fibrosis (OR 2.94, 95%CI 1.25-6.95, p=0.01).
Conclusions: Our work suggests that the MMP-12 rs2276109 polymorphism contributes to the susceptibility to SSc and might be an indicator of severe skin and pulmonary fibrosis in the disease course. Modulation of MMP-12 expression and activity might offer new targeted therapeutic strategies to control the progression of fibrosis in SSc.

Keywords: systemic sclerosis, MMP-12, gene polymorphism, pulmonary fibrosis.

40. The Wnt signalling inhibitor gene, WIF1, is silenced in fibroblasts derived from Systemic Sclerosis patients: role of oxidative stress.

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Background: Systemic sclerosis is an autoimmune disease characterized by extensive fibrosis and vascular lesions. Primary fibroblasts derived from

systemic sclerosis (SSc) patients contain high level of cytoplasmatic and peri-nuclear free radicals (ROS) and breaks in the genomic DNA.
We have identified stimulatory IgG auto-antibodies to the PDGF receptor that are capable of converting normal fibroblasts into SSc-like cells inducing excessive oxygen species (ROS) production by activating membrane NADPH oxidase complex.Wnt family constitutes a large group of highly conserved glycoproteins that are implicated in developmental processes and recently in carcinogenesis, aging and fibrosis. Wnt signaling is tightly controlled by several groups of negative regulators that interfere either with receptor-ligand binding or with intracellular signaling.
Wnt inhibitor factor 1, WIF1, is frequently silenced in human cancer by DNA methylation. Recently, its inhibition has been associated to ageing of mesenchymal stem cells and fibrosis, induced by unrestrained Wnt signaling. In order to identify specific markers of the disease, we have decided to analyze WIF1expression in cells derived from patients affected by systemic sclerosis and explore the mechanism of WNT signaling regulation.
Materials and Methods: Human skin fibroblasts were obtained from punch biopsies taken from the forearms of normal volunteers and from the involved skin of scleroderma patients. We have investigated WIF-1 expression by reverse transcription and quantitative real-time PCR. Total RNA isolation from normal and sclerodermic fibroblasts was performed with total RNA mini kit (BioRad) and reverse transcription PCR was performed using iScript cDNA synthesis kit from BioRad. Quantitative PCR was performed in triplicate with SYBR Green (Biorad).
Results: WIF gene expression was significantly down-regulated in cells derived from systemic sclerosis patients. Moreover, the gene was not methylated, as in breast cancer cells, and its expression was reactivated by inhibiting histone de-acetylase enzymes, trichostain (TSA). PDGF or the immunoglobulin fraction from systemic sclerosis patients in 24 hours robustly stimulated WIF-1 expression prevented by inhibiting NADPH oxidase. Prolonged oxidative stress by IgG SSc silenced WIF1 by inducing DNA damage. Inhibition of ATM kinase by the specific inhibitor KU-55933 rescued WIF expression in fibroblasts exposed to long term oxidative stress.
Conclusions: These data indicate that in SSc fibroblasts a persistent oxidative stress triggered by PDGF or anti PDGF receptor autoantibodies, induce WIF-1 downregulation. Our data indicate that the ultimate cause of silencing WIF-1 is DNA damage.

41. Organotelluride Catalyst (DAM030) as a new treatment for HOCL-induced Systemic Sclerosis in the mice

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Objective: Systemic sclerosis (SSc) is a connective tissue disorder characterized by microvascular damage and fibroblasts activation leading to massive fibrosis of skin and visceral organs. HOCL-induced SSc is a newly described murine model that mimics the main features of the human disease. The aim of this study was to demonstrate the efficiency of the redox sensitive catalyst DAM030 in a murine model of SSc. DAM030 is a tellurium-based, pro-oxidative molecule whose mechanism of action has already been described for cancer cells. DAM030 combines with the Reactive Oxygen Species (ROS) overproduced in tumor cells to induce a lethal oxidative stress. This phenomenom does not occur in normal cells with a normal intracellular level of ROS. Since SSC fibroblast displayed an increase level of intracellular ROS compared to normal fibroblast, we hypothesized that DAM030 could favor their selective apoptosis and thus decrease disease severity in SSc mice.

Methods: The antifibrotic effect of DAM030 was evaluated in a nearly described model of SSc induced by injection of hypochlorous acid in BALB/c mice every day for 6 weeks. DAM030 was administrated intravasculary once per week. Skin thickness was measured. Skin and lung fibrosis were assessed by histological and biochemical methods. Anti-topoisomerase-1 Abs level and concentration of advanced oxidation protein products (AOPP) a marker of systemic oxidative stress were also measured. To assess the mechanism of action of DAM30 on disease development, the anti-proliferative and pro-apoptotic effect of DAM30 was compared on normal and on SSc skin fibroblasts.

Results: SSc mice treated with DAM030 presented significant decrease in dermal thickness and collagen concentration compared to untreated SSc mice. The same results were observed for the lung. Western blot and

immunofluorescence analysis showed a decrease of Alpha-Smooth Muscule Actin (alpha-SMA) expression in the skin of SSc mice treated with DAM030 compare to untreated SSc mice. Levels of anti-DNA topoisomerase I autoantibodies and of AOPP in the serum were lower in the mice treated with DAM030 compare to untreated SSc mice.

Conclusion: In our SSc model, DAM030 decrease fibrosis progression in both skin and lung. Due to its low toxicity profil, DAM030 might be an interesting candidate for clinical trials in patients with scleroderma disease.

42. MMP-7 serum levels as marker for tissue remodeling and fibrosis in systemic scleroderma as a model disease

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Fibrosis is characterized by an excessive accumulation of connective tissue due to a disturbed balance between synthesis and degradation of extracellular matrix proteins (ECM). This is regulated by matrix metalloproteinases (MMPs) and their inhibitors (TIMPs).

Matrix Metalloproteinase-7 is involved in the degradation of ECM in many physiological situations as well as in other disease processes, like tumor invasion, wound healing and lung fibrosis. It was the aim of the present study to investigate whether serum MMP-7 levels might reflect the tissue remodeling going on during the course of fibrotic diseases, using systemic sclerosis (SSc) a prototypic fibrotic disease as a model.

Serum samples were obtained from 110 patients with systemic sclerosis. MMP-serum levels of all SSc patients were compared with patients suffering from idiopathic pulmonary arterial hypertension (PAH) and age matched healthy controls that had no rheumatic disease by using a commercial enzyme immunoassay kit.

An increased mean serum MMP-7 level was found in SSc patients when compared to controls and to idiopathic PAH-patients ($p<0.0001$). Male patients showed a significant higher level of MMP-7 compared to female patients ($p<0.017$). Patients suffering from the limited form of SSc showed lower MMP-7 serum levels compared to the diffuse form ($p<0.06$). Furthermore higher serum levels of MMP-7 correlate well with the degree of skin involvement as determined by the modified Rodnan Skin Score and pulmonary arterial hypertension. Interestingly, there was no significant correlation of MMP-7 levels with chronic digital ulcerations.

These results indicate the correlation between high MMP-7 levels and the more progressive course of fibrosis. Further analyses seem to be interesting and important, whether MMP-7 activity is directly involved in the pathophysiology of this disease and whether it naturally reflects tissue remodelling.

43. PDGF receptor as therapeutic and diagnostic target in systemic sclerosis

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Background: Systemic sclerosis or scleroderma (SSc) is a disease characterized by fibrosis of skin and visceral organs. We have provided evidence that the serum of SSc patients contains stimulatory auto-antibodies (auto-abs) directed to the PDGF receptor (PDGFR) that elicit Ha Ras-ERK 1/2 signaling and collagen production in normal human fibroblasts *in vitro* (Baroni SS et al, NEJM 2006). A recent study (Olson LE, Soriano P, Dev Cell 2009) has confirmed the central role of increased PDGFR activation and signaling in driving systemic fibrosis *in vivo* in transgenic mice.
Objectives: To identify the epitopes of PDGFR extracellular domains bound by stimulatory auto-abs. This information will be used to generate i) PDGFR selective inhibitors and ii) binding assays for detection of functional auto-abs in serum.
Methods and Results: IgG-positive memory B cells obtained from peripheral blood of SSc patients were immortalized by EBV infection. Supernatants of individual lymphocyte clones were screened by immunofluorescence and flow cytometry for the ability to react selectively with F alpha cells (mouse fibroblasts expressing the human PDGFR alpha), but not with F-/- cells (mock-transfected mouse fibroblasts). Positive clones were further screened for the production of antibodies stimulating reactive oxygen species (ROS) generation in normal human fibroblasts *in vitro*. Positive clones were expanded in serum-free medium, IgG were purified from supernatants and tested to confirm both binding and biological activity on fibroblasts. mRNA was obtained from such positive B cell clones for sequencing and cloning of antibody variable regions into a human IgG expression vector. Human IgG constructs were expressed in CHO cells, recombinant (rec.) monoclonal antibodies (mAbs) were purified from medium and tested by immunoprecipitation and stimulation experiments. These rec. monoclonal human IgG showed different extents of PDGFR binding and stimulation. Few mAbs displayed the properties identified in total IgG pools purified from serum of SSc patients, since they bound to PDGFR and induced ROS, p-ERK and type I collagen gene expression in normal human fibroblasts. Molecular docking simulation indicated that the PDGFR epitopes bound by stimulatory mAbs differ from those of non-biologically active mAbs. Epitope mapping was confirmed by binding competition experiments using a rec. PDGFR immobilized onto a biosensor. A peptide library is under construction to further define the map of PDGFR epitopes targeted by the different mAbs.
Conclusions: We identified the specific epitopes bound by functional PDGFR auto-abs that trigger PDGFR signaling. This information is shedding light on the structure of active PDGFR domains, on SSc pathogenesis, and can be used to devise new therapeutic strategies to block PDGFR signaling and, specifically, the progression of SSc. Moreover, synthetic polypeptides corresponding to these functional PDGFR domains will be employed to selectively detect stimulatory auto-abs in serum, possibly implicated in the early phases of SSc pathogenesis.

44. Activation of stress induced signaling pathways in the epidermis in systemic sclerosis

Joanna Nikitorowitcz, Nima Aden, Xu Shiwen, Carol Black, Christopher Denton, Robert Unwin, David Abraham, Richard Stratton

Background Epidermal cells activated by injury orchestrate tissue repair and regulate local fibroblasts during wound healing. Changes in the epidermal cell layer have not been extensively investigated in SSc. Previously, we found using a proteomic analysis of diffuse SSc forearm skin biopsy material and healthy control skin biopsy material, altered abundance of proteins critically involved in keratinocyte maturation. Because wound epidermal cells regulate local fibroblasts and initiate fibroblast differentiation in wounds, we became interested in the idea that epidermal cells are capable of promoting fibroblasts in the disease.

Methods Skin biopsy material from recent onset diffuse SSc patients and from healthy controls were stained for markers of proliferation (Ki-67), apoptosis (cleaved caspase 3), keratinocyte differentiation (cytokeratin 14, involucrin, loricirn, purinergic receptor P2X7), and for the presence of wound associated proteins (cytokeratins 6 and 16). Signalling pathway induction in SSc and control epidermal biopsies was analysed further by phosphorylation array analysis using phosphokinex arrays, and by immunohistochemistry. Whole explants of SSc and control epidermis were maintained in 3 dimensional co-culture with healthy control dermal fibroblasts. Gel contraction and induction of CTGF were used as markers of fibroblast induction by co-culture

Results Immunostaining of SSc and control sections revealed that SSc keratinocytes are committing to an activation / injury response pathway, rather than differentiating in the normal way. Keratinocytes in SSc epidermis express cytokeratins 6 and 16, normally associated with wound healing epidermis, and fail to lose cytokeratin 14 expression as they migrate upwards. Terminal differentiation marker and calcium channel P2X7 was absent in SSc epidermis but present in cornified layer of control We found using 3- dimensional co-culture that SSc epidermis promotes fibroblast contraction of collagen lattices, and induces CTGF, ET-1 and TGFβ in co-cultured fibroblasts. These effects are mediated by increased levels of IL-1α in the keratinocyte layer and are antagonised by exogenous IL-1ra. c-Met receptor for HGF emerged as the most abundantly increased phosphoprotein in SSc versus control epidermis. In addition stress activated MAPKs c-Jun N-terminal kinase, and p38 were among the induced phosphoproteins in SSc. Immunostaining revealed induction of stress activated MAPKs in basal cells of the epidermis in SSc and confirmed increased c-Met phosphorylation throughout the SSc epidermis.

FOLD IN-CREASE	PHOSPHOR-YLATION SITE	FULL TARGET PROTEIN NAME	SWISS PROT #	P VALUE (STUDENT'S T)
2.89	Y1003	Hepatocyte growth factor receptor (c-Met)	P08581	0.05
2.63	Pan-specific	Protein-serine phosphatase 4 - regulatory subunit (PPX/A'2)	Q8TF05	0.0005
2.57	S674	Protein-serine kinase C eta	P24723	0.04
2.37	S63	Jun proto-oncogene-encoded AP1 transcription factor	P05412	0.02
2.29	Pan-specific	NIMA (never-in-mitosis)-related protein-serine kinase 7	Q8TDX7	0.003
2.18	Y705	Signal transducer and activator of transcription 3	P40763	0.05
2.18	Pan-specific	Dual specificity protein kinase	P33981	0.02
2.18	T674	Protein-serine kinase C gamma	P05129	0.04
2.17	Pan-specific	Integrin-linked protein-serine kinase 1	Q13418	0.05
2.13	T180+Y182	Mitogen-activated protein-serine kinase p38 alpha	Q16539	0.05
2.10	Pan-specific	PCTAIRE-1 protein-serine kinase	Q00536	0.02

Conclusions In SSc epidermis keratinocytes are taking on an activated phenotype associated with wound repair. SSc keratinocytes appear capable of promoting fibroblast contractility and CTGF induction. These changes may be initiated through activation of stress signaling pathways in the basal keratinocytes (environmental stress) or maintained by HGF/c-Met signaling (HGF release by dermal fibroblasts). Keratinocyte-fibroblast interactions may be important in SSc fibrosis.

45. Cytokines, chemokines and growth factors present in conditioned media from epidermal and dermal explants of healthy controls and systemic sclerosis patients.

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Background Systemic sclerosis (SSc) is a severe disease of unknown aetiology characterised by cellular injury and activation in early stage, followed by autoimmunity and fibrosis. We found that SSc epidermal cells show signs of injury and activation similar to changes observed during the wound healing process. Keratinocytes are known to be able to secrete chemo-attracting agents as well as growth factors influencing phenotype and proliferation rate of fibroblasts. We hypothesized that in SSc injured epidermal cells are releasing chemokines and cytokines capable of recruiting immune cells to the skin and promoting fibrosis. Thus we wanted to determine the array of cytokines and growth factors released by SSc epidermal cells and draw comparison with the array of factors released by SSc dermis, and control samples.

Materials and methods In the preliminary study we used 4 mm forearm biopsies taken from 6 healthy controls and 6 SSc patients with various stage and type of disease. Dermis and epidermis were separated after 2hr incubation with trypsin/EDTA. The explants were then incubated overnight with 1ml of serum free media (DMEM, Gibco) at 37˚C and 5% CO_2. Then the media were collected and analysed using LegendPLEX (BioLegend) for presence of G-CSF, GM-CSF, VEGF, PDGF-AA, PDGF-BB, MCP-1, FGF-2, IL-8, IL-1α, IL-1β, and IL-1ra. Additionally, HGF and CCL20 were measure by ELISA (R&D Systems). The statistical analysis was performed using Wilcoxon rank-sum test.

Results Predominant amongst growth factors released by the epidermal explants were chemokines MCP-1 and IL-8 (Table 1). A trend towards increased IL-8 release by SSc epidermis comparing to control epidermis was observed, similar with MCP-1. Also HGF was abundantly present in both epidermis and dermis with trend towards significant increase in the SSc dermis. Moreover, elevated levels of IL-1ra were detected in SSc dermis when matched to controls. VEGF was produced in higher amounts by SSc dermis, while no striking differences were observed with G-CSF, FGF-2, PDGF-AA or IL-1 β when compared with concentration in healthy skin media. Levels of GM-CSF in the epidermis conditioned media were below detection, as were PDGF-BB for both epidermis and dermis conditioned media.

Table 1. Mean and standard error of the mean (SEM) concentration of growth factors, cytokines and chemokines in the conditioned media

	Control epidermis		SSc epidermis		Control dermis		SSc dermis	
Growth factor	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
HGF	664.0	30.20	813.2	124.80	740.9	61.70	1221.2	472.20
G-CSF	64.5	18.47	64.5	18.47	57.3	29.82	37.6	21.69
GM-CSF	0.0	0.00	0.0	0.00	0.2	0.17	0.6	0.57
VEGF	84.0	31.02	84.0	31.02	22.4	4.64	62.9	35.63
PDGF-AA	1.3	0.54	1.3	0.54	3.2	1.11	4.7	1.41
PDGF-BB	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00
MCP-1	930.5	348.79	930.5	348.79	2081.3	1028.47	745.5	297.72
FGF-2	0.4	0.43	0.4	0.43	0.9	0.43	1.0	0.59
IL-8	904.6	251.81	904.6	251.81	1575.5	752.34	2116.5	1124.92
IL-1α	50.9	26.90	50.9	26.90	1.4	0.84	1.3	0.84
IL-1β	0.4	0.23	0.4	0.23	0.2	0.13	0.8	0.57
IL-1ra	123.8	84.85	123.8	84.85	2.0	1.68	16.3	14.25

Conclusions Growth factor and cytokine release by SSc explants can be assessed using Luminex cytokine arrays. Chemokines IL-8 and MCP-1 predominate in the conditioned media from epidermal biopsies, whereas HGF release was prominent in the dermal conditioned media. The epidermis is confirmed as a possible source of chemokines in SSc. It may be possible to categorise SSc patients based on cytokine profiling arrays of explant skin biopsy material.

46. A Clinically Defined Subset of DcSSc with Thrombocytosis is Associated with High Serum IL-6 Level

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Purpose: IL-6 is a pleiotropic cytokine that has a wide range of biological activities in various target cells and regulates immune responses. We examined the relationship between serum IL-6 levels and acute phase response and modified Rodnan skin score (mRSS) in SSc. Based upon the known stimulatory effect of IL-6 on thrombopoiesis, we also hypothesised that a subgroup of diffuse cutaneous systemic sclerosis (dcSSc) patients with elevated platelet counts might have higher levels of IL-6.

Method: We compared IL-6 and soluble IL-6 receptor (sIL-6r) levels in a cross sectional study with defined subsets of 68 patients with SSc and 15 healthy controls. The three subgroups of SSc cases included dcSSc with high platelets (n=20, mean platelet count: 458 x 10^9/L), dcSSc with normal platelets (n=19, mean platelet count: 276 x 10^9), limited cutaneous SSc (lcSSc; n=22, mean platelet count: 264 x 10^9) and 15 controls. Serum IL-6 and sIL-6r levels (in pg/ml) were determined by ELISA. Associations between serum IL-6 and CRP, platelet count, peak and concurrent skin score were determined by Pearson's correlation coefficient. IL-6 levels were subdivided into three categories: High (>10 pg/ml), Low (<10 pg/ml and > 3.12 pg/ml) and undetectable (below quantitation limit, < 3.12 pg/ml). Categorical data were analysed by Chi-square test.

Results: A majority of the SSc cases were female: 77% and 96% dcSSc or lcSSc respectively compared to 53% in controls. The age of the subjects was equally distributed in all cohorts (mean±SD, years): 55.1±10.3 dcSSc, 59.1±11.4 lcSSc and 53.7±11.4 controls. Duration of disease (mean±SEM,months) for lcSSc and dcSSc was 152.6±23.9 and 52.4±7.0 respectively. Disease duration (mean±SEM,months) was longer in dcSSc with elevated platelets (57.0±11.9) than those with normal platelets (47.3±6.9).

More than a third of patients with dcSSc have high IL-6 and a majority of these patients (73%) have thrombocytosis with median platelet count above 450 ($p<0.001$). In contrast, a majority of the lcSSc (75%) and control (87%) cohorts had undetectable IL-6 levels. Moreover, IL-6 level positively correlated with platelet count in SSc ($r=0.5$, $p<0.001$). However, there were no significant differences in sIL-6R levels across all cohorts ($p=0.16$) and no correlation was observed between sIL-6R levels and platelet count ($r= -0.15$, $p=0.23$). There was strong association between serum IL-6 and CRP in the total cohort ($r =0.74$, $p<0.001$) and this correlation remained significant in the dcSSc with elevated platelets cohort ($r =0.5$, $p<0.001$). In addition, serum IL-6 levels positively correlated with concurrent mRSS ($r=0.48$, $p=0.02$) but not with peak mRSS ($r=0.19$, $p=0.40$). There was moderate correlation between platelet count and concurrent mRSS ($r=0.33$, $p=0.05$).

Conclusion: These results suggest that IL-6 may be a potential surrogate marker for skin disease in SSc and that thrombocytosis in dcSSc patients may selectively identify those with high IL-6, and higher mRSS. Together these data support further exploration of the biological significance of IL-6 in SSc and suggest that targeted therapeutic strategy against IL-6 ligand-receptor axis may be relevant in SSc.

47. Cardiovascular involvement in scleroderma, case study

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Background and purpose of the study:
The heart is one of the major organs involved in scleroderma, the involvement of which can be manifested by coronary disease, myocardial disease, conduction system abnormalities or arrhythmias . Many studies shows that cardiac involvement in scleroderma is one of the strongest predictive factor for death . We aimed to present the case study of a scleroderma patient with history of myocardial infarction.
Material and methods:
A 59 year-old male patient suffering from systemic scleroderma, psoriasis and hypertension was referred to our Cardiologic Department with

myocardial infarction. . He had no history of cardiac pain until the recent MI. The patient has undergone PCI with stent implantation to Cx and RCA. Additionally various diagnostic tools such as ECG, cardiac ECHO, Holter ECG, IMT measurement were performed. Intensive drug treatment of hypertension, hyperlipidaemia were established and after week of follow-up, his medical condition was improved.

Short discussion:

Our study proved that patients suffered from scleroderma are likely to develop atherosclerosis. It is important to assess cardiovascular condition in every patients with connective tissue disease.

48. The evaluation of the ultrasonic intima-media complex thickness in connective tissue disease patients.

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Introduction: Small changes in internal layer of artery (intima-media complex thickness IMT) predate developing of atherosclerosis plaque. Structural change in this complex is a well-known determinant of developing atherosclerosis in the future. The evaluation of the ultrasonic intima-media complex thickness -it's a non-invasive technique that can point borders between layers in artery, detect the presence of an atherosclerosis and follow it's progress, by multiple examination. Many studies has proved that patients suffered from connective tissue disease has also cardiovascular involvement.

Aim: The evaluation of the ultrasonic intima-media complex thickness in connective tissue disease patients.

Material and Method: We assessed carotid intima-media complex thickness in 32 patients with connective tissue disease and correlated with the control group. The patients suffers from: SLE, Scleroderma and MCTD. The average age was: 54 years, the group consist of 25 female and 7 male. The examination was carried out using USG- Vivid 7; transducer: 3,6 MHz, We assessed carotid intima-media complex in both carotid arteries about 1cm from its bulb. The results were averaged, and compared with the results of the control group.

Results: IMT in the examination group : the median carotid IMT: 0,8 mm; 21 (65,6%) patients IMT exceeded 1,0 mm; 11 (34,4%) patients IMT was within normal limits. IMT in control group: the median carotid IMT: 0,6 mm; 7 (23,3%) patients IMT exceeded 1,0 mm; 23 (76,7%) patients IMT was within normal limits.

Conclusion: The measurement of an ultrasonic IMT is quick and easy to perform diagnosis tool that can be carried out in every patient with high cardiovascular risk factors. Early detection of atherosclerosis can institute proper treatment and improve patient's quality of life. It has been proved that developing atherosclerosis and the ultrasonic IMT are inextricably connected. The evaluation and the control of IMT in patients with CTD are important part of making diagnosis and evaluating of patient health condition (screening test in cardiovascular complications);

49. Genome-Wide Association Study in UK Systemic Sclerosis cohort.

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Introduction: Systemic sclerosis (SSc) is a complex disease which involves endothelial and immune dysfunction together with an abnormal extra cellular matrix deposition. There is a definitive genetic component underlying the susceptibility to the disease, with strong associations with several genes involved in autoimmunity.

Objectives: To assess the potential usefulness of GWAS in a small group of SSc patients with clearly defined disease and biochemical phenotype to detect meaningful genetic associations.

Methods: We analysed 150 SSc and 50 controls (UK Caucasians) using Affymetrix Human 6.0 array. The SSc group was selected using stringent clinical criteria and follow-up data in order to obtain a clearly defined SSc phenotype. Control data was also obtained from convenient controls including the UK Wellcome Trust Case Control Consortium and from the NIMH Bipolar disease and Schizophrenia cohort. We verified the top 50 SNPs identified by GWAS in an additional group of 415 phenotyped SSc patients and 231 control samples.

Results: We found strong associations with SNPs in different gene regions which have been previously related to SSc. These regions included HLA (rs7763822, 7764491), STAT4 (rs932169) and XKR4 (rs6988356). Other SNPs, rs1455688, rs12147597 and rs396 were also found to have significant association with SSc. All of these SNPs had a p-values < 10^{-6}. The original association of these SNP from GWAS was sustained in the verification group (p = 0.04 to 0.006)

Conclusion: The pilot GWAS performed in UK SSc patients with a clearly defined phenotype was able to detect areas of association previously reported in SSc, in addition to new areas of association. These associations were verified in an expanded SSc group.

50.The novel TGF-β co-receptor, CD109, regulates extracellular matrix production in scleroderma skin fibroblasts

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Objective: Recent evidence indicates that dysregulation of TGF-β action plays an important role in the pathophysiology of scleroderma (systemic sclerosis, SSc). Skin fibrosis represents a clinical hallmark of SSc and TGF- is the most potent pro-fibrotic cytokine known. We have shown previously that the TGF-β co-receptor, CD109, plays an important role in regulating TGF-β action in skin cells. The specific objective of the current study was to determine whether CD109 plays a role in regulating TGF-β action and the fibrotic process in SSc

Methods Fibroblasts from arms or abdomens of 16 SSc patients and 9 normal controls were studied. The expression of CD109 in normal and SSc skin was analyzed by immunofluorescence and Western blot analysis. The effect of CD109 on extracellular matrix (ECM) synthesis was determined by blocking CD109 expression using CD109-specific siRNA. In addition, recombinant CD109 protein was used to determine its effect on ECM production.

Results We demonstrate that CD109 is expressed in normal and SSc skin fibroblasts. Importantly, CD109 protein expression is upregulated in SSc fibroblasts, while CD109 mRNA expression is similar in both SSc and normal fibroblasts. Furthermore, blocking the expression of CD109 using siRNA results in a marked upregulation of fibronectin, collagen type I and CTGF protein levels as compared to control siRNA treated cells in both normal and SSc skin fibroblasts. In addition, blocking CD109 expression leads to enhanced basal and TGF- -induced Smad2 and Smad3 activation. Furthermore, exogenous recombinant CD109 decreases TGF-β1-induced production of fibronectin, collagen type I and CTGF in normal and SSc skin fibroblasts.

Conclusions We demonstrate that CD109 is upregulated in SSc skin sections and cultured SSc skin fibroblasts. CD109 is an important regulator of ECM production in SSc fibroblasts with blocking CD109 expression leading to an increase and addition of recombinant CD109 protein resulting in a decrease in ECM production. CD109 may exert these effects by regulating Smad2/3 activation since blocking CD109 expression leads to an increase in Smad2/3 phosphorylation. Thus, the upregulation of CD109 in SSc may represent an adaptive response to aberrant activation of TGF- signaling pathways in SSc. Our finding that CD109 is able to decrease excessive ECM production in SSc fibroblasts suggests that this molecule has potential therapeutic value for the treatment of SSc.

51. Dysregulated pro- and antiangiogenic chemokine expression in systemic sclerosis

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Purpose. Systemic sclerosis (SSc) is characterized by fibrosis and microvascular abnormalities including dysregulated angiogenesis. Chemokines, in addition to their chemoattractant properties, have the ability to modulate angiogenesis. Chemokines lacking the ELR motif, such as monokine induced by interferon-γ (IFN-γ) (MIG/CXCL9) and IFN-inducible protein 10 (IP-10/CXCL10), inhibit angiogenesis by binding CXCR3. In addition, CXCL16 promotes angiogenesis by binding its unique receptor CXCR6. In this study, we determined the expression of these chemokines and receptors in SSc skin and serum and correlated their expression with clinical disease parameters.

Methods. Biopsies from proximal (less involved) and distal (more involved) skin and serum were obtained from patients with SSc and normal (NL) volunteers. Clinical data was gathered at the time of biopsy and includes the presence or absence of interstitial lung disease, renal disease, nonspecific interstitial pneumonia, pulmonary arterial hypertension, digital ulcers, gastrointestinal disease, gastric antral vascular ectasia, and disease duration. Immunohistochemistry was performed to determine the expression of MIG, IP-10, CXCL16, CXCR3, and CXCR6 in SSc and NL skin. ELISAs were performed to determine the concentration of MIG, IP-10, and CXCL16 in SSc and NL serum. Correlations were made between chemokine and chemokine receptor expression and clinical characteristics using the Pearson correlation test and SPSS software.

Results. MIG is highly expressed in the stratum spinosum of the epidermis of distal SSc skin (52%, n=21 patients), proximal SSc skin (52%, n=21), and normal skin (48%, n=10). Similarly, IP-10 is also highly expressed in the stratum basalis layer of the epidermis of distal SSc skin (100%, n=21 patients), proximal SSc skin (100%, n=21), and normal skin (100%, n=10). The levels of MIG and IP-10 are also significantly increased in SSc serum (876 pg/ml and 495 pg/ml, respectively, n=21 patients) compared to normal serum (126 pg/ml and 263 pg/ml, respectively, n=8, both $p<0.05$). In contrast, the expression of their receptor, CXCR3, is significantly decreased on ECs in distal SSc skin (21%, n=21) and proximal SSc skin (25%, n=21) compared to NL skin (54%, n=10, both $p<0.05$). In addition, the expression of CXCL16 is significantly decreased on the surface of ECs in distal SSc skin (20%, n=21) and proximal SSc skin (18%, n=21) compared to NL skin (45%, n=10, both $p<0.05$), while being significantly increased in SSc serum (4.6 ng/ml, n=21) compared to normal serum (3.3 ng/ml, n=9, $p<0.05$). However, the expression of its receptor, CXCR6, is significantly increased on the surface of ECs in distal SSc skin (15%, n=19) and proximal SSc skin (16%, n=19) compared to NL skin (4%, n=8, both $p<0.05$). Importantly, the increased expression of CXCR6 on ECs in proximal SSc skin is positively associated with the involvement of pulmonary arterial hypertension (Pearson correlation coefficient=0.590, p=0.010).

Conclusions. Antiangiogenic MIG and IP-10 are highly expressed in SSc skin and serum, whereas the expression of their receptor, CXCR3, is downregulated in SSc skin. In contrast, angiogenic CXCL16 is upregulated systemically in SSc serum, while the expression of its receptor, CXCR6, is upregulated in SSc skin. In addition, the increased CXCR6 expression on ECs in proximal SSc skin correlates positively with pulmonary arterial hypertension, suggesting a role for CXCL16/CXCR6 in SSc vasculopathy. In all, these results indicate that despite an abundance of pro- and antiangiogenic chemokines in SSc serum, that chemokine receptor expression in SSc skin is regulated in an effort to promote angiogenesis in the avascular SSc skin.

52.Upregulation of angiotensin signalling pathways underlies the increased contractility observed in pulmonary fibrosis lung fibroblasts.

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Background: Several lines of evidence implicate the angiotensin system in the pathogenesis of lung fibrosis, and lung fibroblasts isolated from fibrotic lungs have been shown to secrete increased levels of angiotensin II precursor. However, little is known on whether the Angiotensin axis is involved in the increased contractility of fibrotic fibroblasts. We evaluated the role played by the Angiotensin signalling axis in matrix contraction of lung fibroblasts isolated from idiopathic pulmonary fibrosis (IPF) lungs, used as a model for fibrotic lung diseases.

Methods: Lung fibroblasts isolated from IPF and from control lungs (three each), were used between passage 2-5. Lung fibroblast expression of Angiotensin receptor 1 (AT1) and receptor 2 (AT2), angiotensin converting enzyme-2 (ACE2) and alpha-smooth muscle actin (alpha-SMA) were examined using immunofluorescence staining and Western blot. The ability of Angiotensin II (Ang II; 100 nM) to enhance fibroblast contraction in a collagen gel contraction model was examined. To assess the effect of Ang II on fibroblast migratory ability, a migration scratch assay was performed. AT1 and AT2 specific antagonists Losartan (10 μM) and PD123319 (10 μM), respectively, were used. Ang II-induced signalling pathways were evaluated using selective pharmacological inhibitors, including PI3-kinase/AKT inhibitors wortmanin (0.1 μM) and LY294002 (20 μM).

Results: Angiotensin II significantly enhanced the ability of control lung fibroblasts to contract a collagen gel matrix by inducing the expression of alpha-SMA and by increasing lung fibroblast migratory capability; these features were significantly inhibited by AT1 specific antagonist Losartan, and partially by AT2 specific antagonist PD123319. IPF fibroblasts displayed higher levels of alpha-SMA and increased collagen gel contractility compared to controls; these characteristics were significantly reversed by treatment with Losartan, and partially by treatment with PD123319, as well as by PI3kinase/AKT inhibitors. Compared to controls, IPF fibroblasts had lower ACE2 and higher AT2 expression, while AT1 receptor expression was similar.

Conclusions: Treatment of IPF lung fibroblasts with either Losartan or PI3 kinase/AKT inhibitors reduces IPF fibroblasts contractility to levels seen in control lung fibroblasts, with potential clinical implications for the treatment of fibrotic lung diseases.

53. Involvement of functional autoantibodies against vascular receptors in systemic sclerosis

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BACKGROUND: Systemic sclerosis (SSc) features autoimmunity, vasculopathy, and tissue fibrosis. The effectors of renin-angiotensin and endothelin systems have been implicated in vasculopathy and fibrosis. We hypothesized a role for autoimmune receptor stimulation as a functional link between three major pathophysiologic features consistent with SSc . METHODS: We tested sera of 478 SSc patients (298 in the study cohort and 180 from two independent cohorts), 372 healthy subjects, and 333 control-disease subjects, for antibodies against angiotensin II type 1 receptor (AT1R) and endothelin-1 type A receptor (ETAR) by solid phase assay. Organ involvement and patient survival were also investigated. Binding specificities were tested by immunoprecipitation. The biological effects of autoantibodies were tested in microvascular endothelial cells *in vitro*. RESULTS: Anti-AT1R-and anti-ETAR-autoantibodies were detected in most SSc patients ($p<0.001$ compared to control diseases or healthy subjects). Autoantibodies specifically bound to respective receptors on endothelial cells. Higher levels of both autoantibodies were associated with progressive disease and in particular with vascular complications. Both autoantibodies exert biologic effect as they induced ERK1/2 phosphorylation and increased TGFß-gene expression in endothelial cells which could be blocked with specific receptor antagonists. CONCLUSIONS: Functional autoimmunity directed at AT1R and ETAR identifies SSc patients with more severe fibrotic and in particular vascular disease manifestations and decreased survival. AT1Rand ETAR-autoantibodies participate in disease pathogenesis and may serve as diagnostic tool for risk assessment. Identification of affected patients may lead to improved surveillance and early specific pharmacologic interventions.

54. Autocrine TGFβ signaling contributes to enhanced Erk phosphorylation via modulation of Protein Phosphatase 2A expression in Scleroderma fibroblasts

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Background

During Scleroderma (SSc) pathogenesis, fibroblasts acquire an activated phenotype characterized by enhanced production of extracellular matrix proteins and activation of several major signaling pathways including extracellular signal-regulated kinase (ERK1/2). Constitutive activation of Erk1/2 has been implicated in SSc fibrosis, however the mechanism of this sustained phosphorylation is still unclear. Protein Phosphatase 2A (PP2A) is a key serine threonine phosphatase with a known role in the dephosphorylation of ERK1/2. Recently published microarray data from cultured SSc fibroblasts suggests that the catalytic subunit (C-subunit) of PP2A is down-regulated in SSc. In this study we examine whether PP2A is dysregulated in SSc and could contribute to aberrant Erk1/2 phosphorylation.

Results

SSc fibroblasts isolated from patient skin biopsies and age, race and gender matched controls were used to analyze mRNA and protein expression of PP2A and the phosphorylation status of Erk1/2. These experiments show for the first time that PP2A expression is decreased in SSc fibroblasts at the protein and mRNA levels. Furthermore, TGFβ a major profibrotic cytokine implicated in SSc fibrosis, down-regulates PP2A expression in healthy fibroblasts. Accordingly, blockade of autocrine TGFβ signaling in SSc fibroblasts using soluble recombinant TGFβ receptor II (SRII) restored PP2A levels and decreased ERK1/2 phosphorylation. To confirm that this decreased Erk phosphorylation was a specific effect of PP2A, we used siRNA against the catalytic subunit of PP2A. PP2A blockade resulted in enhanced phosphorylation of Erk1/2, confirming our previous result.

Conclusion

These studies suggest that decreased PP2A levels in SSc is a result of constitutively activated autocrine TGFβ signaling and could contribute to hyper-phosphorylation of ERK1/2 in SSc fibroblasts.

55. Comparison of Estimated Glomerular Filtration Rate (eGFR) and Actual GFR in Patients with Systemic Sclerosis (Scleroderma, SSc)

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Background. Little is known about renal function in patients with SSc who have not experienced scleroderma renal crisis. Renal involvement is likely to be underestimated based on autopsy revealing nephropathology in 80% of cases. Serum creatinine (Cr) and creatinine-based equations like Cockcroft Gault (CrCl) and Modified Diet in Renal Disease Equations (eGFR), although widely used in clinical practice, may not be accurate in evaluation of renal function in SSc. We compared glomerular filtration rates using the iohexol plasma disappearance technique (iGFR) to CrCl and eGFR in a cohort of SSc patients.

Methods. Patients who met ACR criteria for SSc were recruited as part of a pilot study designed to evaluate renal function in patients receiving oral treprostinil or placebo. Subjects with any of the following: Hgb <75% of normal, weight <40 KG, history of diabetes, liver or renal impairment and use of PDE5 inhibitors or prostanoids were excluded. The iGFR procedure involves: hydration of the patients, injection of 5mL of iohexol, blood samples collection at 5, 10, 15, 180, 240 and 300 minutes and estimates clearance using a 2-compartment model for plasma disappearance of iohexol. The CrCl was calculated based on the Cockcroft Gault equation and the eGFR value was obtained using the 4-component MDRD formula.

Results. Thus far, 5 women with SSc were recruited by informed consent into the study and underwent iGFR procedures at baseline. One subject had a unilateral nephrectomy 10 years prior due to renal cell carcinoma*.

	Sex	Age	SSc	Disease Duration (y)	Renal	Weight (KG)	BSA	Cr	CrCl	iGFR	eGFR
A	F	39	LcSSc	1	N	122	2.2	0.9	156.4	73	74
B	F	77	LcSSc	1	N	79.8	1.8	0.6	95.7	105.3	103
C	F	62	LcSSc	33	N	58.7	1.7	0.8	65.4	83.7	77
D	F	66	DcSSc	30	N	75.2	1.8	0.9	66.8	95.4	66
E*	F	59	LcSSc	6	Y	97.3	2.1	1.0	90.0	87.2	57

Conclusions. In this small cohort of SSc patients the iGFR was not similar to the eGFR in all cases. This preliminary data highlights observations in the general population that indicate serum creatinine and creatinine-based equations are not accurate in predicting GFR in subjects with SSc who are at significant risk for renal involvement. Larger longitudinal studies are needed to evaluate the utility of iGFR in SSc patients.
This study was supported with funds provided by United Therapeutics Corp. RTP, NC

56. Pharmacokinetics of Oral Treprostinil in Patients with Systemic Sclerosis (SSc) and Digital Ulcer Disease

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Purpose: Fibrotic vasculopathy with endothelial dysfunction is the hallmark of SSc (scleroderma) and is the underlying cause of clinical complications including Raynaud's phenomenon, digital ulcers, pulmonary hypertension and renal crisis. Prostacyclins are proven effective therapies for pulmonary hypertension, but delivery systems can be either cumbersome (IV, SQ) or targeted (inhalation). Treprostinil diethanolamine (TDE), an oral prostacyclin (PGI_2) analogue, is in development as an oral sustained release (SR) tablet. The availability of a formulation permitting convenient systemic delivery might have applicability to non-pulmonary SSc vascular complications. The objective of this study was to evaluate the disposition of TDE SR in patients with systemic sclerosis with peripheral vascular complications.

Methods: Patients with SSc (as defined by the ACR criteria) and evidence of peripheral vaso-occlusive disease (defined as presence or history of an active digital ulcer within the past 6 months) participated in this study. Subjects with PAH were excluded. A single 1 mg TDE SR oral dose was administered in the morning following a standardized 500 kcal breakfast. Fourteen blood samples were obtained over 24 hrs and plasma concentrations of treprostinil quantified by liquid chromatography/mass spectrometry. Treprostinil pharmacokinetic (PK) parameters were calculated using non-compartmental analysis.

Results: Eight subjects were recruited (7 F / 1 M). The mean age was 48.5 years and the mean disease duration was 12.8 years. Five subjects had limited SSc and three had diffuse SSc. We compared the PK data to those obtained from a pool of healthy volunteers. Results are shown as geometric mean (CV%).

Parameter	SSc N=8	Healthy Volunteers N=128
$AUC_{0\text{-}24hr}$ (hr*pg/mL)	3908 (62%)	2977 (49%)
Cmax (pg/mL)	672 (42%)	601 (48%)
Tmax (hr)	3.5	4.0
Total Clearance (L/hr)	206 (40%)	346(47%)
$T_{1/2}$(hr)	4.3	3.6

SSc exhibited similar mean maximum plasma concentrations and modestly higher AUC values (by mean factors of 1.1 and 1.3). Headache and nausea were the most commonly reported adverse events in three and two SSc subjects, respectively.

Conclusions: TDE SR is absorbed and had no unexpected adverse effects following administration of a single 1 mg dose in SSc patients with peripheral vascular disease. Although the study population was expected to display a variable degree of absorption due to SSc-related gastro-intestinal complications, the pharmacokinetic profile was comparable to that observed in healthy volunteers. Multiple dose PK studies are in progress and will support dosing guidelines in controlled trials in SSc patients.

57. Long-term outcome of patients with an isolated low diffusing capacity

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Objective. To determine the outcome of systemic scleroderma (SSc) patients with isolated decreased diffusing capacity for carbon monoxide (DL_{CO}).
Methods. We screened a large university-based scleroderma specialty center database to select patients with the following profile: 1) DL_{CO} <50% predicted; 2) forced vital capacity (FVC) >70% predicted; 3) no evidence of severe obstructive lung disease on pulmonary function testing (PFT); and 4) no signs of pulmonary hypertension (PH) on echocardiography (echo). Qualifying studies had to be performed within 12 months of each other. Patients in this retrospective cohort were followed to assess for the following primary outcome: pulmonary hypertension defined as a mean pulmonary artery pressure (mPAP) >25 mm Hg or an exercise induced mPAP >30 mm Hg. The secondary outcomes were all-cause mortality, the development of PH according to the echocardiographic (right ventricular systolic pressure >45 mm Hg) or clinical picture, the development of severe restrictive or obstructive lung disease (FVC <60% predicted or FEV_1/FVC <60% predicted), and/or the development of cardiomyopathy (ejection fraction <40%). **Results.** Ninety-five patients met our inclusion criteria and 91 had evaluable follow-up data. Cases were predominantly Caucasian females in their sixth decade of life with limited SSc. The mean disease duration from the onset of Raynaud's phenomenon or symptoms attributable to SSc to the time of study entry was 10 and 7 years, respectively. After a mean follow-up of 4.2 years, twenty-four patients were diagnosed with PH on right heart catheterization (cath), 20 patients had evidence of PH on echo but did not undergo cath, and another four patients had a clinical picture consistent with PH without cath or echo findings. Twenty patients developed severe restriction, one patient developed severe obstruction, and six patients developed cardiomyopathy. Overall mortality was 38%, regardless of specific diagnosis.

Subjects were categorized into one of 4 groups: 1) pulmonary vascular disease (PVD) alone; 2) PVD with interstitial lung disease (ILD defined as severe restriction); 3) ILD alone; or 4) no lung disease. When categorized as such, there were significant differences in baseline characteristics, follow up PFT and echocardiographic data and mortality. Patients with PVD alone and PVD and ILD together had significantly higher rates of mortality than those with ILD alone or no diagnosed lung disease at follow up, although those with PVD were older at study entry. Subjects had a 2- and 5-year cumulative survival of 85% and 60%, respectively. There was a significant difference in 5-year survival between cases with and without PVD (55% and 75% respectively, p=0.0154). Cases had a 45% 5-year cumulative diagnosis rate of PVD, and a 25% 5-year cumulative diagnosis rate of PH on right heart cath.
Conclusion. Systemic scleroderma patients with an isolated decrease in DL_{CO} are at high risk of developing PVD with a 5-year cumulative diagnosis rates of 45%. In this study, patients who develop evidence of PVD had significantly decreased survival compared to those without PVD. However, no significant difference in survival was present between patients diagnosed with PH on right heart cath and those who were not diagnosed, although many patients without right heart cath data had substantial evidence to support a diagnosis of PH. Although systemic scleroderma patients with an isolated decrease in DL_{CO} are at high risk of developing PVD, all cardiopulmonary manifestations may ultimately be seen. Therefore, thorough work-up is warranted. With the development of better medical therapies for pulmonary vascular disease, it is imperative that patients with low DL_{CO} be quickly evaluated and treated as survival appears poor in this group.

58. Can we predict presence of Pulmonary Hypertension in Scleroderma Using Lung Function Data?

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Introduction

The leading causes of mortality in systemic sclerosis are interstitial lung disease and pulmonary hypertension. Pulmonary hypertension in 10-15% of patients. Although right heart catheterisation remains the gold standard test for diagnosing pulmonary hypertension, it is invasive and associated with morbidity and mortality and so other tests are needed as screening tests. Pulmonary function tests are widely used in this population to screen for both interstitial lung disease and pulmonary hypertension, but it is not known which patients require right heart catheterisation on the basis of results of lung function tests.

Methods

We retrospectively reviewed patients with connective tissue diseases who had their first right heart catheter at our institution between September 1996 and May 2010 and who underwent any pulmonary function testing within six months of the right heart catheter.

Results

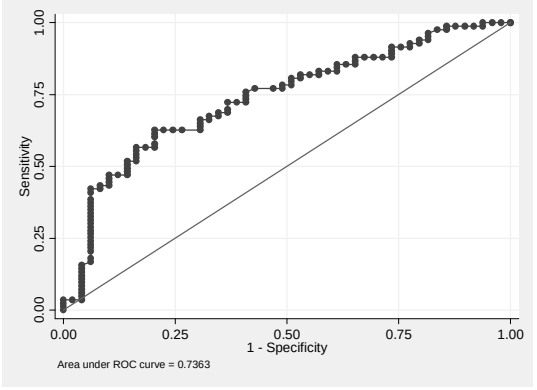
Pulmonary function tests (including a minimum data set of pulse oximetry, FVC% predicted and DLCO% predicted) were available within 6 months of the right heart catheter in 396 patients. Of these patients, 275 had LcSSc, 90 had DcSSc and 31 had SSc not otherwise defined.

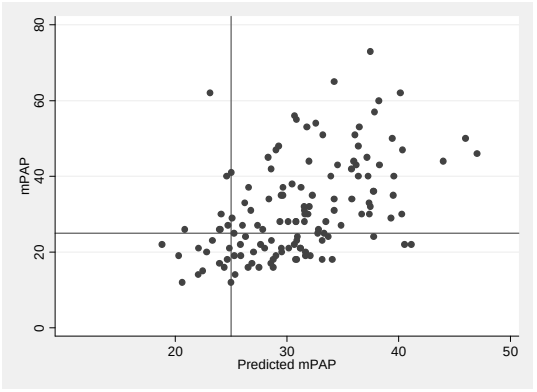
Within the study sample, age and gender did not differ between those with PH (n=193, mean PAP 37.9 mm Hg) and those without (n=108, mean PAP 19.2 mm Hg). The presence of PH was associated with reduced SpO2, FVC, DLCO and KCO.

Multivariable linear regression using oxygen category (given a value of 1 if SpO2>94%, 2 if 88%<SpO2<94%, 3 if SpO2<88%) and Dlco% predicted predicted mPAP with R^2=22.1% (i.e. 22.1% of the variability in mPAP is explained by these two variables). FVC was not significantly associated with mPAP.

Predicted mPAP = 31.04055+6.885172 x Oxygen category - 0.2219308 x DLCO % predicted

This formula gives an area under the curve of 0.7346 (see figure) in the test cohort (n=132, 95% CI 0.64871 - 0.82387). When using a cutoff predicted mPAP of 25 to diagnose pulmonary hypertension, the formula has a sensitivity of 91.6%, specificity of 22.5% and classifies 65.9% of patients correctly.





Discussion

We have derived and validated a formula using oxygen saturation and diffusion of carbon monoxide which predicts presence of pulmonary hypertension with a sensitivity over 90%. Use of this formula may be helpful when screening patients with scleroderma to indicate the need for right heart catheterisation.

59. Sunitinib limits the phosphorylation of platelet-derived growth factor receptors in the skin and abrogated the development of HOCl-induced systemic sclerosis in mice.

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Background: Systemic sclerosis (SSc) is characterized by the fibrosis of various organs, vascular hyperreactivity and immunological dysregulation. Platelet-derived growth factors (PDGFs) are the primary mitogens for the cells of mesenchymal and neuroectodermal origin and are implicated in development of various organ fibrosis and vascular diseases. Increased presence of PDGF and PDGF receptors have been found in scleroderma skin biopsies, and sera from those SSc patients may contain pathological autoantibodies directed against PDGF receptors. Sunitinib, a currently available drug, interferes with PDGF signaling by blocking the tyrosine kinase activity of PDGF receptors. We investigated here the effect of this molecule in a mouse model of diffuse SSc induced by hypochlorite injections.
Materials and Methods: SSc was induced in BALB/c mice by daily subcutaneous injections of hypochlorous acid daily for 6 weeks. Those mice were simultaneously treated intraperitoneally with sunitinib or with PBS. Skin and lung fibrosis were assessed by histological and biochemical methods. The proliferation of fibroblasts purified from diseased skin was assessed by thymidine incorporation. Auto-antibodies were detected by ELISA and spleen cell populations analyzed by flow cytometry. The expression of phosphorylated PDGF receptors in the skin was assessed by western blot and immunofluorescence analysis.
Results: Injections of hypochlorite induced cutaneous and lung fibrosis, increased the proliferation rate of fibroblasts isolated from fibrotic skin, increased splenic B cell counts and anti-DNA topoisomerase-1 autoantibodies in BALB/c mice. High amounts of phosphorylated PDGF receptors were found in fibrotic skin from those mice. Treatment with sunitinib significantly reduced the expression of phosphorylated PDGF receptors in the skin. Moreover, this treatment prevented the accumulation of collagen in the skin and lungs and reduced the dermal thickness ($p=0.030$ and $p=0.012$ for skin and lung collagen content in treated versus untreated mice). In addition, Sunitinib decreased the proliferation rate of fibroblasts isolated from fibrotic skin ($p=0.001$ for treated versus untreated mice). The expansion of the splenic B cell population was also found reduced in treated mice compared to untreated animals ($p=0.002$).
Conclusions: The PDGF pathway is hyperactivated in SSc tissues. Interruption of this perpetual signaling by sunitinib abrogated the development of skin and lung fibrosis in a mouse model of diffuse SSc. Consequently, this tyrosine kinase inhibitor may represent a new alternative for the treatment of SSc that need to be tested in clinical trials.

60. Anti-fibrillarin antibodies (anti-U3-RNP) in African American patients with SSc: Disease correlates and survival in the GENISOS cohort

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OBJECTIVES: To evaluate the survival and clinical correlates of African American Systemic sclerosis (SSc; Scleroderma) patients with anti-

fibrillarin antibody (AFA) in the Genetic versus Environment in Scleroderma Outcome Study (GENISOS) cohort

METHODS: At the time of analysis, 284 patients were enrolled in the multiethnic GENISOS cohort. Recruitment started in January 1998 and continues through the present. Patients were followed 3.9 (±3.6) years; up to 12 years, in 1438 visits. AFAs were determined on patients with anti-nucleolar antibodies by immunoprecipitation of the radiolabeled recombinant protein. We investigated the frequency of AFA in different ethnic groups in GENISOS. Furthermore, we examined survival, different clinical aspects in the African Americans with and without AFA. All the comparisons were conducted using student's t test for continuous and the χ^2 test for the categorical variables. A Cox proportional hazards regression model was used to investigate the effect of anti-fibrillarin on survival in African American patients after adjustment for age at disease onset. The starting point for the survival analysis was the time of 1st non-Raynaud's symptom.

RESULTS: The GENISOS cohort included 133 (46.8%) Caucasian, 83 (28.9%) Hispanic, 58 (20.4%) African American, and 10 (3.5%) other ethnicities in the GENISOS cohort. Twenty-nine (29; 10.2%) patients had AFA. AFA were reported more frequently in African Americans vs. non-African Americans; 14 (24.6%) vs. 15 (6.8%), respectively (P<0.001; OR: 4.45; CI: 1.83-10.6). In the overall population, patients with AFA (n=29) were significantly younger at disease onset (40.1 vs. 46.7 years; p=0.011) and had more frequently diffuse disease (p=0.033) and pericarditis (p=0.009).

In African Americans, patients with AFA were younger at disease onset (34.5 vs. 47.1; p=0.003). Disease duration and type were same in both groups. There was no significant difference in gender, modified Rodnan skin score, gastrointestinal, cardiac, renal, and musculoskeletal involvement between the two groups. Alveolar fibrosis on CT Scan was more frequently reported in those without AFA (p=0.029). However, there was no difference between FVC and DLCO % predicted values. After adjustment for age, African American patients with AFA had better survival in comparison to the remainder of African American patients (HR: 0.21; CI: 0.04-0.98; p=0.05).

CONCLUSIONS: The frequency of AFA was higher in African Americans as compared to Caucasians and Hispanics. African Americans with AFA had less frequently lung fibrosis and a better survival.

KEYWORDS: Systemic sclerosis, anti-fibrillarin antibody, anti U3-RNP, survival, African Americans

61. Fibroblast specific Connective tissue growth factor expression modulates anomalous features of cell fate changes of adjacent epithelia

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Introduction: Scleroderma (Systemic Sclerosis; SSc) is an autoimmune rheumatic disease characterised by immune activation, micro-vascular alterations, and fibrosis of skin and major internal organs including the lungs, kidney, and gastrointestinal tract. Connective tissue growth factor (CTGF), a secreted matricellular protein has emerged as a key pathologic mediator in SSc and other fibrotic disorders. Because of the early infiltration of mononuclear immune cells in SSc, fibroblasts and fibroblast-like cells are activated in the disease. Recent studies have implicated changes within epithelia in SSc, but elucidation of the significance and contribution of the epithelial cells to tissue fibrosis in SSc is unclear.

Objectives: To assess the role of fibroblast-selective expression of CTGF *in vivo* on organ and tissue development and function, and connective tissue remodelling, scarring and fibrosis.

Methods: Dermal and pulmonary tissues was harvested from mice over-expressing CTGF in fibroblasts under the control of the fibroblast-specific collagen alpha2(I) promoter enhancer and wild type control. Tissue samples were examined by immuno-histochemistry for phenotypic markers of fibrosis, epithelial activation and differentiation and stimulation of signal transduction pathways.

Results: Here we show the paracrine effects of CTGF on epithelial cells in skin and lung in transgenic mice over-expressing CTGF targeted to fibroblasts (*Col1a2-CTGF*). As reported *Col1a2-CTGF*-transgenic mice develop pronounced systemic fibrosis affecting the skin, lung, kidney and small blood vessels in kidney. The skin fibrosis was characterized by an expansion of the dermal compartment; associated with increased -SMA (myofibroblasts) expressing cells. The skin was also characterized by focal hyperplasia of the epidermis determined by activated keratinocyte markers K16 and K6. Unlike wild type mice, basal cells in the epidermis of *Col1a2-CTGF* mice were hypertrophic and stained positive for -SMA, Snail, and S100A4, indicating that these cells changed their genetic program. The transcription factor Sox9, is expressed in outer root sheath and in the stem cell compartment in hair follicles. Increased expression of Sox9 was

observed in hair follicles as well as in the basal cells of the epidermis suggestive of cells that are not fully differentiated. The change in the genetic program of the epithelial cells was associated with activation of p-p38 and pErk1/2 in the granular and cornified layers. Abnormal Snail, S100A4 and Sox9 expression patterns strongly suggested that over-expression of CTGF in the dermal compartment results in major cell fate changes in the basal layer of the epidermis. Activation of the Erk MAPK pathway may mediate epidermal hyper-proliferation and perturbed differentiation of keratinocytes in the epidermis.

Col1a2-CTGF-transgenic mice develop extensive fibrosis and focal fibrotic lesions within the lung parenchyma. A marked increase in myofibroblasts exemplified by expression of -SMA was evident in the lesional and unaffected lung tissue of *Col1a2-CTGF*-transgenic mice. To determine if these cells are derived from epithelial cells, co-immunostaining with -SMA and TTF-1, a transcription factor normally expressed in alveolar epithelial cells revealed TTF-1 and -SMA co-staining of cells around the alveolar walls with a similar staining pattern as in the fibrotic foci. Only TTF-1 expressing cells were evident in control lung sections. Therefore it appears that -SMA expressing myofibroblasts are probably derived from epithelial cells and that these cells are in transition, expressing both epithelial and mesenchymal characteristics.

Conclusion: Our data supports the notion that CTGF expression in fibroblastic cells in the skin and lungs not only causes extensive fibrosis and myofibroblast transformation, but also causes changes in the differentiation program of adjacent epithelial cells, which might directly contribute to fibrogenesis.

62. Role of Fli1 in proliferation and migration of human dermal microvascular endothelial cells

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Background

Systemic sclerosis is an autoimmune disease of unknown etiology that is characterized by obliterative vasculopathy and excess production and deposition of extracellular matrix proteins resulting in fibrosis of both skin and internal organs. Although the mechanisms of fibrosis in SSc are poorly understood, there is strong evidence that transcription factor Fli1, which is expressed at the reduced levels in endothelial cells in affected skin of patients with SSc, plays a pivotal role in development and maintenance of SSc phenotype. However specific mechanisms of its role especially in endothelial cells are still unclear.

Our previous study showed that mice with endothelial Fli1 deficiency display disorganized dermal vascular network with greatly compromised vessel integrity and markedly increased vessel permeability, suggesting that Fli1 is involved in regulation of genes critical for vascular remodeling (1). The aim of this study was to further investigate the biological role of Fli1 in endothelial cells.

Materials and methods

Human dermal microvascular endothelial cells (HDMECs) were isolated from foreskin tissues. Fli1 knockdown was performed using Fli1siRNA adenovirus. Expression levels of Fli1 were determined by qPCR. Proliferation was examined with CellTiter 96® AQueous One Solution Cell Proliferation Assay. Cell migration activity was performed by “scratch assay”. Angiogenic factors: VEGF 50ng/ml, CTGF 100ng/ml, CYR61 100ng/ml for 24 hours. Angiostatic factors: IFN-α 1000U/ml, TLR (Toll-like receptor) ligands: Pam3CSK4 1μg/ml (TLR2), Poly(I:C) 2.5μg/ml (TLR3) and LPS 10μg/ml (TLR4) for 24 hours.

Results

To investigate whether growth factors or other immune mediators modulate expression level of Fli1 HDMECs were treated with VEGF, CTGF, CYR61, IFN-α, and TLR2, -3 and -4 ligands. After 24h of treatment, Fli1 mRNA expression was increased in response to VEGF, CTGF and CYR61 (6.8-fold, 4.37-fold and 3.18-fold, respectively). In contrast, mRNA expression level of Fli1 was decreased in response to IFN-α, TLR2, -3 and -4 ligands (7.14-fold, 1.26-fold, 2.79-fold and 4.78-fold, respectively). In the absence of Fli1 basal proliferation index of HDMECs was markedly decreased and

no further significant responses to angiogenic or angiostatic factors was observed. However, in control cells treated with scrambled RNA, responses to the above treatments were preserved indicating that the proliferative responses of HDMECs are Fli1-dependent. To better understand how Fli1 regulates the behavior of endothelial cells, we also examined the effects of Fli1 knockdown on migration of HDMECs in response to a known inducer of endothelial cell migration, sphingosine-1 phosphate (S1P). We observed that Fli1 deficiency inhibits HDMEC migration in response to S1P.

Conclusion

This work demonstrates that Fli1 function is required for proliferation and migration of HDMECs. Furthermore, Fli1 expression level closely correlates with proliferative responses of HDMECs. IFN- is a potent inhibitor of Fli1 expression, suggesting that activation of innate immune pathways in endothelial cells may be responsible for the reduced level of Fli1 observed in patients with SSc.

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63. Altered Salivary Redox Homeostasis in Patients with Systemic Sclerosis

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Objectives: Oxidative stress has been implicated in the pathogenesis of systemic sclerosis (SSc). The objective of the current study was to determine whether SSc is associated with altered redox homeostasis in human saliva.

Methods: Participants in this study were 70 female scleroderma patients and 120 female controls. 8-hydroxy-2'-deoxyguanosine (8-OHdG), 8-epi-prostaglandin F2α (8-epi-PGF2α), and total protein carbonyls were assayed by ELISA to quantify oxidative damage to nucleic acids, lipids and proteins, respectively, in whole nonstimulated saliva. Logistic regression analyses were used to assess the association between scleroderma and oxidative stress.

Results: We observed a significantly positive association between salivary protein carbonyls and SSc in a crude statistic (OR = 9.06, $p < 0.0001$), and multivariable model adjusted for log 8-OHdG, log 8-epi- PGF2α and antioxidant exposure (OR = 9.26, $p < 0.0001$). No significant association was noted between SSc and salivary 8- epi-PGF 2α or 8-OHdG. Scleroderma subjects were significantly more likely to report antioxidant ingestion (35/70, 50%) than the control group (52/350, 15%, $P< 0.0001$). We noted a strong association between antioxidant ingestion and scleroderma (OR= 5.73, 95%CI: 3.30-9.97, $P<0.0001$) in crude analyses. This effect remained in a multivariate logistic regression analysis that took into account the difference of age and gender between scleroderma and control groups (OR= 4.20, $P<0.0001$). Age and female gender covariates were significantly associated with scleroderma in the crude analyses (OR_{age}= 1.03, 95%CI: 1.02-1.05, $P<0.0001$; $OR_{females}$= 8.54, 95%CI: 3.61-20.23, $P<0.0001$) and multivariable logistic regression analyses (OR_{age}= 1.03, 95%CI: 1.00-1.04, $P=0.007$, $OR_{females}$= 6.61, 95%CI: 2.73-15.98, $P<0.0001$).

Conclusions: The results obtained in the present study indicate i) significantly higher levels of 8-OHdG and protein carbonyls in whole non-stimulated saliva of scleroderma subjects relative to control values, ii) a positive correlation between salivary TAC and SSc status and iii) no significant change in levels of 8-epi-PGF2alpha in SSc saliva. A previous study demonstrated diurnal variations in salivary protein carbonyl levels. Thus, all salivary samples were obtained between 9:00am - 12:00pm to minimize temporal fluctuation in salivary redox homeostasis. Salivary redox homeostasis is perturbed in patients with SSc and may inform on the pathophysiology and presence of the disease (biomarkers) and efficacy of therapeutic interventions. The current data set extends these observations to saliva and provides strong evidence of perturbed redox homeostasis in the oral cavity of SSc subjects. Our observations further attest to the role of oxidative stress and possible up-regulation of antioxidant defenses in the pathophysiology of scleroderma.

64.A preliminary investigation of the predictive value and response to thereapy with cyclophosphamide of interleukin-16 in bronchoalveolar lavaage from patients with interstitial lung disease in systemic sclerosis

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RATIONALE. Interstitial lung disease (ILD) in systemic sclerosis (SSc) is present in 80% of patients with SSC and is a common cause of mortality. Disease activity predicting progression to fibrosis has previously been determined from the percentage of eosinophils or neutrophils in bronchoalveolar lavage (BAL) fluid. Cyclophosphamide (CYC), has been shown to lessen decline in FVC among patients with SSc and active lung inflammation. No cytokines in BAL form patients with SSc have been reliably demonstrated to predict disease progression or response to CYC. Interleukin-16 (IL-16) is an immunoregulatory cytokine produced by leukocytes, epithelial cells, and fibroblasts and is present in lungs of patients with a variety of inflammatory lung diseases. IL-16 may act by in conjunction with other cytokines to promote Th-1 development, which has been shown to be increased in SSc patients with active ILD. We examined IL-16 concentrations in the BAL of patients with SSc to determine if IL-16 could identify patients with active ILD who responded to CYC.

METHODS. 24 (16 diffuse, 8 limited) SSc patients with inflammatory BAL defined as $\geq$ 3 % neutrophils or $\geq$ 2 % eosinophils were treated with monthly intravenous CYC for 6-12 months and 15 patients without inflammatory BAL (untreated) were followed with serial PFTs and HRCT scans. IL-16 ELISA was performed on archived BAL fluid. IL-16 levels in patients without BAL inflammation who did not develop SSc-ILD on HRCT were compared to those who did develop ILD and to patients who had a positive BAL using a non-paired students t test. Results from patients treated with CYC who had a prolonged remission (>3 years) determined by stable FVC were compared to results from i) patients who relapsed within one year of therapy, and ii) non-responders.

RESULTS. Patients with a negative BAL had significantly lower IL-16 levels (17.9 $\pm$ 16.5 pg/ml, range 0 to 36.4 pg/ml) compared with both patients who had a negative BAL but then developed fibrosis (157.8 $\pm$ 93.8 pg/ml, range 73 to 447.4 pg/ml; p=0.009) and to patients with an inflammatory BAL (97.9 $\pm$ 57.1 pg/ml, range 1 to 231.5 pg/ml; p=0.003). There was no difference in IL-16 levels between patients who developed fibrosis regardless of BAL inflammation presence (p=0.08). Patients who received CYC and developed a prolonged remission had significantly lower baseline BAL IL-16 (46.9 $\pm$32.7 pg/ml, range 1 to 121.8 pg/ml) levels compared with non-responders (146.1 $\pm$ 70.5 pg/ml, range 46.3 to 231.5,; p=0.005) or those w who relapsed within one year of therapy (133.9 $\pm$ 35 pg/ml, range 37.7 to 180.9 pg/ml; p=0.00017). The latter two groups had similar BAL IL-16 levels (p=0.4).

CONCLUSION. High concentrations of IL-16 in BAL are associated with active SSc-ILD and may be a better predictor of disease activity and loss of FVC than an elevation in inflammatory cell percentage. Patients with low BAL IL-16 levels are less likely to have active SSc-ILD but those who do have a longer remission with CYC than patients with higher concentrations. IL-16 levels in BAL may be useful in identifying patients with SSc-ILD who would benefit from treatment with CYC. IL-16 may represent a marker of fibroblast or epithelial cell activity in active SSc-ILD and contribute to the dominance of Th-1 cells which could be mediating chronic inflammation.

65.Comparative analysis of sclerodermoid-Graft Versus Host Disease and Systemic Sclerosis skin tissues reveals insights in the specific pathways involved in bridging immune activation and tissue fibrosis.

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Systemic Sclerosis is a chronic disease involving autoimmune activation, fibroproliferative vasculopathy and tissue fibrosis. Microarray analysis of scleroderma skin biopsies identified an intrinsic gene signature associated with Scleroderma. Besides the putative role that can be hypothesized by the known gene product function, it is not clear which genes within this signature are specifically involved in the immunopathogenesis of skin fibrosis. Sclerodermoid GVHD (scl-GVHD) is a form of chronic GVHD

that shares with Scleroderma a similar skin involvement, clearly induced by the immune trigger of allogeneic bone marrow transplant. Though scl-GVHD patients do not develop autoantibodies typical of Systemic Sclerosis, neither a similar lung involvement, histopathological analysis conducted on 8 scl-GVHD and 3 non sclGVHD skin biopsies confirmed in scl-GVHD a tissue fibrosis and a fibroproliferative vasculopathy similar to SSc, with the remarkable difference of an increased drive towards neo-angiogenesis. Dense, non-specific tissue infiltrate was the main histopathologic finding of non-scl-GVHD. Furthermore, immunohistochemistry analysis followed by confocal laser scanner microscopy of scl-GVHD confirmed a specific pattern of expression of Allograft Inflammatory Factor-1 and caveolin-1, both previously identified by our group as involved in different aspects of Scleroderma pathogenesis. Specifically, AIF-1 expression was increased by 6.3 fold in Scl-cGVHD (±1.06; p= 0.0146) compared both with healthy and GVHD skin. The pattern of expression was mostly perivascular, in tissue infiltrating mononuclear cells, whereas microvascular endothelial cells did not show any AIF-1 expression despite what has been previously shown in SSc. Cav-1 immunofluorescence was decreased in Scl-GVHD (- 2.68 fold; p=0.0289), mostly due to lack of expression in stromal, pro-collagen positive cells, since endothelial and epithelial cells showed a conserved expression for Cav-1. To further indentify communal pathways in scl-GVHD and SSc skin involvement we analyzed the level of expression of the scleroderma gene signature within scl-GVHD and non scl-GVHD transcriptomes, by pathway focused qPCR array. Of the 80 genes analyzed, 46 were differentially expressed in cGVHD biopsies and 34 remained peculiar of Scleroderma. 78.3% of the differentially expressed genes had a similar pattern of regulation in SSc. 25% were similarly expressed in both cGVHD variants, whereas 16.6% were specific of Scl GVHD. Remarkably, this analysis allowed to identify specific chemokines (CCL5, CXCL9-10-11) involved specifically in the fibrotic versus non fibrotic response in GVHD and a specific modulation of WNT and Hedge-Hog pathway peculiar of Scleroderma and scl-GVHD skin biopsies. Identification of the source of expression of these genes by immunohistochemistry will shed light in unraveling the events bridging immune activation and skin fibrosis.

66. Degos Disease, possible lessons for Scleroderma treatment?

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Background: Degos Disease (DD) or Malignant Atrophic Papulosis (MAP) is a rare thrombo occlusive vasculopathy with increased deposition of C5b-9 deposition in affected tissues. Small vessel obliterative vasculopathy is a shared feature of MAP and Slceroderma. Increased C5b-9 deposition in tissue has also been seen in Scleroderma. This similarity might significantly influence future treatment for Scleroderma patients with Degos like lesions. We wish to present two patient histories, one with DD and a second one with Scleroderma, SLE overlap with new onset DD like lesions. The first patient was febrile, hypertensive, tachycardic and had required laparotomy for acute abdomen with evidence of perforation and peritonitis. After the first dose of eculizumab was given he immediately showed signs of improvement, vital signs normalized; eventually was discharged home and he restarted school. Eculizumab is a monoclonal Antibody against C5, which prevents C5b-9 deposition in tissues. The classical evolution of Systemic DD is death from repeated small bowel perforation within two to three years. It almost always presents with quite characteristic cutaneous leisons. On histology the lesions look similar independent of the organ involved. The CNS, pericardium and other organs have been affected to a lesser degree. There are about 200 cases reported in the literature and there are a few cases with Scleroderma and DD like lesions proven by biopsy, concomitantly in the same patient. Both diseases tend to be histologicaly pauci immune, they have been linked to a possible endotheliotropic virus triggering the complement cascade with subsequent MAC deposition and tissue destruction, along with autoantibody formation. There is evidence of ischemia in both diseases. The primary aim of this report is to compare MAP and Scleroderma with DD like lesions, with the hypothesis that that patients with scleroderma-DD overlap may benefit from C5 inhibitor therapy.

Materials and Methods: Case Reports: A 17 year old male presented with acute abdomen and a 2 year history of skin lesions typical for MAP. He had a small bowel obstruction with multiple DD like lesions seen on exploratory laparotomy. Due to peritonitis and impending perforation he went through 3 exploratory laparotomies during the same hospitalization; had abdominal compartment syndrome from severe swelling and was critically ill until the eculizumab was started. From then on very quickly and consistently he improved.

More recently a 40 year old female with a prior history of Scleroderma and lupus overlap has developed DD like lesions over the course of one year along with more recent abdominal pain which is getting progressively worse. Her systemic sclerosis was diagnosed in 2006 followed by lupus in 2009. Both diagnoses are supported by serology and biopsy. A year ago she developed pericardial effusion leading to tamponade without clear etiology. The biopsy of pericardial tissue was revised very recently and it does show evidence of a possible Degos like reaction.

Conclusion: The primary Degos patient had an immediate, dramatic and sustained response to eculizumab. The second patient displays very similar pathology but in the setting of an already diagnosed connective tissue disease. She does have C5b-9 deposition and confirmed DD like lesions on biopsy. Lessons learned from treatment of a very rare disease with similar vascular pathology may have applications to some patients with systemic sclerosis. Efforts are underway to identify patients with systemic sclerosis who may be appropriate candidates for eculizumab therapy**.**

67. Caveolin-1 Regulates CXCR4/CXCL12-dependent Monocyte Recruitment in Scleroderma Patients and in a Murine Model of Interstitial Lung Disease

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Reduced caveolin-1 levels in scleroderma (SSc) lung fibroblasts and in the lungs of bleomycin-treated mice promote collagen overexpression and lung fibrosis. Here we report for the first time that caveolin-1 is also deficient in peripheral blood monocytes (PBM) from bleomycin-treated mice and from SSc patients. Further, we examine the consequences of this deficiency and its reversal.

In PBM from bleomycin-treated mice, caveolin-1 expression was reduced by more than 50% and CXCR4 expression was increased by more than 40%. Systemic administration of the synthetic caveolin-1 scaffolding domain (CSD) peptide one day prior to bleomycin almost completely blocked monocyte accumulation in lung tissue. To examine the relationship between caveolin-1 and CXCR4 in human disease, we examined their expression in lung tissue and PBM from SSc patients. We found a massive increase in CXCR4 and its ligand CXCL12 in the lung tissues of patients with SSc-ILD. SSc PBM contain nearly 60 % less caveolin-1 and three times as much CXCR4 as their normal counterparts. The overexpression of CXCR4 in SSc PBM is dependent on their underexpression of caveolin-1, given that CSD peptide treatment inhibits their expression of CXCR4. TGF-β causes normal PBM to adopt the SSc phenotype, i.e. low caveolin-1 and high CXCR4 expression. To further investigate the consequences of high CXCR4 expression in SSc we studied monocyte migration. The percentage of SSc PBM that migrated in response to CXCL12 was more than four-fold higher than for control PBM ($p < 0.0001$). Treatment with CSD peptide inhibited migration by > 80 % for PBM from both normal and SSc subjects. We also evaluated the effect of TGFβ on the migration of control PBM in response to CXCL12. TGFβ increased monocyte migration more than twelve-fold ($p < 0.0001$). Again, migration was inhibited > 80 % by CSD peptide. In summary, our results support the notion that using the CSD peptide to compensate for low caveolin-1 levels may be a useful treatment strategy for SSc and other inflammatory/fibrotic lung diseases.

68. The effect of oxidative stress on protein tyrosine phosphatase 1B in scleroderma fibroblasts

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Purpose. Skin fibrosis is a main characteristic of systemic sclerosis (scleroderma, SSc). Platelet-derived growth factor (PDGF) and its receptor (PDGFR) have been shown to play a key role in promoting fibrosis in SSc fibroblasts, which produce excessive superoxide compared to normal (NL) fibroblasts. Upon PDGF stimulation, the PDGFR is phosphorylated, and its downstream signaling pathways, including extracellular signal-regulated kinases 1 and 2 (ERK1/2), are activated. The PDGFR is dephosphorylated by phosphatases, including protein tyrosine phosphatase 1B (PTP1B), and the signaling cascade is hence terminated. In this study we sought to determine whether the thiol-sensitive PTP1B is affected by oxidative stress in these cells, enhancing the extracellular signal-regulated kinases 1 and 2

(ERK1/2) signaling pathway of PDGFR that results in excessive fibrosis. The effect of a thiol antioxidant, n-acetylcysteine (NAC), on PTP1B activity was also investigated.

Methods. SSc and NL fibroblasts were isolated from skin biopsies. SSc fibroblasts were established from affected skin from patients with less than 12 months of clinically detectable skin induration. Cells were stimulated with 30 ng/ml PDGF-BB at various time points (unstimulated [US], 10 min, 20 min, 45 min, 2 hr, and 4 hr) and phosphorylation of ERK1/2 was measured by Western blotting. PTP1B levels were assessed by quantitative polymerase chain reaction and Western blotting. ELISA coupled with phosphate release assay was used to determine PTP1B activity. Superoxide production was measured using dihydroethidium (DHE). Student's t-test was used for statistical analysis.

Results. Activation of PDGFR by PDGF-BB resulted in phosphorylation of ERK1/2. In NL fibroblasts, ERK1/2 phosphorylation was maximal at 45 min (~3 fold increase vs. US, $p<0.05$, n=5 donor samples) and returned to baseline at 4 hr. In contrast, in SSc cells, ERK1/2 was significantly phosphorylated at 10 min and remained phosphorylated at 4 hr (~2 fold increase vs. US, $p<0.05$, n=5). PDGF-BB increased PTP1B protein expression significantly at 45 min and 2 hr ($p<0.05$, n=5) in NL fibroblasts. In contrast, PTP1B expression in SSc fibroblasts remained the same with PDGF-BB incubation, and at 45 min the level of PTP1B in NLs was significantly higher than that in SSc (~2 fold increase, $p<0.05$, n=5). PTP1B activities were 20.1 ± 2.7 and 11.9 ± 1.3 nmoles (phosphate released, $p<0.05$, n=5) in NL and SSc fibroblasts, respectively. In the presence of NAC, PTP1B activity was restored in SSc fibroblasts (from 11.9 ± 1.3 nmoles without NAC to 19.2 ± 2.1 nmoles with NAC, $p<0.05$). In contrast, the activity of PTP1B was unaffected with NAC treatment in NLs (20.1 ± 2.7 nmoles without NAC vs. 16.2 ± 0.4 nmoles with NAC, $p>0.05$). Superoxide was significantly higher in SSc than in NL dermal fibroblasts as measured by DHE, and the level in SSc dermal fibroblasts was reduced significantly after incubation with NAC ($p<0.05$, n=5).

Conclusions. We showed that the profile of ERK1/2 phosphorylation, which indicated PDGF-induced PDGFR activation, was different in NL and SSc dermal fibroblasts. The inability to dephosphorylate ERK1/2 in SSc fibroblasts suggests that the phosphatases that are responsible for ERK1/2 dephosphorylation are deficient in SSc. Additionally, the ability to produce PTP1B after PDGF stimulation, and hence terminate PDGFR signaling, was hampered in SSc dermal fibroblasts. Moreover, PTP1B activity was significantly inactivated in SSc fibroblasts, which may have resulted from higher levels of superoxide than in NL fibroblasts. NAC treatment both restored the low PTP1B activity and decreased superoxide levels in SSc dermal fibroblasts. Thus, we introduce a new class of proteins dysregulated in SSc. Our study also provides a novel molecular mechanism by which NAC therapy may act on these proteins to benefit SSc patients.

69. Cross-sectional evaluation of chemokine profile in early and definite systemic sclerosis

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Background: Few studies investigated chemokines in serum (1), skin, upper gastrointestinal tract mucosa (1,2) or bronchoalveolar lavage (3) from Systemic Sclerosis (SSc) patients, reporting their increase respect to healthy controls. In all cases, patients suffering from both diffuse cutaneous (dc) and limited cutaneous (lc) SSc were investigated. Patients with early SSc, i.e. Raynaud's phenomenon (RP) plus SSc marker antibodies and/or scleroderma pattern at capillaroscopy (4) without any evidence of skin or internal organ fibrosis, have never been investigated under this aspect.
Objectives: To assess serum chemokine profile in patients with early SSc, including CCL2, CCL3, CCL4, CCL5, CCL11, CXCL10 and CXCL9; to compare serum chemokine levels from early SSc to those from definite SSc patients.
Methods: Sera from 54 patients (8 early SSc, 21 lcSSc and 25 dcSSc) and 10 healthy controls were tested. Clinical features were assessed, then patients and controls underwent blood donation. Chemokines were measured in 50 µl of serum per sample by a suspension protein immunoassay, with spectrally encoded beads as the solid support, allowing simultaneous evaluation of multiple analytes. Each sample was tested in duplicate. Data were analyzed by a dedicated software. Serum chemokines in SSc patients were correlated to clinical features.
Results: Mean serum levels of the following chemokines were significantly higher in all the 3 SSc groups than in controls: CCL2 (772.8±248.7 in dcSSc, 786.6±193.6 in lcSSc, 863.9±374.5 in early SSc vs 122.1±61.7

pg/ml in controls; p<0.001); CCL3 (313.1±22.2 in dcSSc, 121.4±32.9 in lcSSc, 109.5±9.8 in early SSc, 18±1.4 pg/ml in controls; p<0.001); CCL4 (164.5±231 in dcSSc, 175.6±6 in lcSSc, 147.2±82.9 in early SSc vs 41.2±3 pg/ml in controls; p<0.05, p<0.001, p<0.01 respectively); CXCL9 (134.3±233.5 in dcSSc, 255.9±37 in lcSSc, 247.3±30 in early SSc vs 13.8±15.9 pg/ml in controls; p<0.01). CCL3 was higher in the dcSSc vs early and lcSSc subsets (p<0.001). CXCL9 was higher in early and lcSSc than in dcSSc subset (p<0.001). Mean serum levels of CCL11 and CXCL10 were higher only in dcSSc patients (609.5±266.1 and 265.6±286.3 pg/ml respectively). For CCL11 p values were <0.0001vs controls (203±106.8) and <0.001 vs early SSc (224±142.7) and lcSSc patients (218.2±66.33). For CXCL10 p values were <0.0001 vs controls (48.14±40) and lcSSc patients (51.24±10.93) and <0.001 vs early SSc patients (50.83±12). By linear and stepwise regression analysis, a correlation between CCL-2 (p=0.028) and CXCL10 (p=0.0025) and modified Rodnan skin score (mRSS) was pointed out (R2=0.68).

Conclusion: Our data confirm the increase of serum CCL2, CCL3 and CCL4 in definite SSc (CCL3 being higher in dcSSc with respect to lcSSc patients); support the role of Th2 polarized response in tissue remodeling (correlation of CCL2 with the extent of skin sclerosis) and chronic inflammation (CCL3 and CCL4); and, most importantly, point out an increase in profibrotic chemokines in early SSc patients, suggesting that they may play a pathogenic role in early disease.

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70.Effect of Selective and Dual Endothelin Receptor Antagonists (ERAs) on SSc and Normal Endothelial Cells Apoptosis.

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***OBJECTIVES*:** microvascular endothelial cells (MVEC) apoptosis is a critical event in the pathogenesis of SSc vascular disease. Endothelin 1 (ET) signaling in MVEC favors cell survival and antagonizes apoptotic signals. In this study we examined the effects of ERAs on ET mediated resistance to apoptosis.

METHODS: MVEC apoptosis was induced by oxidative stress or by growth factor withdrawal (GFW) and was assessed by flow cytometry, caspase-3 activity and cell viability. We tested the selective and non-selective ERAs: PD145065 a dual ERA, BQ 788 a selective B, and the selective type A ERAs FR139317 and Ambrisentan. We also investigated the effects of si-RNA knockdown of EDNRA, B or A&B mRNAs on EC apoptosis. Finally, a focused apoptosis gene expression microarray was utilized to investigate the effect of ET and ERAs on MVEC apoptotic gene expression profile.

RESULTS: significant upregulation of both type A and B ET receptors in SSc-MVEC was noted, particularly the A receptors. SSc MVEC apoptotic responses to oxidation or GFW were significantly higher in SSc than in control cells. The addition of ET resulted in a significant, albeit incomplete, reversal of MVEC apoptosis, particularly in control cells. The addition of ERAs alone did not alter the level of apoptotic responses. Selective type A-ERAs maintained ET antiapoptotic effects, while selective type B-ERA and dual-ERA completely blocked the antiapoptotic effect of ET. ET effects were also blocked when selective A-ERAs concentrations were increased beyond the concentrations associated with selectivity of the tested agent. To confirm the conclusion that signaling through type B receptor mediates ET protective effects, we performed si-RNA knock down experiments and tested the cell using the same experimental design. Type A receptor mRNA knock down had no effect on ET protective effect, while type B receptor knock down and the combined A&B mRNAs knock down completely abolished ET antiapoptotic effects. Next, we performed focused apoptosis microarray gene expression profiling of MVEC after GFW and the effects of ET and ERAs on gene expression were examined. GFW induced an upregulation of the pro-apoptotic gene *BAX* expression and the addition of ET inhibited *BAX* expression. Selective type A-ERA maintained ET induced downregulation of *BAX* while the nonselective ERAs and selective type B-ERA prevented ET effect on *BAX* expression.

CONCLUSIONS

- Oxidation and growth factor withdrawal induced MVEC apoptosis is prevented by ET.
- ET antiapoptotic effects are mediated by inhibition of *BAX* gene expression.
- Selective A-ERAs preserve ET anti-apoptotic effects, while the dual and selective B-ERAs negate ET protective effect.
- Therapeutic strategies utilizing selective A-ERAs may be superior to the non-selective approaches from a vascular prospective.

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71. Adipocyte-targeted Wnt activation results in spontaneous dermal fibrosis and subcutaneous lipoatrophy

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Background: Fibrosis and subcutaneous lipoatrophy are hallmarks of scleroderma, and are thought to reflect mesenchymal cell differentiation into activated fibroblasts. The Wnts are a family of extracellular ligands that signal through canonical beta-catenin pathways as well as non-canonical pathways, and are involved in development and cell fate determinations. Canonical Wnt signaling drives preadipocyte differentiation into osteoblasts at the expense of adipogenesis, in part through negative modulation of PPAR-gamma (PPAR-γ) expression and function. Recent studies implicate abnormalities in the canonical Wnt pathway in scleroderma and pulmonary fibrosis. Because Wnt signaling has profound effects on regulating mesenchymal cell lineage, we examined the effect of adipocyte-specific ectopic Wnt10b expression on skin homeostasis and differentiation in transgenic mice.

Methods: Female transgenic mice harboring Wnt10b under the control of the adipocyte-specific FABP4 promoter were studied. Serum adiponectin levels were determined by ELISA. Dermal thickness was measured. Collagen accumulation in the skin was determined using Masson's trichrome and Picrosirius Red stains, and by colorimetric assays. Mast cells were identified by Astra Blue staining. Fibroblasts were explanted from the skin and evaluated in vitro at early passage. Gene expression was assessed by real-time quantitative PCR and Western blot analysis.

Results: At six months of age, female FABP4-Wnt10b transgenic mice showed a marked loss of subcutaneous and visceral adipose tissue. Serum levels of adiponectin were >80% lower than in wildtype littermates. In the skin from Wnt10b transgenic mice, a dramatic increase in Wnt10b mRNA expression was noted, and mRNA level for axin 2, a transcriptional target of canonical Wnt signaling, was significantly elevated. The dermis showed a >60% increase in thickness, with a striking reorganization of the collagenous matrix, and a >80% increase in soluble collagen content per punch biopsy. Degranulating mast cells were seen in the reticular dermis and among muscle bundles in Wnt10b transgenic mice but not in wildtype littermates. mRNA levels for Type I collagen and α-smooth muscle actin were elevated. Explanted dermal fibroblasts showed elevated Wnt10b expression. Expression of the adipogenic markers PPAR-γ and FABP4 were reduced, whereas mRNA levels for Type I collagen α-smooth muscle actin were elevated, compared to wildtype fibroblasts examined in parallel.

Conclusion: Ectopic Wnt10b expression targeted to adipocytes results in time-dependent progressive loss of cutaneous and visceral adipose tissue accompanied by the spontaneous development of dermal fibrosis with increased expression of fibrotic markers. Dermal fibroblasts explanted from Wnt10b transgenic mice show sustained activation of Wnt10b-driven canonical signaling and upregulation of collagen gene expression in vitro, suggesting that ectopic Wnt10b drives a shifts in mesenchymal cell fate toward myofibroblasts by induction of fibrotic genes while simultaneously suppressing adipogenic gene expression. This shift appears to be driven, at least in part, by suppression of the adipogenic master regulator transcription factor PPAR-γ. The results implicate that Wnt signaling plays an important role in the pathogenesis of scleroderma. Modulating Wnt activity may therefore represent a novel therapeutic approach for the treatment of scleroderma.

72. Osteopontin in Systemic Sclerosis and its Role in Dermal Fibrosis

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Purpose: Osteopontin (OPN) is a multifunctional cytokine produced during inflammation and tissue repair. OPN has been implicated in autoimmune diseases such as rheumatoid arthritis and fibrosis in diseases such as idiopathic pulmonary fibrosis and mouse models of pulmonary fibrosis. Given the importance of autoimmunity and fibrosis in systemic sclerosis (SSc), we hypothesized that OPN is elevated in SSc patients and plays a critical role in the development of dermal fibrosis.

Methods: Plasma from a large cohort of SSc patients (n= 319) and controls (n= 144) were used to determine OPN levels by ELISA. Skin biopsies from SSc patients and healthy controls were used for immunohistological (IHC) analyses. To determine if OPN is a mediator of dermal fibrosis, OPN deficient (def) and wild type (WT) mice were compared in the bleomycin (bleo)-induced dermal fibrosis model.
Results: Circulating levels of OPN were elevated in SSc patients compared to healthy controls. In addition OPN levels were elevated in patients with limited and diffuse disease as well as anticentromere, anti-topoisomerase and anti-RNA polymerase III positive SSc patients relative to controls. Compared to control, SSc skin biopsies expressed OPN that localized to both fibroblast-like cells and macrophages on IHC analyses. Similarly skin biopsies from mice treated with subcutaneous bleo had increased levels of OPN compared to saline injected mice. Interestingly, using the bleo-induced dermal fibrosis model, OPN def. mice had markedly attenuated dermal fibrosis as quantitated by skin thickness, collagen levels, and myofibroblasts accumulation in the lesional skin, relative to WT mice. Furthermore, OPN def. mice had decreased dermal inflammation, including decreased number of Mac-3 positive macrophages. Lastly, Col1a1, IL-6, PAI-1 and CTGF mRNA levels were significantly decreased in lesional skin of OPN def. compared to WT mice.
Conclusions: These data demonstrate that OPN levels are increased in SSc patients. Furthermore, OPN def. mice have decrease inflammation and dermal fibrosis in the bleomycin induced dermal fibrosis model, demonstrating that OPN is a critical mediator of dermal fibrosis. These data suggest that OPN may be a biomarker as well as a therapeutic target in SSc patients.

73. Expression of activation markers on eosinophils in patients with Systemic Sclerosis

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Objective: Eosinophils have been associated with fibrosis. In Systemic Sclerosis (SSc), increased percentage of eosinophils in broncho alveolar lavage has been related to an increased mortality in patients with interstitial lung disease. Knowledge of eosinophil activation is lacking in SSc. Our study aimed to analyse eosinophil activation markers in SSc patients.

Method: In a prospective study, forty-three consecutive patients with a median disease duration of 1.5 year from onset of first non-Raynaud symptom were enrolled. Twenty-nine patients had limited cutaneous SSc, nine patients had diffuse cutaneous SS and five patients had limited SSc. Patients were further divided into one group with short disease duration (<3 years, n=26) and one with longer disease duration (≥3 years, n=17). Twelve age matched controls were enrolled. Whole peripheral blood was analysed by flow cytometry. Eosinophils were identified in the granulocyte population of the forward/sideward scatter and staining as CD16-/CD9+ cells. Eosinophils were analysed for the activation markers CD11b, CD44, CD48, CD54, CD69, CD81 and HLA-DR. Alveolar nitric oxide in exhaled air was calculated as fractional exhaled nitric oxide at flow rate 50, 100, 200 and 400 ml/s.

Results: SSc patients with longer disease duration had significantly higher eosinophil counts compared to patients with early SSc ($p<0.05$) or healthy controls ($p<0.01$). Eosinophils from SSc patients with either short or long disease duration had lower surface expression of CD81 compared to healthy controls ($p<0.01$). In patients with short disease duration, eosinophils had higher expression of CD48 compared to both SSc patients with longer disease duration ($p<0.05$) and healthy controls ($p<0.05$). The expression of CD48 and the ratio of CD48/CD81, CD48/CD9 as well as the ratio CD11b/CD9 correlated to alveolar inflammation as measured by the production of alveolar nitric oxide in early SSc ($r=0.53$, $r=0.56$, $r=0.58$, respectively $r=0.53$; $p<0.01$ for all correlations).

Conclusion: Activation markers on eosinophil granulocytes may be used to in the assessment of patients with SSc and may reflect alveolar inflammation in patients with early disease.

74. Epithelial to mesenchymal switching in the pathogenesis of Systemic Sclerosis

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Introduction and Objectives: Systemic Sclerosis (SSc) is a complex disorder of uncertain etiology characterised by progressive vascular and interstitial fibrosis. Recently, we found that the epidermal compartment of SSc skin exhibits abnormalities, taking on an activated phenotype reminiscent of that observed during the wound healing response. Fibroblast-epithelial cell interactions are believed to be important during normal tissue repair and aberrant cellular cues may underlie important aspects of scarring and fibrosis including epithelial to mesenchymal transition (EMT). Transgenic mice in which fibroblasts express a constitutively-active Transforming growth Factor β (TGFβ) type I receptor (ALK5) or which over-express Connective tissue growth factor (CTGF), develop progressive tissue fibrosis most prominent in the skin and lung. Our *in vivo* data also suggests that activation of TGFβ signalling or CTGF over-expression by fibroblasts not only causes stromal cell activation, but also induces pathological changes in the adjacent epithelium. Here, we focus on the role of CTGF in EMT in the lung.

Methods: Human and rat type II alveolar epithelial cell lines (A549 and T2) were grown in DMEM containing 10% foetal calf serum and serum-starved over-night. Cells were stimulated with TGFβ (2ng/ml) in the presence and absence of siRNA specific for CTGF or scrambled siRNA control for 24 hours. Markers of EMT, namely decreased E-cadherin (the prototypic epithelial marker) and Thyroid transcription factor-1 (TTF-1); increased CTGF, Snail, αSMA, collagen type I and fibronectin were examined by Western blotting.

Results: A549 and T2 epithelial cells exposed to TGFβ develop a mesenchymal-like morphology and express molecular markers associated with EMT (including Snail, CTGF, αSMA). In A549 cells, CTGF-specific siRNA dose-dependently suppressed TGFβ-induced Snail and CTGF protein expression towards basal levels. Transgenic mouse lung fibroblasts over-expressing CTGF showed significantly higher levels of expression of extracellular matrix genes and proteins including collagen type I, fibronectin and also increased αSMA.

Conclusion: These data suggest that CTGF is an important mediator of EMT in lung epithelial cells. In SSc lung disease, over-expression of CTGF by stromal fibroblasts may drive the phenotypic switching increasing the number of pro-fibrotic, matrix-producing mesenchymal cells.

75. dsRNA Activation of TLR3 in Human Endothelial Cells Stimulates Endothelin-1 Expression and Markers of Vascular Activation

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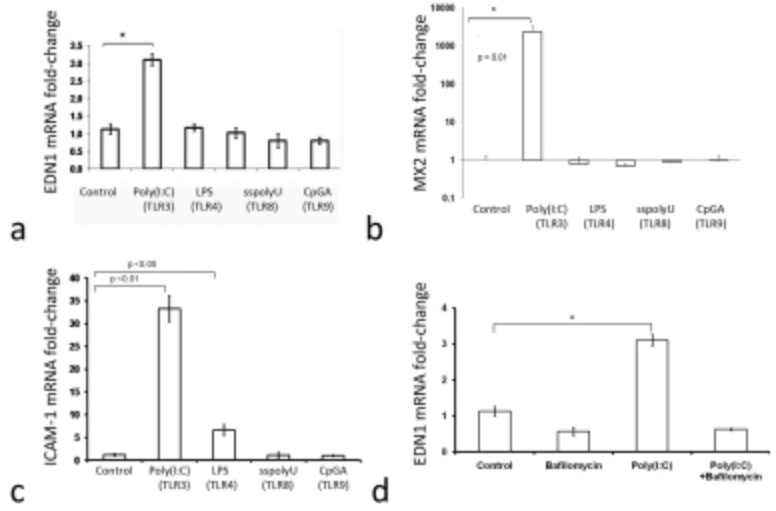
Body: Purpose:
The relationship between immune deregulation and vascular changes in systemic sclerosis (SSc) is uncertain. Certain toll-like receptors (TLRs) strongly induce IFN-regulated genes that have been identified as upregulated in SSc and SLE patient blood. Activation of certain TLRs by endogenous ligands such as ssDNA (TLR9), ssRNA (TLR7,8) and dsRNA (TLR3) have been implicated in mouse models of autoimmune diseases. We hypothesized that TLR receptors might play a role in autoimmunity and vascular disease characteristic of SSc. We investigated the effect of TLR ligands *in vitro* on human dermal endothelial cells (HDMEC) and in vivo using an osmotic pump to continuously infuse the synthetic dsRNA and TLR3 ligand, poly(I:C) in mice over 7 days.

Methods:
The effect of known toll-like receptor (TLR) ligands was tested *in vitro* on HDMEC. Cells were treated for 18 hours and harvested. Gene expression was then quantified by RT-PCR for the IFN-regulated gene, MX2, the vascular activation marker, ICAM-1, endothelin-converting enzyme ECE,

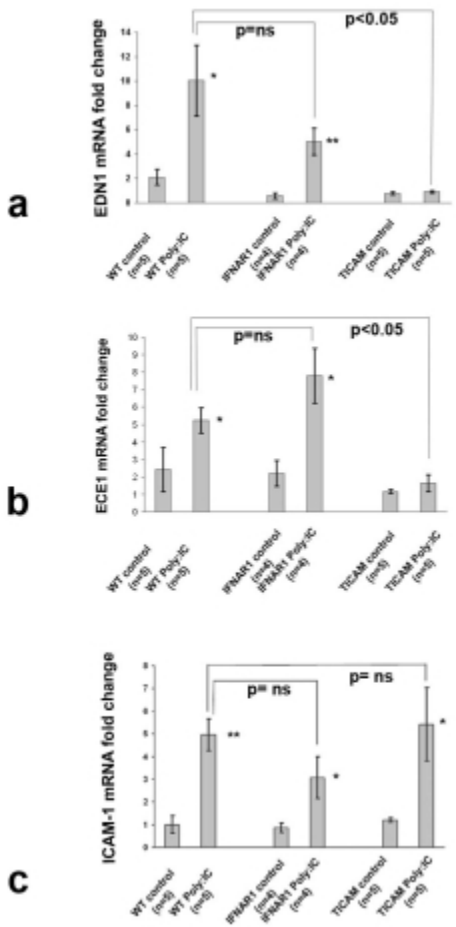
and EDN1. Expression was normalized to vehicle-treated wells. To test the effect of dsRNA on vascular activation/injury *in vivo*, PBS or the TLR3 ligand, poly(I:C), a synthetic dsRNA and known TLR3 ligand, was administered continuously over 7 days by subcutaneous osmotic pump. Gene expression was quantified by RT-PCR in proximal and distal skin. TLR3 specificity was determined by using mice deficient in the adaptor protein for TLR3 signaling, TRIF/TICAM1 (TRIF -/-). As several TLRs stimulate type-I interferon (IFN), mice deficient in the common IFN receptor (IFNAR -/-) were studied.

Results:

HDMEC were stimulated with a panel of TLR ligands. Poly(I:C), but not other TLR ligands, induced EDN1, MX2, and ICAM-1 mRNA expression HDEM (figure 1). Poly(I:C)-induced EDN1 expression was inhibited by bafilomycin, demonstrating the importance of endosomal acidification (figure 1d).



To study the effect of poly(I:C) *in vivo*, we measured EDN1, ECE1 and ICAM-1 mRNA expression in skin proximal and distal to a pump releasing poly(I:C) or PBS. Poly(I:C) induced EDN1, ECE-1 and MX2 expression and was not blocked in mice deleted of the type I IFN receptor (figure 2). However, poly(I:C)-induced EDN1 and ECE1, but not poly(I:C)-induced ICAM-1 expression was blocked in TRIF -/- mice.



Conclusion:

In this work we show that dsRNA increases markers of vascular activation expression in HDMEC such as EDN1 and ICAM1. Interestingly, poly(I:C) induction of EDN1 is TLR3 dependent, whereas that of ICAM-1 is TLR3 independent, suggesting other dsRNA receptors are responsible for ICAM-1 induction.