PS82

HUMAN ADIPOSE-DERIVED STROMAL CELLS FOR CELL-BASED THERAPIES IN THE TREATMENT OF CUTANEOUS MANIFESTATIONS IN PATIENTS AFFECTED BY SYSTEMIC SCLEROSIS (SSC)

N. Scuderi, P. Fino, M.G. Onesti
Department of Plastic, Reconstructive and Aesthetic Surgery, University of Rome Sapienza, Policlinico Umberto I, Rome, ITALY.

The present study was designed to evaluate the clinical outcome of cell-based therapy with cultured adipose derived stromal cells (ASCs) for the treatment of cutaneous manifestations in patients affected by systemic sclerosis (SSc). ASCs have an extraordinary developmental plasticity, including the ability to undergo multilineage differentiation and self-renewal. Moreover, ASCs can be easily harvested from small volumes of liposuction aspirate, showing great in vitro viability and proliferation rate. Here we isolated, characterized, and expanded ASCs, assessing both their mesenchymal origin and their capability to differentiate towards the adipogenic, osteogenic, and chondrogenic lineage. We developed an effective method for ASCs transplantation into sclerodermic patients by means of a hyaluronic acid (HA) solution, which allowed us to achieve precise structural modifications. ASCs were isolated from subcutaneous adipose tissue of six sclerodermic patients and cultured in a chemical-defined medium before autologous transplantation to restore skin sequelae. The results indicated that transplantation of a combination of ASCs in HA solution determined a significant improvement in tightening of the skin without complications such as anechoic areas, fat necrosis, or infections, thus suggesting that ASCs are a potentially valuable source of cells for to improve dermal repair in rare diseases such as SSc and generally in skin disorders.

Poster Tours – *Basic*

Poster Tour 13: *Pathogenesis*

PS83

ANGIOTENSIN RECEPTOR TYPE 1 AND ENDOTHELIN RECEPTOR TYPE A ON IMMUNE CELLSMEDIATE MIGRATION AND THE EXPRESSION OF IL-8 AND CCL18 WHEN STIMULATED BY AUTOANTIBODIES FROM SYSTEMIC SCLEROSIS PATIENTS

J. Günther[1,2], A. Kill[1,2], M.O Becker[1], E. Siegert[1], M. Radic[1,3], G. Riemekasten[1,2]
[1]University Hospital Charité, Department of Rheumatology and Clinical Immunology, Berlin, GERMANY; [2]German Rheumatism Research Centre, Berlin, GERMANY; [3]University Hospital Split, Department of Rheumatology and Clinical Immunology, Split, CROATIA.

Background. Agonistic autoantibodies against the angiotensin II receptor type 1 (AT1R) and the endothelin receptor type A (ETAR) have been identified in patients suffering from systemic sclerosis (SSc). Here we examined the expression of AT1R and ETAR in human immune cells and pathological effects mediated through these receptors by corresponding autoantibodies (Aabs).
Methods. AT1R and ETAR protein expression on peripheral blood mononuclear cells (PBMCs) from healthy individuals and SSc patients was analyzed using flow cytometry, and mRNA expression was examined by real-time PCR in PBMCs from healthy donors. In addition, PBMCs from healthy donors were stimulated in vitro with affinity-purified immunoglobulin G (IgG) fractions from SSc patients positive for AT1R- and ETAR-Aabs, and with IgG from healthy donors serving as control. Alterations in chemotactic motility and cytokine secretion were analyzed using chemotaxis assays and ELISA, respectively. Results were correlated with characteristics/clinical findings of the IgG donors.
Results. Both AT1R and ETAR were expressed on human peripheral lymphocytes and monocytes. Protein expression of both receptors was decreased in SSc patients when compared to healthy donors and correlated negatively with disease duration. In addition, IgG fractions of SSc patients induced T cell migration in an anti-AT1R and anti-ETAR Aab level-dependent manner. Moreover, IgG of SSc patients was capable of stimulating PBMCs to produce more IL-8 and CCL18 than IgG of healthy donors. All effects could be significantly abrogated by the application of selective AT1R and ETAR antagonists. Statistical analysis revealed a negative correlation between SSc IgG-induced IL-8 concentrations and disease duration, between SSc IgG-induced CCL18 concentrations and time since onset of lung fibrosis as well as an association of CCL18 concentrations with vascular complications of the corresponding SSc IgG donors.
Conclusion. We demonstrated the expression of both, AT1R and ETAR, on human peripheral T cells, B cells and monocytes, and found a decreased receptor expression on cells from SSc patients suggesting downregulation due to chronic activation. The inflammatory and profibrotic effects upon Aab stimulation in vitro, and their associations with clinical findings indicate a role for autoantibody-mediated activation of immune cells mediated through the AT1R and ETAR in the pathogenesis or even the onset of the disease.

PS84

ANTI-AT1R AND ANTI-ETAR AUTOANTIBODIES FROM PATIENTS WITH SSCANDTHEIRAGONISTIC EFFECTS

A. Kill[1], C. Tabeling[2], A. Kühl[3], J. Günther[1,4], M. O. Becker[1,4], H. Heidecke[5], D. Dragun[6], G.R. Burmester[1], G. Riemekasten[1,4]
[1]University Hospital Charité, Rheumatology and clinical Immunology, Berlin, GERMANY; [2]University Hospital Charité, Department of Infectious Diseases and Pulmonary Medicine, Berlin, GERMANY; [3]University Hospital Charité, Department of Inner Medicine, Berlin, GERMANY; [4]German Rheumatism Research Center (DRFZ), a Leibniz Institute, Berlin, GERMANY; [5]CellTrend GmbH, Luckenwalde, GERMANY; [6]University Hospital Charité, Department of Nephrology, Transplantology and Intensive Care, Berlin, GERMANY.

Background. Functional autoantibodies to angiotensin II type 1 receptor (AT1R) and endothelin 1 type A receptor (ETAR) are found in elevated levels in systemic sclerosis (SSc) and show association to increased risk of SSc-related manifestations and reduced cumulative survival. Biologic effects of these autoantibodies (anti-AT1R and anti-ETAR autoantibodies) have been demonstrated in vitro. Here, the functional effects were studied in vivo using animal models.

Objectives. To analyse functional effects of anti-AT1R and anti-ETAR autoantibodies *in vivo* using animal models.
Methods. Healthy C57Bl/6J mice were subjected to passive transfer treatment either with anti-AT1R and anti-ETAR autoantibody-positive IgG of SSc patients or with IgG of healthy donors as control. Experiments were performed with short term IgG transfer over seven days and with long term transfer over three months. Bronchoalveolar lavage fluid (BALF) was performed at the end of experiments and the cellular composition was analysed by microscopic differentiation in a blinded fashion. Lung architecture was visualized by staining with Hematoxylin and Eosin (H&E) of paraffin embedded sections and assessed by light microscopy. Plasma of treated mice was analysed for cytokines and chemokines using a bead array system (BioPlex®).
Results. Mice treated with anti-AT1R and anti-ETAR autoantibody-positive IgG of SSc patients (SSc-IgG group) showed distinct differences compared to mice that were treated with IgG of healthy donors (NC-IgG group). Cellular composition of the BALF revealed an increase of neutrophils in the BALF of the SSc-IgG group compared to the control NC-IgG group in the short and long term transfer. In the plasma elevated levels of the murine chemokine KC (functional homologue to human interleukin-8) were found in the short term transfer. Long term transfer resulted alteration of lung architecture featuring increased immune cell infiltrates showed by H&E staining of lung sections of SSc-IgG group compared to NC-IgG group.
Conclusions. Our findings demonstrate the potential to induce features of SSc pathogenesis in animal models *in vivo* by autoantibody positive IgG of SSc patients. Previous *in vitro* studies indicate direct receptor activation by anti-AT1R and anti-ETAR autoantibody-positive SSc-IgG and activation of the angiotensin and endothelin receptors by these autoantibodies could account in part for in vivo effects seen here. Therefore, receptor inactivation studies will be performed to assess a deeper knowledge of anti-AT1R and anti-ETAR autoantibody-mediated effects *in vivo* which could help to improve our current understanding of SSc pathogenesis.

PS85

SYSTEMIC SCLEROSIS SERA AFFECT ANGIOGENESIS, WOUND HEALING CAPACITY ANDMIGRATION OF DERMAL BLOOD MICROVASCULAR ENDOTHELIAL CELLS: THERAPEUTIC IMPLICATIONS OF CYCLOPHOSPHAMIDE

A. Borghini[1], M. Manetti[2], F. Nacci[2], E. Romano[2], A.F. Milia[2], S. Guiducci[2], M. Matucci-Cerinic[2], L. Ibba-Manneschi[2], E. Weber[1]
[1]Department of Molecular and Developmental Medicine, University of Siena, Siena, ITALY; [2]Department of Experimental and Clinical Medicine, University of Florence, Florence, ITALY.

Objective. Systemic sclerosis (SSc) is a complex connective tissue disease characterized by extensive fibrosis and vascular abnormalities. Dermal capillaries are progressively reduced in number with consequent chronic tissue hypoxia insufficiently compensated by angiogenesis. In SSc, clinical studies reported that cyclophosphamide (CYC) treatment may improve nailfold capillary damage. In the present study, we evaluated the effects of sera from naïve or CYC-treated SSc patients on the in vitro capacity of human adult dermal blood microvascular endothelial cells (B-MVECs) to perform angiogenesis, and to migrate and proliferate in response to injury.
Methods. Dermal B-MVECs were challenged with sera from SSc patients (n=21; n=13 limited SSc (lSSc), n=8 diffuse SSc (dSSc)), naïve (n=8) or under CYC treatment (n=13), and healthy controls. Angiogenesis was evaluated after 24 hours of cell seeding on Geltrex (reduced growth factor basement membrane matrix) in EBM containing 2% fetal bovine serum and 10% control or SSc serum. The number of branching points was quantified. Wound healing assay was performed on confluent cells grown in 12-well plates and evaluated at 24 hours after wounding. Chemotaxis was assessed by using the Boyden chamber assay.
Results. Angiogenesis was significantly reduced upon challenge with sera from naïve SSc patients compared with healthy controls (p<0.005). Moreover, angiogenesis was significantly lower in the presence of naïve dSSc sera compared with naïve lSSc sera (p=0.02). Upon challenging of B-MVECs with sera from CYC-treated SSc patients, the angiogenic capacity was comparable to that of cells treated with healthy sera. Wound healing capacity was significantly decreased upon challenge with sera from both naïve and CYC-treated SSc patients compared with healthy controls (both p<0.005), with no difference between naïve and CYC-treated SSc sera. The Boyden chamber assay gave similar findings with significantly lower migration of B-MVECs in the presence either of naïve SSc or CYC-treated SSc sera compared with healthy sera (both p<0.001). Furthermore, both wound healing capacity and chemotaxis were significantly reduced upon challenge with naïve dSSc sera compared with naïve lSSc sera (p<0.001).
Conclusions. Naïve SSc sera have significant inhibitory effects on angiogenesis, wound healing capacity and chemotaxis of dermal B-MVECs. Challenge with CYC-treated SSc sera effectively maintained B-MVEC angiogenesis on Geltrex at levels comparable to those of healthy control sera. Conversely, it was not able to specifically restore B-MVEC wound healing capacity and migration. Therefore, in SSc CYC treatment might foster angiogenesis mainly through the normalization of the endothelial cell invasive capacity and cell-matrix interactions.

PS86

FROM MICROVASCULATURE TO FIBROBLAST: CONTRIBUTION OF ANTI-ENDOTHELIAL CELL ANTIBODIES (AECA) IN SYSTEMIC SCLEROSIS

C. Corallo, B. Franci, A. Montella, F. D'onofrio, R. Nuti, N. Giordano
Department of Medicine, Surgery and Neurosciences, University of Siena, Siena, ITALY.

Systemic Sclerosis (SSc) is an autoimmune disease characterized by skin and internal organ fibrosis, caused by microvascular dysfunction. In the last years, the hypothesis that anti-endothelial cell antibodies (AECA) could play a key role in microvascular damage seems to be always more convincing. Some of these AECA are capable of causing antibody-dependent cellular apoptosis and of stimulating the microvasculature in the release of pro-inflammatory and profibrotic cytokines at the same time. In the present study, AECA contribution in the development of microvasculature damage was evaluated by stimulating human-microvascular-endothelial-cells (MVECs) with SSc sera (with and without AECA), and with sera from healthy donors. The conditioned MVECs culture media were then added to control (CTR), not affected-skin (NA) and affected-skin (SSc) fibroblast cultures respectively. The presence of AECA contributed to MVECs over-release of endothelin-1 (ET-1) in the culture medium and finally to cell apoptosis. Fibroblast (CTR, NA and SSc) proliferation resulted increased after treatment with AECA-positive conditioned media compared to AECA-negative and control conditioned media. Moreover, both AECA-positive (in major contribution) and AECA-negative conditioned media were responsible of alpha-smooth-muscle-actin (αSMA) over-expression in fibroblasts compared to control conditioned media. Moreover, fibroblast type-I-collagen synthesis chanced in presence of AECA. Finally, the synthesis of fibroblast transforming-growth-factor-beta (TGF-β) was statistically higher in AECA-positive conditioned media compared to AECA-negative and control conditioned media. These findings support the concept that AECA may mediate the crosstalk between endothelial damage and dermal-fibroblast activation in SSc.

PS87

CIRCULATING ANGIOGENIC FACTORS IN SSC PATIENTS – ASSOCIATION WITH CLINICALMANIFESTATIONS

M. Madej[1], A. Luczak[1], B. Nowak[1,2], E. Morgiel[1], L. Korman[1], P. Wiland[1]
[1]Department and Clinic of Rheumatology and Internal Medicine, Wroclaw Medical University, Wroclaw, POLAND; [2]Department of Pharmacology, Wroclaw Medical University, Wroclaw, POLAND.

Background. The involvement of the small arteries and capillaries, causing SSc vasculopathy belongs to the pathological background of systemic sclerosis (SSc). Recent studies have shown elevated levels of angiogenic molecules in plasma from SSc patients, which may reflect the dysregulation of the endothelium function during the disease (1). Scleroderma interstitial lung disease (ILD) is bound up with VEGF deficiency (2). Other data suggest increased serum levels of VEGF in SSc patients with higher pulmonary blood pressure (3).
Objectives. To evaluate the serum concentrations of angiogenic factors and the relationships among them. To assess the relations of angiogenic cytokines with organ involvement in patients with SSc.
Methods. Serum levels of VEGF, fibroblast growth factor 2 (FGF-2), angiopoietn-1 (Ang-1), angiopoietin-2 (Ang-2) and endostatin were assessed by ELISA in a group of 27 patients with SSc and 25 healthy controls. Some basic diagnostic procedures including laboratory tests, HRCT, echocardiography, capillaroscopy were performed to assess organ involvement due to SSc.
Results. A total of 27 SSc patients (19 women, 8 men) with a mean age of 53.7±12.0 years were enrolled in the study. Mean disease duration was 6.3±6.3 months. The levels of VEGF (53.3vs39.3; p<0.01), Ang-2 (8.3 vs 3.4) and endostatin (177.2vs126.0) were significantly higher in SSc patients than in the healthy population (p<0.0001). No differences between FGF-2 and Ang-1 concentrations among SSc patients and the control group were noted. A significant increase of Ang-2 was revealed in a subgroup of patients with limited SSc (lSSc) (13.5±8.9 vs 7.3±4.4; p<0.05). Elevated concentrations of endostatin

were observed in SSc patients with confirmed arterial pulmonary hypertension (243.8±120.3 vs 158.8±48.5; p=0.007). No correlations of other features of organ involvement (capllaroscopic changes, digital ulcers, interstitial lung disease) and angiogenic factors were noted.
Conclusions. Angiogenic profile observed in our study showed a domination of angiostatic factors including Ang-2 and endostatin. Our results confirm earlier data suggesting that elevated levels of angiogenic factors reflect a pro-inflammatory state in SSc endothelium and may contribute to the development of clinical symptoms of the disease.
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PS88

CIRCULATING ENDOTHELIAL MICROPARTICLES REFLECT MICROVASCULAR IMPAIRMENT IN PATIENTS WITH SYSTEMIC SCLEROSIS

O. Kowal-Bielecka[1], M. Bielecki[2], M. Michalska-Jakubus[3], D. Krasowska[3]
[1]Department of Rheumatology and Internal Medicine, Medical University of Bialystok, Bialystok, POLAND; [2]Department of Orthopedics and Traumatology, Medical University of Bialystok, Bialystok, POLAND; [3]Department of Dermatology, Venereology and Pediatric Dermatology, Medical University of Lublin, Lublin, POLAND.

Objective. Vascular injury is believed to play an essential role in the development of systemic sclerosis (SSc). However, the pathogenesis of SSc-related microangiopathy is not well understood. In addition, reliable assessment of endothelial state in vivo in SSc patients still remains a challenge. Endothelial microparticles (EMPs) are considered markers of the endothelial state.
Purpose. We aimed to assess possible relationships between circulating EMPs and clinical features including microvascular impairment in patients with SSc.
Methods: Forty seven patients fulfilling the ACR classification criteria for SSc and 27 age- and sex-matched healthy controls were included into the study. Clinical evaluation of patients was obtained, including nailfold capillaroscopy. Based on the capillaroscopic findings patients were classified into 3 groups showing an early, active or late pattern, according to the criteria proposed by Cutolo et al [Rheumatol 2004; 43: 719]. EMPs were identified with flow cytometry after staining platelet-poor plasma with combinations of fluorescent cell-specific monoclonal antibodies (anti-CD31, -51, -42b, -62E and AnnexinV). The following types of EMPs were evaluated: total EMPs (CD31+/CD42b-), activated EMPs (CD62E+/AnnV-,) and apoptotic EMPs (CD62E+/AnnV+ or CD51+).
Results. All types of EMPs were significantly elevated in SSc patients as compared with healthy controls. The concentrations of total EMPs tended to be lower in SSc patients with digital ulcers as compared with those without digital ulcers (p=0.09).
The mean concentration of total EMPs in SSc patients with late pattern in capillaroscopy was significantly lower as compared with SSc patients with early capillarscopic pattern (p<0.05), and tended to be lower as compared with SSc patients with active pattern (p=0.1). There were no significant differences in the levels of total EMPs between SSc patients with early and those with active patterns in capillaroscopy (p>0.05). Moreover, total EMPs and activated EMPs showed opposite correlations with the number of ramified capillaries (R=-0.40 and R=0.37, respectively, p<0.05 for both).
No other statistically significant associations or correlations could be found between the concentrations of total EMPs or any of EMPs' subpopulations and other clinical or immunological parameters including disease subtype, disease duration, the presence of interstitial lung disease, severity of skin or lung involvement, the presence of specific autoantibodies (ACA or anti-Scl70) or ESR values.
Conclusions. Our results suggest that quantity and phenotype of circulating EMPs might reflect microvascular changes in SSc. Further studies are required to reveal the role of EMPs in the development of microangiopathy in SSc.

PS89

IGG SUBCLASSES OF AUTOANTIBODIES DIRECTED AGAINST THE ANGIOTENSIN RECEPTOR TYPE 1 AND THE ENDOTHELIN RECEPTOR TYPE A AND THEIR CLINICAL RELEVANCE

J. Günther[1,2], A. Kill[1,2], M.O. Becker[1], E. Siegert[1], H. Heidecke[3], G. Riemekasten[1]
[1]University Hospital Charité, Department of Rheumatology and Clinical Immunology, Berlin, GERMANY; [2]German Rheumatism Research Centre, Berlin, GERMANY; [3]CellTrend GmbH, Luckenwalde, GERMANY.

Background. IgG4-related diseases are often characterized by a generalized inflammatory fibrosis which is also present in patients suffering from systemic sclerosis (SSc). Recent findings indicate the importance of autoantibodies (Aabs) against the angiotensin II type-1 receptor (AT1R) and the endothelin type-A receptor (ETAR) in the pathogenesis of SSc. Therefore, we analysed the levels of anti-AT1R/ETAR IgG sublasses in patients with SSc to determine a possible role of IgG subclasses as markers for disease manifestations in SSc.
Material and Methods. Sera from 91 SSc patients, 59 patients suffering from systemic lupus erythematosus (SLE), and 199 healthy donors were analysed for the levels of anti-AT1R and anti-ETAR Aabs as well as for the different anti-AT1R and anti-ETAR IgG subclasses by ELISA. The results were associated with clinical manifestations using Mann-Whitney test and correlated with the time since onset of disease manifestations by Spearman correlation test.
Results. IgG3 followed by IgG1 was found to show highest anti-AT1R/ETAR Aab levels in all analyzed groups, in which SSc patients as well as SLE patients had higher IgG1 and IgG3 anti-AT1R/ETAR Aab levels as compared to healthy donors. Comparing SLE and SSc patients IgG1 and IgG3 showed a bit higher anti-AT1R/ETAR Aab levels in SLE.
Within the SSc group patients with diffuse SSc had the higher anti-AT1R/ETAR IgG3 levels as compared to those with limited disease or overlap forms.
Correlation analysis with SSc-related clinical manifestations revealed that levels of anti- AT1R/ETAR IgG3 negatively correlated with time since onset of Raynaud's phenomenon, and with time since first detection of PAH. Of note, there were negative correlations between levels of anti-AT1R/ETAR IgG3 levels and diffusion capacity for carbon monoxide (DLCO) as well as between anti-AT1R/ETAR-IgG3 levels and forced vital capacity (FVC) values (p=0.02/0.07 and p=0.01/0.03, respectively).
Conclusion. Interestingly, not IgG4 but IgG3 showed highest anti-AT1R/ETAR Aab levels when compared to other IgG subclasses. However, in SSc patients, anti-AT1R/ETAR IgG3 levels are strongly correlated to certain disease manifestations, whereby high anti-AT1R/ETAR IgG3 levels are associated with low DLCO and FVC indicating deterioration of lung function. According to these findings high anti-AT1R/ETAR IgG3 levels could predict for lung function deterioration and represent a new marker for SSc complications.

PS90

DECREASED EXPRESSION OF NEUROPILIN-1 IN SYSTEMIC SCLEROSIS: POTENTIAL CONTRIBUTION TO IMPAIRED ANGIOGENESIS

E. Romano[1], M. Manetti[1,2], C. Mazzotta[1], I.J. Da Silva Chora[3], S. Bellando-Randone[1], J. Blagojevic[1], L. Ibba-ManneschI[2], M. Matucci-Cerinic[1], S. Guiducci[1]
[1]Department of Experimental and Clinical Medicine, Section of Rheumatology, University of Florence, Florence, ITALY; [2]Department of Experimental and Clinical Medicine Section of Anatomy and Histology, University of Florence, Florence, ITALY; [3]Department of Centro Hospitalar Sao Joao, Universidade do Porto, Porto, PORTUGAL.

Objectives. In SSc vascular involvement is a primary event characterized by vascular tone dysfunction and increased circulating levels of vascular endothelial growth factor (VEGF). Neuropilin-1 (NRP1) is a receptor for both class-3 semaphorin (sema) family of axon guidance molecules and VEGF. NRP1 is required for optimal VEGF/VEGFR-2 signaling, and NRP1-deficient mice exhibit vascular defects including disorganized blood vessels, lack of normal branching and missing capillary networks. Sema3a controls physiological and pathological angiogenesis. In the present study, we investigated the possible involvement of sema3a/NRP1 axis in SSc.
Methods. Soluble NRP1 (sNRP1) and sema3a levels were measured by quantitative colorimetric sandwich ELISA in serum samples from SSc patients (n=49) and age- and sex-matched healthy controls (n=39). Patients were classified according to nailfold videocapillaroscopy (NVC) patterns (early, active and late). NRP1 and sema3a protein expression was evaluated by immunofluorescence and western blot in skin biopsies from SSc patients (n=10) and healthy controls (n=8).

NRP1 expression was also evaluated in human dermal microvascular endothelial cells from SSc patients (SSc-MVECs) and healthy controls (H-MVECs) at basal level, and in H-MVECs after stimulation for 24 hours with recombinant human VEGF165 (10 ng/ml), SSc sera (n=3) and healthy sera (n=3).
Results. Circulating sNRP1 levels were significantly reduced in SSc patients (median 0.22 ng/ml) compared with healthy controls (median 0.69 ng/ml) (p=0.005). In particular, sNRP1 levels were significantly lower in either SSc patients with active or late NVC patterns than in controls (both p<0.05). Moreover, sNRP1 levels were significantly decreased in SSc patients with digital ulcers compared both with patients without digital ulcers (p=0.009) and controls (p=0.001). No significant differences in sema3a levels were detected between patients and controls. NRP1 expression was decreased in clinically affected skin biopsies from SSc patients compared with healthy skin, especially in dermal endothelial cells and stromal cells. H-MVECs showed higher NRP1 protein expression compared with SSc-MVECs. Stimulation with recombinant VEGF165 strongly upregulated NRP1 expression in H-MVECs. NRP1 expression in H-MVECs increased after treatment with healthy sera compared with basal condition, while it decreased after challenging with SSc sera (both p<0.005 vs basal H-MVECs). Sema3a expression was not different in skin biopsies from SSc patients compared with controls.
Conclusions. NRP1 expression is significantly decreased in SSc, and lower sNRP1 levels correlate with the severity of nailfold capillary modifications and presence of digital ulcers. NRP1 might play a role in the vascular damage and in the impairment of the angiogenic process in SSc.

PS91

INVOLVEMENT OF PLEXIND1/SEMAPHORIN 3E PATHWAY ON THE DYSREGULATION OF VASCULAR TONE CONTROL IN SSC PATIENTS

C. Mazzotta[1], E. Romano[1], C. Bruni[1], M. Manetti[1,2], G. Lepri[1], S. Bellando-Randone[1], J. Blagojevic[1], I. J. Da Silva Chora[3], A. Radicati[1], M. Matucci- Cerinic[1], S. Guiducci[1]
[1]Department of Experimental and Clinical Medicine, Section of Rheumatology, University of Florence, Florence, ITALY; [2]Department of Experimental and Clinical Medicine, Section of Anatomy and Histology, University of Florence, Florence, ITALY; [3]Department of Centro Hospitalar Sao Joao, Universidade do Porto, Porto, PORTUGAL.

Objective. The main hallmark of SSc is vasculopathy characterised by dysregulation of angiogenesis and vascular tone leading to loss of capillaries. The vascular and nervous system have several anatomic similarities that extend to level of the molecular mechanisms. Emerging evidence suggests that proteins involved in transmitting axonal guidance cues, including class III semaphorin families, also play a critical role in blood vessel guidance during physiological and pathological vessel development. Sema3E acts through its receptor plexin-D1 to control endothelial cell positioning and patterning of the developing vasculature. Sema3E is a natural antiangiogenic molecule that causes filopodial retraction in endothelial cells inhibiting cell adhesion by disrupting integrin-mediated adhesive structures. The aim of the present study was to investigate if plexin-D1/Sema3E axis could be involved in dysregulated vascular tone control (RF) characteristic of SSc.
Methods. Sema3E levels were measured by quantitative colorimetric sandwich ELISA in serum samples from 45 subjects with primary Raynaud's phenomenon (PRF) without ANA, Scl70, ACA positivity, 48 SSc patients and 48 age- and sex-matched healthy controls. Patients were classified according to nailfold videocapillaroscopy (NVC) patterns (early, active and late). Sema3E levels were expressed as median and range and compared by Mann-Whitney U test. Differences were considered significant for p values less than 0.05. Western blot was used to evaluate plexin-D1/Sema3E axis in human dermal microvascular endothelial cells from healthy subjects (H-MVECs) at basal condition and after stimulation with recombinant human VEGF165 (10 ng/ml), lcSSc sera (n=3) and healthy sera (n=3) for 24h.
Results. Sema3E sera levels were significantly higher both in PRF subjects (median 0.54 ng/ml) and SSc patients (median 0.67 ng/ml) respect to healthy controls (median 0.19 ng/ml) (both p<0.001). In particular, sema3E levels were significantly higher in SSc patients with early NVC pattern both respect to active/late pattern and PRF (both p<0.05). Moreover, sema3E levels were significantly increased in SSc patients without ulcers compared with patients with digital ulcers (p=0.018). H-MVECs stimulated with SSc sera showed higher levels of the activated plexin-D1 form and sema3E protein expression in respect to basal H-MVECs and healthy sera. No differences were found in plexin-D1/Sema3E axis after challenging with VEGF.
Conclusions. Circulating sema3E is significantly increased both in PRF and SSc. Higher sema3E levels are increased in the early stages of SSc without digital ulcers. Our findings suggest that plexin-D1/Sema3E axis might have a role in the dysregulation of vascular tone control.

PS92

DECREASED EXPRESSION OF THE ENDOTHELIAL CELL-DERIVED FACTOR EGFL7 CONTRIBUTES TO IMPAIRED ANGIOGENESIS AND VASCULOGENESIS IN SYSTEMIC SCLEROSIS

M. Manetti[1], S. Guiducci[1], E. Romano[1], J. Avouac[2], I. Rosa[1], B. Ruiz[2], G. Lepri[1], S. Bellando-Randone[1], L. Ibba-Manneschi[1], Y. Allanore[2], M. Matucci-Cerinic[1]
[1]Department of Experimental and Clinical Medicine, University of Florence, Florence, ITALY; [2]Cochin Institute, Paris Descartes University, INSERM U1016 and CNRS UMR8104, Paris, FRANCE.

Objective. Microvascular damage and defective angiogenesis and vasculogenesis play a major role in the pathogenesis of systemic sclerosis (SSc). Epidermal growth factor-like domain 7 (EGFL7) is a proangiogenic molecule predominantly expressed and secreted by endothelial cells and their progenitors which controls vascular development and integrity. In the present study, we investigated the possible involvement of EGFL7 in SSc.
Methods. Serum EGFL7 levels from 60 patients with SSc and 35 age- and sex-matched healthy volunteers were examined by colorimetric sandwich enzyme-linked immunosorbent assay. The expression of EGFL7 in forearm skin biopsies (n=16 SSc, n=10 controls), cultured dermal microvascular endothelial cells (MVECs) (n=3 SSc, n=3 controls) and late-outgrowth peripheral blood endothelial progenitor cell (EPC)-derived endothelial cells (n=15 SSc, n=8 controls) was investigated by immunofluorescence and Western blotting. Anti-CD31/pan-endothelial cell marker antibodies were used in double immunofluorescence experiments to specifically investigate endothelial EGFL7 expression in skin sections.
Results. Serum EGFL7 levels were detectable in 68.6% of healthy controls and 45% of SSc cases (p<0.05). Circulating levels of EGFL7 were significantly decreased in SSc patients compared with healthy controls (p=0.01). Serum levels of EGFL7 were significantly lower both in limited cutaneous SSc and diffuse cutaneous SSc patients than in controls (p=0.02 and p=0.04, respectively). In SSc, decreased serum EGFL7 levels were significantly correlated with the severity of nailfold capillary abnormalities. Patients with most severe capillary changes and digital ulcers had serum EGFL7 levels significantly lower than healthy controls (p=0.006 and p=0.002, respectively), while the EGFL7 levels did not differ significantly between controls and SSc patients with less capillary damage and lack of digital ulcers. Endothelial EGFL7 expression was strongly downregulated or even almost completely undetectable in SSc affected dermis compared with controls (p<0.001). In cultured SSc dermal MVECs and late-outgrowth peripheral blood EPC-derived endothelial cells, EGFL7 was significantly downregulated compared with cells obtained from healthy subjects (p<0.01 and p<0.001, respectively).
Conclusions. Our findings suggest that the loss of EGFL7 expression in endothelial cells and their progenitors might play a role in the development and progression of peripheral microvascular damage and defective vascular repair process characteristic of SSc.

PS93

NAILFOLD CAPILLAROSCOPIC ASSESSMENT AND VASCULAR BIOMARKERS IN SYSTEMIC SCLEROSIS: LOW CD40L LEVELS IN PATIENTS WITH LATE SCLERODERMA PATTERNS

Y. Yalcinkaya[1], S. Cinar[2], S. Kamali[1], O. Pehlivan[1], N. Alpay[1], L. Ocal[1], G. Deniz[2], M. Inanc[1]
[1]Istanbul University, Istanbul Medical Faculty, Department of Internal Medicine, Division of Rheumatology, Istanbul, TURKEY; [2]Istanbul University, DETAE, Department of Immunology, Istanbul, TURKEY.

Objectives. To determine the relationship between vascular biomarkers reflecting the vascular injury and neoangiogenesis with capillaroscopic changes in systemic sclerosis (SSc).
Methods. Seventy-two SSc patients (66 female) fulfilling Leroy and Medsger classification criteria were evaluated, including clinical findings nailfold videocapillaroscopy (NVC) was performed qualitatively (early, active and late scleroderma patterns) in all patients (Cutolo M, *et al*. J Rheumatol 2000). Serum samples of patients were collected for flow-cytometric analysis of CD40L, tPA, MCP-1, sE-selectin, IL-8, IL-6, VEGF, sP-selectin, TGF-β1 ve VCAM levels (Bender MedSystems, Vienna, Austria) at the same time with NVC. Results were compared with Pearson chi-square / Fischer's, Mann Withney U ve Kruskal Wallis tests.
Results. The mean age of the patients was 44.9 and disease duration from the appearance of Raynaud's and non-Raynaud symptoms were 5.8±5.9 and 3.2±2.4

years. Of the patients 23(%32) had diffuse and 49(%68)limited cutaneous involvement, 15(%21) were anti-centromere(+) and 34(%47) were anti-Scl70(+). When we compared with healthy subjects; tPA (p=0.02), MCP-1 (p=0.001), sE-selectin (p=0.008) and TGF-β1 (p=0.001) levels were significantly higher, sP-selectin (p=0.011) ve IL-8 (p=0.001) levels were lower in SSc patients. SSc patients grouped according to NVC patterns as 'early'(n=10), 'active'(n=37) and 'late'(n=25). Between groups according to NVC patterns, only sCD40L(pg/ml) levels were significantly lower in the 'late' group (p=0.043), higher in patients with limitted cutaneus involvement (p=0.01) and smoking history (n=32,%44) (p=0.033). The other markers were similar between NVC groups.
Conclusions. There was lower sCD40L serum levels in patients with late NVC patterns, although the levels were similar to healthy controls in patients with early, active NVC pattern.CD40L may be a key molecule in the early/active phase of vascular involvement. Higher concentrations of sCD40L in patients with limited cutaneous disease and smoking history might be related to its role in vascular pathology. NVC is a useful method for investigating the vascular pathogenesis in SSc.

PS94

DIAGNOSTIC TARGETS REVEALED BY HIGH-RESOLUTION PROTEIN PROFILING OF HUMAN PLASMA MICROPARTICLES

L. Iversen[1], O. Østergaard[2], S. Jacobsen[3], N. Heegaard[2]
[1]Department of Dermatology, Bispebjerg Hospital, Copenhagen University, Copenhagen, DENMARK; [2]Department of Clinical Biochemistry & Immunology, Statens Serum Institut, Copenhagen, DENMARK; [3]Department of Rheumatology, University Hospital Rigshospitalet, Copenhagen, Denmark, Copenhagen, DENMARK.

Background. Microparticles (MP) are small membranous vesicles shed from cells undergoing apoptosis or activation. They are found in the circulation and they carry information about cellular origin and events which lead to their formation. In addition, they may reflect disease processes in the body.
Methods. MPs were obtained from 1 mL platelet poor citrate plasma by repeated ultracentrifugation (five times 20.000 x g, 30 min at room temperature). Proteins present in the MP preparations were identified and quantified followed by data-analysis using MaxQuant for protein ID and label-free quantitation. In this study, MPs from a cohort of 38 uniformly collected samples from patients with systemic sclerosis (SSc) and 25 healthy controls (HC) were analyzed.
Results. Altogether more than 530 unique proteins were identified. Univariate statistics, hierarchical clustering, and principal components analysis were applied to analyze the protein intensity to search for disease classifiers. Thirty proteins showed highly significant differences between SSc and HCs (p<0.05 after Benjamini-Hochberg correction for multiple hypothesis testing). Among these, TGF-beta was found increased (p=0.003) and several mitochondrial proteins were reduced (p=0.003). The protein concentration of MPs from SSc patients was correlated with soluble markers of vascular activation analysed regarding association with SSc subgroups or specific organ manifestations in SSc patients.
Conclusion. MPs in the circulation are a valuable reservoir of information on disease states. The data from the present study of SSc patients show that both highly specific and sensitive diagnostic novel target molecules and markers associated with disease severity may be present in the MP protein profiles.

Poster Tour 14: *Pathogenesis*

PS95

S100A4 SERUM LEVELS CORRELATE WITH SKIN FIBROSIS, LUNG INVOLVEMENT AND DISEASE ACTIVITY IN SYSTEMIC SCLEROSIS

M. Tomcik[1], L. Andres Cerezo[1], S. Skacelova[1], M. Komarc[2], R. Becvar[1], M. Grigorian[3], J.H.W. Distler[4], L. Senolt[1]
[1]Institute of Rheumatology, Department of Rheumatology, 1st Faculty of Medicine, Charles University, Prague, CZECH REPUBLIC; [2]Institute of Biophysics and Informatics of the First Faculty of Medicine, Charles University, Prague, CZECH REPUBLIC; [3]Institute of Cancer Biology, Danish Cancer Society, Copenhagen, DENMARK; [4]Department of Internal Medicine III and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, GERMANY

Background. In our previous study we demonstrated that S100A4 is overexpressed in scleroderma (SSc) skin, fibroblasts and preclinical models of SSc in a TGF-beta dependent manner. We showed that S100A4 is a new regulator of TGF-beta signalling and its inhibition prevents the stimulatory effects of TGF-beta. Inactivation of S100A4 prevented dermal fibrosis induced by bleomycin and in Tsk-1 mice.
Objectives. To evaluate S100A4 in sera of SSc patients and characterize its potential association with SSc-related features.
Methods. A total of 33 patients (29 females; mean age 52.8; disease duration 4.2 years; dcSSc/lcSSc = 8/25) who met the ACR classification criteria for SSc and 20 healthy individuals matched by age and sex were included in this study. Serum S100A4 levels were measured using ELISA (CycLex Co., Ltd., Nagano, Japan). CRP, ANA and ENA complex were evaluated. SSc-related manifestations were obtained from the Czech Registry Database of SSc patients. Skin changes were assessed using the modified Rodnan skin score (mRSS) and EUSTAR SSc activity score was determined. Data are presented as mean ± SEM.
Results. S100A4 serum levels were significantly increased in SSc patients compared with healthy controls (119.2±23.4 vs. 43.9±3.3ng/ml, p=0.011). Patients with diffuse cutaneous SSc had significantly higher levels of serum S100A4 compared with patients with limited cutaneous SSc or healthy controls (201.8±53.1 vs. 92.7±24.0ng/ml, p=0.017 and 201.8±53.1 vs. 43.9±3.3ng/ml, p=0.001, respectively). Levels of S100A4 positively correlated with mRSS (r=0.556, p=0.001). Furthermore, S100A4 levels negatively correlated with forced vital capacity (FVC) and saturation of peripheral oxygen (SPO2) (r=- 0.362, p=0.038 and r=-0.414, p=0.029, respectively). Of particular interest, S100A4 levels positively correlated with EUSTAR SSc activity score (r=0.750, p=0.0001). However, only correlations between S100A4 and mRSS, and S100A4 and EUSTAR SSc activity score were approved at corrected level of statistical significance after Bonferroni's correction (p<0.01). The presence of autoantibodies (ANA, anti-centromere, anti-Scl70), pathological capillaroscopic pattern (early, active or late), and presence of the main individual clinical symptoms of SSc did not significantly affect levels of serum S100A4.
Conclusions. We demonstrate that S100A4 serum levels are significantly increased in SSc patients compared with healthy controls. Higher levels of S100A4 are associated with dcSSc subset, skin involvement, deteriorated parameters of lung involvement and higher disease activity. These data support our previous findings on the role of S100A4 as a regulator of TGF-beta induced fibroblast activation and dermal fibrosis in SSc.
Acknowledgements. This study was supported by IGA NT 13698-4.

PS96

SECRETED FRIZZLED-RELATED PROTEIN 4 CAN BE INDUCED BY TRANSFORMING GROWTH FACTOR-BETA, IS REGULATED BY CAVEOLIN-1 AND CAN INDUCE NON-CANONICAL WNT SIGNALING IN FIBROBLASTS

J. Gillespie[1], A Giuseppina[1], P Emery[1,2], M.F. McDermott[1,2], F Del Galdo[1,2]
[1]Leeds Institute of Rheumatic and Musculoskeletal Medicine, Leeds, UNITED KINGDOM; [2]Leeds Musculoskeletal Biomedical Research Unit, LTHT, Leeds, UNITED KINGDOM.

Background. Systemic Sclerosis (SSc) is a heterogeneous disease characterized by autoimmune activation, fibroproliferative vasculopathy, and tissue fibrosis of skin and multiple internal organs. Several studies have indicated that both caevolin-1 (CAV-1) and WNT/β-catenin signaling play important roles in the pathogenesis of tissue fibrosis. Indeed, CAV-1 is downregulated by 40% in SSc skin compared to healthy controls and, intriguingly, tissue expression studies with SSc skin biopsies show both upregulation of canonical WNT ligands1,2 and

consistent upregulation of Frizzled-Related Protein 4 (SFRP4), a putative WNT antagonist, at both mRNA at protein level 3,4.
Methods. Immortalized primary healthy (HC) and SSc fibroblasts were cultured in 10% DMEM and starved in 0.5% DMEM for 24hrs prior to stimulation with recombinant TGFβ (10ng/ml), Wnt-5a (100ng/ml) and/or SFRP4 (100-1000ng/ml). Gene expression was quantified by SYBRgreen RT-PCR and by western blot. CAV-1 siRNA was transfected at a final concentration 10nM. Canonical WNT signaling was assessed by TOPflash TCF/LEF reporter activity. ELISA was used to measure both the levels of Phospho-c-Jun from whole cell lysates.
Results. In SSc fibroblasts, the basal expression of SFRP4 is increased at both protein level and also by 264% at mRNA level compared to HC [p<0.001]. TGFβ stimulation upregulated SFRP4 mRNA by 170% [p<0.01] at 48hrs and by 348% [p<0.01] at 72hrs. TGFβ also induced a time-dependent increase of both SFRP4 and α-SMA protein expression, while reducing CAV-1. siRNA-mediated silencing of CAV-1 was sufficient to induce a time-dependent increase in SFRP4 protein expression. Wnt-3a induced a 600% increase in TOPFlash activity, co-treatment with SFRP4 decreased this activity by 283%. In contrast, SFRP4 induced a 260% increase [p<0.001] in c-JUN phosphorylation at 10min in both HC and SSc fibroblasts. This was similar to non-canonical Wnt-5a stimulation. Interestingly, basal c-Jun phosphorylation was increased by 180% [p<0.005] in SSc compared to HC fibroblasts. However, SFRP4 treatment did not affect collagen or α-SMA protein levels within a dose range of 100-1000ng/ml.
Conclusions. Indeed, the increased expression of SFRP4 observed in SSc may be a direct consequence of CAV-1 downregulation by TGFβ in tissue fibroblasts. Given the non-canonical WNT activity of SFRP4, a TGFβ primed microenvironment may be responsible for shaping the phenotype of both fibroblasts and neighboring cells, through aberrant WNT pathway activation. Investigation of the mechanisms linking CAV-1 expression and SFRP4 function will improve our understanding of the pathogenetic role aberrant WNT activation plays in SSc.

PS97

TARGETING IL-6 BY BOTH PASSIVE OR ACTIVE IMMUNIZATION STRATEGIES PREVENTS INFLAMMATION-DRIVEN SKIN FIBROSIS

J. Avouac[1], L. Desallais[2], M. Fréchet[3], M. Elhai[1], J.F. Zagury[2], Y. Allanore[1]
[1]Rehumatology A department and INSERM U1016, Cochin Hospital, Paris, FRANCE; [2]Conservatoire National des Arts et Métiers, Paris, FRANCE; [3]INSERM U1016, Cochin Hospital, Paris, FRANCE.

Objective. Interleukin 6 (IL-6) is a pleiotropic cytokine involved in inflammatory and autoimmune processes. Preliminary data have suggested that IL-6 might contribute to systemic sclerosis (SSc). Our aim was to compare the efficacy of both passive and active immunization against IL6 to reduce skin fibrosis in complementary mouse models of scleroderma.
Methods. We first evaluated the monoclonal IL-6R antibody MR16-1 in the mouse model of bleomycin-induced dermal fibrosis, reflecting early and inflammatory stages of SSc. Six-week-old DBA/2 mice received in parallel subcutaneous injections bleomycin (0,5 mg/ml) and intraperitoneal (ip.) injection of MR16-1 or control antibody at a dose of 2 mg at day 0 followed by one ip. injection of 1 mg at day 7 and 14. Then, we assessed the merit of MR-16 in the tight skin (Tsk-1) mice, an inflammation-independent mouse model of skin fibrosis. Tsk-1 mice received a first ip. injection of 2 mg of MR16-1 or control antibody at the age of 5 weeks followed by one ip. injection of 1 mg once a week for 5 weeks. Thereafter, because of the drawbacks of anti-cytokine monoclonal antibodies, we developed an innovative strategy using active immunization against a small peptide derived from murine IL-6, which was performed in the mouse model of bleomycin-induced dermal fibrosis.
Results. Passive immunization with MR16-1 exerted antifibrotic effects in the mouse model of bleomycin-induced dermal fibrosis: dermal thickness, hydroxyproline content and myofibroblast counts were reduced by 25±4% (p=0.02), 30±6% (p=0.007) and 45±7% (p=0.005) respectively, compared to mice receiving control antibody. MR16-1 demonstrated no efficacy in Tsk-1 mice. Mice immunized against the mIS200 peptide derived from murine IL-6 exhibited in the bleomycin mouse model similar antifibrotic effects as passive immunization. We observed a significant reduction of dermal thickness by 20±3% (p=0.02), hydroxyproline content by 25±4% (p=0.005) and myofibroblast counts by 41±9% (p=0.01), compared to the group immunized against the carrier protein alone.
Conclusion. We demonstrated that passive and active immunization targeting IL-6 had similar antifibrotic properties in a mouse model of inflammation-driven dermal fibrosis. Translation to human disease is now required, and targeting early and inflammatory stages of SSc sounds the most appropriate. This strategy is currently under investigation in a phase-3 clinical trial assessing the efficacy of tocilizumab to improve skin involvement in patients with early diffuse SSc. Our results also highlight the relevance of active immunotherapy that might be an avenue for IL6 axis in immunotherapy in a near future.

PS98

ENDOTHELIN-1 MEDIATES DOWNSTREAM PROFIBROTIC EFFECTS BY TRANSFORMING GROWTH FACTOR-BETA 1 IN SYSTEMIC SCLEROSIS SKIN FIBROBLASTS

T. Higuchi, Y. Kawaguchi, K. Takagi, Y. Ota, Y. Katsumata, T. Gono, H. Yamanaka
Institute Of Rheumatology, Tokyo Women's Medical University, Tokyo, JAPAN.

Background. Systemic sclerosis (SSc) is an autoimmune connective tissue disorder characterized by excess collagen deposition, vascular changes and production of autoantibodies that affect multiple organs. Transforming growth factor β1 (TGF-β1), which promotes collagen synthesis, extracellular matrix (ECM) remodeling and myofibroblast differentiation, is thought to play a key role in the pathogenesis of SSc. The vasoconstrictive peptide endothelin-1 (ET-1) is known to be a potent fibrotic factor similar to TGF-β1. ET-1 binds to two distinct subtypes of G protein coupled receptors, ET receptor A (ETRA) and ET receptor B (ETRB). The fibrotic functions of each ET receptor remain unclear partially because ET receptor distribution and expression differ according to the disease, affected organ and cell type. Our study aimed to examine the ET-1-mediated effects of TGF-β1 on the fibrogenic phenotype of SSc skin fibroblasts using a single and/or dual ET receptor antagonist.
Methods. Human SSc skin fibroblasts (SSc fibroblasts) were obtained from SSc patients. Recombinant TGF-β1, recombinant ET-1, SIS3 as an inhibitor of Smad3 phosphorylation, BQ123 as a single ETRA antagonist, BQ788 as a single ETRB antagonist and bosentan as a dual ETRA/ETRB receptor antagonist were used in this study. The SSc fibroblasts were incubated with TGF-β1 in the presence of SIS3. In addition, the effects of BQ123, BQ788 and bosentan were explored. Expression of ET-1, CTGF and type I collagen was evaluated using ELISA and real-time RT-PCR. ETRA and ETRB expression were assessed using immunohistochemistry and fluorescence activated cell sorting (FACS) analysis.
Results. Both ETRA and ETRB were expressed in SSc fibroblasts. TGF-β1 increased ET-1 mRNA and protein expression, and this increase in ET-1 was suppressed by SIS3. Upregulation of COL1A1 and CTGF by TGF-β1 was reduced by an ETRA or ETRB antagonist, and a dual ETRA/ETRB antagonist had an additive inhibitory effect.
Conclusions. TGF-β1 produced ET-1 through Smad3 phosphorylation, and a dual ETRA/ETRB antagonist decreased COL1A1 and CTGF mRNA levels in fibroblasts. These findings suggest that both ETRA and ETRB signaling are associated with a fibrotic phenotype in SSc skin fibroblasts and that dual ETRA/ETRB might be a novel therapeutic target for SSc skin fibrosis.

PS99

THE ROLE OF MCSF AND ENDOTHELIN 1 IN FIBROCYTE DIFFERENTIATION

S. Trinder[1], X. Shi-wen[1], B. Ahmed-Abdi[1], R. Good[1], A. Gilbane[1], C. Denton[1], D. Budd[2], D. Abraham[1], A. Holmes[1]
[1]Centre for Rheumatology & Connective Tissue Diseases, UCL, London, UNITED KINGDOM; [2]Respiratory Drug Discovery, Inflammation, Hoffmann-La Roche, Nutley, USA.

Introduction. Systemic sclerosis (SSc) is a complex autoimmune fibrotic disease, characterised by elevated deposition of extracellular matrix (ECM) proteins, including collagen type I. The disease is heterogeneous; organs commonly affected by fibrosis are the skin, kidney, lung and heart. Vascular complications include pulmonary arterial hypertension (PAH), occuring in 12-40% of patients. CD14+ monocytes are a functionally heterogeneous cell type able to differentiate into a number of cell phenotypes including macrophages and fibrocytes. In culture fibrocytes adopt a spindle shape, co-express haematopoietic - CD45RO and 25F9, along with mesenchymal markers including aSMA and collagen type I. Fibrocytes amplify the inflammatory/immune response through distinct mechanisms, including antigen presentation, cytokine and chemokine secretion, and the production of MMPs. We and others have shown fibrocyte differentiation is enhanced by fibrogenic cytokines. Here we seek to understand the mechanism by which SSc fibrocytes influence the local microenvironment of the tissue.
Methods. CD14+ peripheral blood mononuclear cells (PBMCs) were isolated from SSc patient and healthy control blood. PBMCs were cultured in the presence of macrophage colony stimulating factor (MCSF; n>10) and/or endothelin-1 (ET-1; n>10); after 14 days of culture number of fibrocytes was assessed. The effect of pharmacological inhibitors including ETRA and ETRB antagonism on fibrocyte differentiation (n=6 SSc and control) was investigated. Secreted factors in culture media from SSc and control fibrocytes were assessed by ELISA (n=6), and the effects of conditioned media explored in 3D-collagen gel

Results. MCSF and ET-1 significantly induced fibrocyte differentiation, in combination differentiation was significantly augmented (p<0.05) in comparison to mono-treatment. SSc fibrocytes more readily differentiated from CD14+ PBMCs than healthy control donors in response to MCSF (p<0.05), ET-1 (p<0.05) as well as MCSF with ET-1 in combination (p<0.01). ETRA and ETRB antagonists, BQ123 and BQ788 (respectively), and Bosentan (a dual ETR antagonist) inhibited MCSF induced fibrocyte differentiation in a concentration dependant manner. Furthermore SSc fibrocytes secreted significantly more CTGF than control fibrocytes (p<0.05) cultured with MCSF. Consistent with fibrocytes acting in a paracrine manner, conditioned media from SSc fibrocytes promoted fibroblast gel contraction by control cells (p<0.05).
Discussion. Here we show CD14+ SSc PBMCs more readily differentiate into fibrocytes and that activation via the ETRA/B is essential for ET-1 and MCSF induced fibrocyte differentiation. Suggesting MCSF acts indirectly via ET-1 release; possibly resulting in a positive feedback loop. Our data suggests fibrocytes may contribute to the development of a pro-fibrotic environment through influencing tissue resident fibroblasts in a paracrine manner.

PS100

SIMVASTATIN MODULATES AORTIC INTIMA/MEDIA THICKNESS IN AN ANIMAL MODEL OF SYSTEMIC SCLEROSIS

G. Bagnato[1], A. Bitto[1], G. Pizzino[1], W.N. Roberts[2], D. Altavilla[1], F. Squadrito[1], G. Bagnato[1], A. Saitta[1]
[1]University of Messina, Messina, ITALY; [2]University of Louisville, Louisville, USA.

Background/Purpose. Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by vasculopathy and organ fibrosis. Although many previous studies highlighted microvascular alterations in SSc, a growing body of evidence exists for structural and functional abnormalities in the macrovascular circulation. Recent reports shows that in SSc patients macrovasculopathy occurs preferentially at the forearm and aorta.
Aim of the study was therefore to evaluate the effect of simvastatin administration on aortic intima-media (IM) thickness and ratio in a murine model of systemic sclerosis.
Methods. SSc-like illness was induced in BALB/c mice by daily subcutaneous injections of HOCl as an oxidant stress for 6 weeks. Mice (n=24) were randomized in three arms to treatment w11ith either HOCl (n=10), HOCl plus simvastatin (n=9); or vehicle alone (n=5). Simvastatin treatment was initiated 30 minutes after HOCl subcutaneous injection (40 mg/kg) continuing daily for the 6 weeks. Thoracic aorta was evaluated by histological methods. IM thickness and ratio were measured for statistical analysis.
Results. In HOCl treated mice aortic IM thickness was significantly higher than controls, showing an increase of 104% (p<0.0001). Treatment with simvastatin diminished this increase by 92% (p<0.0001). Simvastatin treated animals had a significantly thinner intima layer (-9%, p<0.0001) and media layer (-197%, p<0.0001) compared to HOCl group. IM ratio was also decreased in HOCl treated mice compared to controls (0.75 vs 1.74, p<0.0001) and significantly increased by simvastatin administration (1.61 vs 0.75, p<0.0001).
Conclusion. Administration of simvastatin moderates the increase of IM thickness in this animal model of SSc. Further analysis on IM ratio suggests that aortic media layer is thickened in HOCl treated animals and this increase can be prevented by simvastatin.

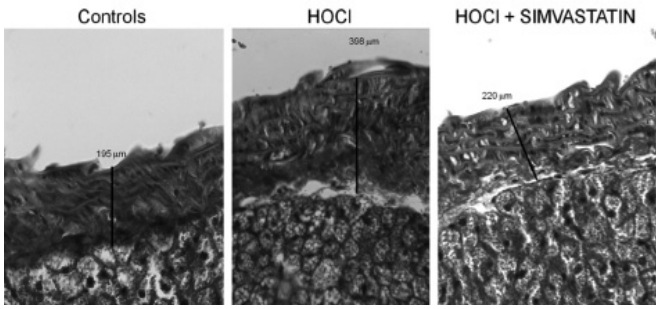

Fig. 1.

PS101

SUSCEPTIBILITY OR RESISTANCE TO EXPERIMENTAL LUNG FIBROSIS IS PREDICTEDBYRESIDENT LUNG FIBROBLAST GENE EXPRESSION SIGNATURE

E. Derrett-Smith, R. Hoyles, K. Khan, D. Abraham, C. Denton
UCL Medical School Centre for Rheumatology and Connective Tissue Diseases, London, UNITED KINGDOM.

Background. In scleroderma (SSc), lung fibrosis is linked to epithelial damage and dysregulated repair mechanisms. Resident lung fibroblasts may affect multiple cell types including epithelium, endothelium, smooth muscle cells and fibrocytes. We have used two complementary transgenic mouse strains with altered TGFβ signalling to better understand the regulatory role of resident lung fibroblasts in defining susceptibility to fibrosis.
Methods. The TβRIIδk-fib mouse model of SSc, in which TGFβ signalling is upregulated in fibroblasts, is susceptible to fibrotic lung injury whereas the TβRII-null-fib strain, in which TβRII is conditionally knocked out in fibroblasts, is resistant to bleomycin-induced lung fibrosis. We have used an illumina® microarray platform to profile lung or skin fibroblasts from these two strains and identified a cohort of genes that determine susceptibility or resistance to experimental lung fibrosis, comparing to a control group using whole lung from TβRIIδk-fib animals and wildtype littermates (n=3) on the same microarray platform. Technical validation of data and additional quantitation of gene expression was performed using quantitative RT-PCR assays with replicate samples.
Results. The TβRIIδk-fib lung fibroblast gene expression signature includes key genes that are implicated as pathogenic drivers of fibrosis and inflammation and potential biomarkers in SSc. Conversely, many of these genes are downregulated in TβRII-null-fib mice, including BMP4 (fold reduction in TβRII-null-fib 31.8, p<0.02; fold upregulation in TβRIIδk-fib compared with WT 2.01, p<0.6); elastin (TβRII-null-fib 17.8, p<0.14; TβRIIδk-fib 1.86, p<0.09); CCL2 (TβRII-null-fib 56.8, p<0.09; TβRIIδk-fib 1.72, p<0.03)and MMP13 (TβRII-null-fib 13.2, p<0.08; TβRIIδk-fib 3.6, p<0.4).

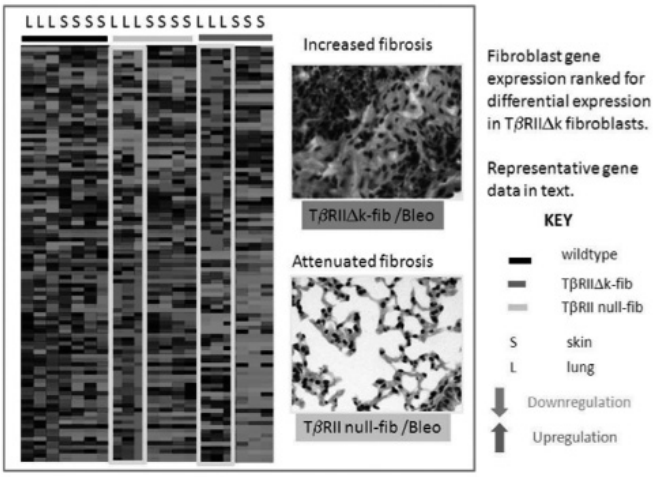

Fig. 1.

CTGF (CCN2) was strongly upregulated in TβRIIδk-fib lung fibroblasts, but showed less downregulation than other genes in the TβRII-null-fib, probably reflecting multiple pathways of activation. No signature of overexpression was present in the whole lung analysis suggesting that fibroblast-specific differences in gene expression determine altered fibrotic response.
Conclusion. These data define a cohort of genes differentially expressed in fibroblasts that associate strongly with susceptibility or resistance to experimental lung fibrosis. These transcripts include many that are important in tissue repair and that have previously been shown to be over expressed in SSc skin samples. They suggest that the same resident fibroblast gene expression signature may govern fibrosis in both lung and skin.

PS102

EXCESSIVE FIBROSIS AND PULMONARY VASCULAR REMODELING IN FRA-1 TRANSGENIC MICE

H. Yasuoka, Y. Okazaki, A. Tam, Y. Tamura, I. Takada, K. Matsuo, T. Takeuchi, M. Kuwana
Keio University School of Medicine, Tokyo, JAPAN.

Introduction. Excessive fibrosis and microvasculopathy are typical pathogenic processes of systemic sclerosis (SSc). It has been reported that mice overexpressing Fra-2, which is a component of a transcription factor AP-1, spontaneously develop vascular remodeling with obliteration of pulmonary small arteries as well as generalized fibrosis found predominantly in the lung. We accidentally found that mice transgenic for another AP-1 component Fra-1 (Fra-1-TG mice) died early due to cardiopulmonary insufficiency. Detailed histologic findings in the lung and skin of this mouse strain were evaluated.
Methods. We examined 8 pairs of Fra-1-TG and wild-type mice (age between 5-16 weeks). In Fra-1-TG mice, murine fra-1 gene was overexpressed ubiquitously under the control of the major histocompatibility complex class I antigen H2Kb promoter. Paraffin-embedded tissue sections were subjected to Hematoxylin and Eosin, Masson-Trichrome, and Elastica-van Gieson staining. Expression of CD31 and α-smooth muscle actin (SMA) was further evaluated by immunohistochemistry. Right ventricular overload was evaluated by transthoracic echocardiography with Doppler technique and the ratio of the right to left ventricle size (RV/LV ratio) by postmortem examinations.
Results. Fra-1-TG mice died at a median age of 14 weeks with signs of cardiopulmonary insufficiency. At age of 5 weeks, diffuse thickening of alveolar walls was apparent with infiltration of mononuclear inflammatory cells in perivascular area and in alveolar walls..Subsequently, active deposition of extracellular matrix (ECM) progressed serially and uniformly, leading to diffuse fibrosis in the parenchyma. The Fra-1-TG mice also developed excessive ECM deposition in the dermis with loss of subcutaneous fat tissue. .In terms of pulmonary vasculature, intimal and medial thickening in small-to-medium-sized pulmonary arteries were already present at the age of 5 weeks. These changes progressed with age and resulted in concentric laminar fibrosis, resulting in narrowing of the vascular lumen. Neo-muscularization of small arterioles was also detected after age of 10 weeks. At 16 weeks, Fra-1-TG mice represented pulmonary hypertension confirmed by typical echocardiographic findings of tricuspid regurgitation and an increased RV/LV ratio. These histologic and functional changes were not found in wild-type mice.
Conclusions. Fra-1-TG mice spontaneously developed excessive fibrosis in the lung and skin as well as pulmonary vascular remodeling, characteristic of patients with SSc. Mice overexpressing Fra-1 and Fra-2 exhibit the similar fibrotic/vasculopathic phenotype, indicating involvement of the AP-1 pathway in pathogenesis of SSc.

Poster Tour 15: *Pathogenesis*

PS103

INCREASED FREQUENCY OF INTERLEUKIN-2 PRODUCTION BY TH17 LYMPHOCYTES IN PERIPHERAL BLOOD OF SYSTEMIC SCLEROSIS PATIENTS

M.G. Santiago[1], M. Raposo[2], C. Duarte[1], M.J. Salvador[1], A. Paiva[2], J.A.P. Da Silva[1]
[1]Rheumatology Department,, Centro Hospitalar e Universitário de Coimbra - Hospitais da Universidade de Coimbra, Coimbra, PORTUGAL; [2]Centro do Sangue e da Transplantação de Coimbra - Instituto Português do Sangue e da Transplantação, Coimbra, PORTUGAL.

Background/Purpose. The pathogenesis of systemic sclerosis (SSc) is largely unknown, although proinflammatory cytokines are considered to play a central role. We hypothesized that Th17 cell populations and cytokine expression may be altered in SSc.
Our purpose was to investigate the pattern of expression of proinflammatory cytokines by peripheral blood (PB) IL-17+ T cell populations in SSc and to explore clinical associations.
Methods. This study included 41 SSc patients and 20 age- and sex-matched healthy controls (HC). All SSc patients fulfilled the American College of Rheumatology Criteria for the classification of SSc and were classified according to LeRoy et al. as having limited cutaneous SSc (lSSc, n=29) or diffuse cutaneous SSc (dSSc, n=12). Clinical evaluation included disease duration, modified Rodnan skin score (mRSS), digital necrosis and target organs' involvement. The autoantibody profile was collected from medical records.
Each participant was submitted to a blood sample collection, which was processed in order to separately analyze the intracellular expression of IL-2, TNF-α,and IFN-γ in Th17 cells.
Data was statistically analyzed using the SPSS® version 20.0. Mann-Whitney test was used to evaluate differences between groups. Correlations between continuous variables were assessed by Spearman's correlation coefficient. P values < 0.05 were considered statistically significant.
Results. The mean age was 56.1±11.8 and 52.0±9.9 years for SSc patients and HC respectively. Females represented 78% of the SSc group and 80% of the HC. The patients had a mean mRSS of 11.32±7.76 and mean disease duration of 9.5±8.5 years.
The frequency of PB Th17 cells was not statistically different in SSc patients when compared to HC, neither between lSSc, dSSc and HC. A difference between lSSc, dSSc and HC regarding the frequency of IL-2-producing Th17 cells was found. We also found differences between lSSc and HC regarding the frequency of TNF-α-producing Th17 cells. There were no differences between groups regarding the frequency of IFN-γ expression among Th17 cells. We also have negative findings regarding disease duration and internal organs' involvement. The frequency of IL-2-producing Th17 cells showed a positive correlation with mRSS (p=0.002).
Conclusion. IL-2-producing Th17 cells frequency is higher in SSc than in HC. The frequency of IL-2-producing Th17 cells was correlated with the extension of skin involvement. These findings support the hypothesis that IL-2 produced by Th17 cells may be involved in the pathological process of SSc, regardless of the disease subset.

PS104

ROLE OF IL-13-PRODUCING CD8+ T CELLS IN THE PATHOGENESIS OF SYSTEMIC SCLEROSIS

P. Fuschiotti[1], A. T. Larregina[2], C. Feghali-Bostwick[3], J. Devlin[4], D.B. Stolz[4], T. A. Medsger[5]
[1]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, USA; [2]Department of Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, USA; [3]Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh School of M, Pittsburgh, USA; [4]Cell Biology and Physiology Center for Biological Imaging, University of Pittsburgh School of Medicine, Pittsburgh, USA; [5]Division of Rheumatology and Clinical Immunology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, USA.

T lymphocytes play an important role in systemic sclerosis (SSc), a connective tissue disease characterized by inflammation, fibrosis and vascular damage. Its most characteristic feature is cutaneous fibrosis that is attributable to excessive deposition of collagen and other connective tissue components by activated dermal fibroblasts. Although the pathogenesis is still unclear, this fibroblast activation is believed to result from their interaction with immune mediators, such as T cell-derived cytokines, and other growth factors. We recently found that dysregulated production of the profibrotic cytokine IL-13 by peripheral blood effector CD8+ T cells correlates with more severe forms of cutaneous SSc and is associated with defects in the molecular control of IL-13 production, such as the aberrant expression of the transcription factor GATA-3. Here we report our most recent results. Firstly, we found that CD8+ T-cell supernatants from SSc patients induce collagen production by normal skin fibroblasts and that this is inhibited by the addition of an anti-IL-13 antibody. Secondly, we established that increased numbers of CD8+ T cells expressing skin homing receptors and producing IL-13 are found in the peripheral blood of SSc patients compared to normal controls. Thirdly, we demonstrated that high numbers of CD8+IL-13+ T cells are present in the sclerotic skin of SSc, particularly in the early stages of the disease. Furthermore, we found that CD8+ T cells in the skin lesions of SSc patients express markers of cytotoxicity, such as Granzyme B, and are therefore potentially cytotoxic. We conclude that IL-13-producing CD8+ T cells are directly implicated in driving the pathogenesis of SSc. These new insights into disease pathogenesis suggest novel therapeutic targets that may be exploited for the treatment of SSc.

PS105

TOLL LIKE RECEPTOR 3: A CROSSROAD IN SCLERODERMA ETIOPATHOGENESIS

C.B. Chighizola[1,2], E. Raschi[1], L. Cesana[1], S.C. Uceda Renteria[2], M. Biggioggero[2], M.O. Borghi[1,2], P.L. Meroni[1,2]
[1]IRCCS Istituto Auxologico Italiano, Milan, ITALY; [2]University of Milan, Milan, ITALY.

Background. Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by excessive deposition of extracellular matrix (ECM) components, immune activation and neoangiogenesis. The pathogenesis of scleroderma is still poorly elucidated; however, an increasing burden of evidence suggests (Toll-like Receptor) TLR3 involvement. In SSc fibroblasts, polyinosinic-polycytidylic acid [poly(I:C)], the TLR3 synthetic agonist, was indeed shown to induce type I (interferon) IFN and transforming growth factor-β modulated genes and to be the only TLR ligand up-regulating endothelin-1 expression via IFN-γ. Furthermore, stimulation of lung fibroblasts with poly(I:C) induced myofibroblast differentiation, and ECM production via TGF-β and NF-κB. In fibroblast-like synoviocytes, TLR3 activation enhanced the expression of metalloproteinases and proangiogenic molecules. In our working hypothesis, nucleic acid-containing immune complexes (ICs) isolated from scleroderma patients bearing different autoantibody antigenic specificities might activate TLR3, thus inducing several mediators involved in SSc pathogenesis.
Aim. To characterize the role of TLR3 as potential mediator in the initiator phase of scleroderma.
Methods. Fibroblasts were isolated from skin biopsies obtained from healthy donors and cultured in adequate conditions up to the eight passage. ICs were purified using polyethylene glycol precipitation from sera of healthy controls and scleroderma patients carrying different autoantibody specificities (antibodies against centromere proteins, DNA topoisomerase I, RNA polymerase and Th/T0). Fibroblasts were transiently silenced for TLR3 using a specific small interfering RNA (siRNA); silencing was confirmed by RT-PCR and Western Blotting. TLR3-silenced and un-silenced cells were incubated with ICs of different sources or TLR agonists (poly(I:C) and LPS). Levels of TLR3 mRNA expression in different experimental conditions were analyzed by RT-PCR. Adhesion molecule (ICAM-1) expression was evaluated by cell-ELISA; interleukin (IL)-6 and IL-8 secretion in the supernatants was measured by commercial ELISA assays.
Results. Both mRNA and protein TLR3 levels were significantly reduced by specific silencing. The maximal up-regulation of TLR3 mRNA expression was observed at a poly (I:C) concentration of 1 μg/ml. ICAM-1 expression was significantly increased in cells treated with both TLR agonists and ICs from SSc patients but not healthy controls. Similarly, IL-6 and IL-8 secretion was elevated in the same experimental conditions. TLR3 specific silencing significantly affected ICAM-1, IL-6 and IL-8 levels.
Conclusions. Our data suggest that TLR3 activation by ICs isolated from SSc patients leads to fibroblast activation, with upregulation of adhesion molecule expression and pro-inflammatory interleukin secretion.
Further work is needed to better investigate TLR role as a potential mediator in SSc.

PS106

ENHANCED IL-8 PRODUCTION BY MONOCYTES IN SYSTEMIC SCLEROSIS PATIENTS WITH PULMONARY FIBROSIS

M.G. Santiago[1], S. Horta[2], M.J. Salvador[1], A. Paiva[2], J.A.P. Da Silva[1]
[1]Rheumatology Department,, Centro Hospitalar e Universitário de Coimbra - Hospitais da Universidade de Coimbra, Coimbra, PORTUGAL; [2]Centro do Sangue e da Transplantação de Coimbra - Instituto Português do Sangue e da Transplantação, Coimbra, PORTUGAL.

Background/Purpose. Substantial evidence supports the implication of immune-activated cells, cytokines and chemokines in the pathogenesis of systemic sclerosis (SSc). In fact, interleukin 6 (IL-6) and IL-8 play a crucial role in immunity and fibrosis, both key aspects of SSc. Recent evidence, suggests that an increase of activated circulating monocytes (Mo) on peripheral blood of SSc patients have a potential role on SSc pathogenesis. This could be the source of macrophages that accumulate in injured areas and are active producers of fibrosis-inducing cytokines.
Our purpose was to investigate the pattern of expression of IL-6 and IL-8 cytokines by peripheral blood Mo and to explore clinical associations.
Methods. This study included 43 SSc patients and 20 healthy controls (HC). All SSc patients fulfilled the American College of Rheumatology Criteria for the classification of SSc (limited cutaneous SSc (lSSc, n=30) or diffuse cutaneous SSc (dSSc, n=13), according to LeRoy et al.). A further subdivision was made, based upon the duration of disease, as early- (n=11) and late-stage (n=32), and these groups were individually compared with HC. A clinical evaluation was performed and registered.
Each participant was submitted to a blood sample collection, which was processed according to a protocol, designed to separately analyze the intracellular expression of IL-6 and IL-8 in Mo cells.
Data was statistically analyzed using the SPSS® version 20.0 for windows. Mann-Whitney U-test was used to evaluate differences between groups. Correlations between continuous variables were assessed by Spearman's correlation coefficient. P values < 0.05 were considered statistically significant.
Results. The mean age was 56.7±12.3 and 52.0±10.0 years for SSc patients and HC respectively. Females represented 79% of SSc and 80% of the control group. The mean disease duration was 9.4±8.3 years and the mean mRSS was 12.0±8.1. The frequency of circulating IL-6 and IL-8-producing Mo cells was not statistically different between SSc patients and HC.
The percentage of IL-8-producing Mo cells was significantly higher in patients with pulmonary fibrosis (p=0.009). No statistically significant differences were observed between early and late-stage SSc, concerning IL-6 and IL-8 expression among Mo. There were no significant association between disease subset, history of digital necrosis or mRSS and the frequency of IL-6 and IL-8 expression among Mo cells.
Conclusion. IL-8-producing Mo cells frequency is higher in SSc patients with pulmonary fibrosis. These findings support the hypothesis that IL-8 produced by these cellular type may be involved in the pathological process of SSc, regardless of the disease subset.

PS107

MOLECULAR ASPECTS OF IL-13 UP-REGULATION BY CD8+ T CELLS FROM SSC PATIENTS

S. Cascio[1], A. Moss[1], M. Jessup[2], D. B. Stolz[2], C. Milcarek[1], T. A. Medsger[3], P. Fuschiotti[1]
[1]Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, USA; [2]Cell Biology and Physiology Center for Biologic Imaging, University of Pittsburgh School of Medicine, Pittsburgh, USA; [3]Division of Rheumatology and Clinical Immunology, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, USA.

Systemic sclerosis (SSc) has the highest fatality rate among connective tissue diseases and is characterized by vascular damage, inflammation and fibrosis. T cells are important in pathogenesis and produce cytokines that contribute to the induction of fibrosis. We found previously that dysregulated production of the profibrotic cytokine IL-13 by effector CD8+ T cells is associated with more severe skin thickening in SSc, and defects in the molecular control of IL-13 production. We observed that total and naïve CD8+ T cells from the blood of SSc patients present an increased expression of the Th2-specific transcription factor GATA-3, and this is associated with augmented IL-13 production and specific clinical manifestations. Furthermore, silencing of GATA-3 with specific siRNA blocks IL-13 production in CD8+ T cells, demonstrating a causal relationship between GATA-3 and IL-13. GATA-3 is also highly expressed by CD8+ T cells in the sclerotic lesions of SSc patients, where it may be associated with the overproduction of IL-13. GATA-3 function is controlled at different levels, including by interactions with other nuclear proteins expressed in T cells. The Th1-specific nuclear factor T-bet induces IFN-γ production and inhibits Th2 cytokines, including IL-13, by antagonizing GATA-3 expression and/or function. Here we show that peripheral blood CD8+ T cells from SSc patients while expressing higher levels of IL-13 and GATA-3, still maintain similar levels of IFN-γ and T-bet compared to controls. Moreover, we found that the interaction between T-bet and GATA-3 in SSc CD8+ T cells is weaker compared to normal controls, which allowed more GATA-3 to bind to the IL-13 promoter and induce its expression. We conclude that increased IL-13 expression by SSc CD8+ T cells results, at least in part, from reduced down-regulation of GATA-3 by T-bet. Thus, our data provide new insights into SSc pathogenesis and will enable establishment of highly relevant biomarkers of immune dysfunction in patients with SSc that can be used as novel therapeutic targets for this currently incurable disease.

PS108

EXPRESSION OF THE TRANSCRIPTION FACTOR FORKHEAD BOX E3 (FOXE3) IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF PATIENTS WITH SYSTEMIC SCLEROSIS

E. Favoino, I. E. Favia, L. Digiglio, F. Perosa
Rheumatologic and Autoimmune Diseases Unit, University of Bari Medical School, Bari, ITALY.

Introduction. The process of epithelial (or endothelial)-mesenchymal transition (EMT) is at the basis of generation of renal and pulmonary fibrosis, and, in systemic sclerosis (SSc), has been regarded as one of the possible mechanism for accumulation of lymphocyte/monocyte-derived fibrocytes or myofibroblasts, which contribute to tissue fibrosis. Forkhead box E3 (FOXE3) is a transcription factor involved in EMT of lens epithelial cells (LEC). Its expression progressively decreases with the migration of LEC from the anterior to the equatorial region. FOXE3 expression cessation marks initiation of fiber differentiation. No data are available on FOXE3 expression in sites other than LEC. Therefore, in this study, we investigate FOXE3-expression in peripheral blood mononuclear cells (PBMC) of SSc patients, to eventually explore its potential role in the generation of lymphocyte/monocyte-derived fibrocytes or myofibroblasts, hence of tissue fibrosis.
Material and Methods. PBMC were isolated from heparinized peripheral blood of 10 patients with SSc and 7 healthy blood donors (HBD) by Ficoll-Hypaque density gradient centrifugation. Lymphocyte subsets (CD2+, CD19+) and monocytes (CD14+) were isolated by positive selection using microbeads. CD2+ cells (5x105 cells/ml) were stimulated with TGF-β (1μg/ml) and IL-6 (10 ng/ml) for 7 days. Total RNA was extracted and semi-quantitative PCR was performed to assess FOXE3 gene expression. The levels of FOXE-3 mRNA were quantified by normalizing its expression against that of GAPDH. Expression was measured as mean relative expression level (MREL). Variation of expression was measured as mean fold change (MFC).
Results. FOXE3 was expressed in CD2+, CD19+ and CD14+ cells from SSc patients and HBD. Specifically, expression level of SSc was similar to that of HBD in both CD19+ (MREL, SSc= 0.02; HBD= 0.08) and CD14+ (MREL SSc= 0.52; HBD=0.61) cells, while in CD2+ cells, the expression in HBD was higher (MREL=0.58) than in SSc patients (MRE 0.22). FOXE3 expression markedly increased following TGF-β stimulation in CD2+ cells from all HBD (MFC=1.43) and 5 SSc patients (MFC 1.94), whereas it decreased in CD2+ cells from the remaining 5 SSc patients (MFC = 0.71). IL-6 stimulation had no significant effect on FOXE-3 expression in CD2+ cells from both SSc patients and HBD.
Conclusion. This study has shown, for the first time, the FOXE3 expression in PBMC of SSc patients, and an heterogeneity in the expression level changes in SSc CD2+ cells following stimulation with TGF-β but not IL-6. Whether this heterogeneity parallels that of clinical manifestations remains to be determined.

PS109

ANTIBODIES ANTI-RO/SSA IN SYSTEMIC SCLEROSIS

I. Cavazzana[1], M. Fredi[1,2], M. Taraborelli[1,2], P. Airo'[1], A. Tincani[1,3], F. Franceschini[1]
[1]UO Reumatologia e Immunologia, Spedali Civili, Brescia, ITALY; [2]Cattedra di Reumatologia, Università degli Studi di Pavia, Pavia, ITALY; [3]Cattedra di Reumatologia, Università degli Studi di Brescia, Brescia, ITALY.

Antibodies to Ro are reported in systemic sclerosis (SSc), with a variable frequency from 3% to 11% when detected by immunoprecipitation assays and from 12% to 37% with ELISA or line immunoassay. Anti-Ro detected by ELISA have been associated with sicca, polymyositis and, more rarely, interstitial lung disease (ILD).
Methods. we studied the immunological repertoire of 444 patients affected by SSc, diagnosed using ACR and Le Roy criteria. Antinuclear antibodies have been detected by indirect immunofluorescence on HEp-2 cells. Antibodies to ENA were determined by counterimmunoelectrophoresis, using a rabbit thymus and spleen extracts as substrate.
Results. anti-Ro antibodies were found in 23 cases (5.1%), as isolated antibody in 11 patients (48%), while associated to anti-topoisomerase I, anti-U1RNP and anti-La in 6, 2 and 4 cases, respectively.
Anti-Ro+SSc showed a high female to male ratio (22:1), mean age at onset of 44 years (SD: 12) and a limited cutaneous SSc in 13 cases (56%). Digital ulcers and SSc active capillaroscopy pattern were found in 43.5% of cases. Calcinosis and joint anchilosis were rarely detected. By contrast 16 patients (69%) showed arthralgias with arthritis in 22%. Organ complications were diagnosed in high number of cases: esophageal involvement was diagnosed in 69% of patients, ILD in 52%, pulmonary hypertension in 17.4% of cases. Sicca symptoms were found in 17 patients (74%). Myositis, renal crisis were diagnosed only in one patient, each. Three patients died during follow-up, due to renal crisis, lung cancer and heart attack.
Comparing 23 anti-Ro+SSc with a sample of 100 anti-Ro negative SSc, we found that anti-Ro+ SSc showed a lower age at disease onset (44 vs 57.7 years, $p<0.005$), a lower rate of calcinosis (4% vs 32%, p:0.03) and a higher frequency of hypergammaglobulinemia (54% vs 16%, $p<0.005$, OR: 6.2, CI: 2-19).
When compared with 252 ACA+SSc, anti-Ro+SSc showed a higher rate of diffuse cutaneous type (43% vs 1%, $p<0.005$), a lower age at disease onset (44 vs 51.9 years, p: 0.013), higher rate of mortality (13% vs 3.6%), a significant higher rate of ILD (52% vs 8%, $p<0.005$, OR: 15.2, CI: 5-45) and a higher rate of pulmonary hypertension (17% vs 5.5%, p: 0.05). No significant difference was found with 125 anti-topoisomerase I+ patients.
Conclusions. anti-Ro antibodies are rarely found in SSc, usually associated with sicca, lung involvement and articular symptoms. Compared with ACA+SSc, they showed a more frequent pulmonary complications and earlier disease onset.

PS110

MEMORY (CD27+) B CELL IMPAIRMENT IS A CHARACTERISTIC FEATURE OF PATIENTS WITH SYSTEMIC SCLEROSIS AND PULMONARY FIBROSIS

L. Sakkas, A. Mavropoulos, T. Simopoulou, A. Varna, C. Liaskos, C. Katsiari, D.P Bogdanos
Department of Rheumatology, Faculty of Medicine, School of Health Sciences, University of Thessaly, Larissa, GREECE.

Introduction. B cells are likely to be involved in the pathogenesis of systemic sclerosis (SSc). Yet, the phenotypic and functional features of memory (CD27+) and naïve (CD27-) B cell sub-populations expressing the stimulatory CD19 or the inhibitory CD22 molecule have not been studied in detail.
Aim. The aim of the present study was to properly characterize the heterogeneous B cell subsets expressing CD19, CD22, and CD27 by flow cytometric analysis.
Methods. A total of 31 individuals were studied, including 14 SSc, 5 Sjögren's syndrome, 5 psoriatic arthritis patients and 7 normal controls (NCs). Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Hypaque procedures and the expression of CD19, CD22 and CD27 on B cells was examined by flow cytometry.
Results. CD19 mean fluorescence intensity (MFI) expression was significantly higher in naïve or memory B cells of untreated SSc patients, compared to treated SSc patients or controls (pathological or normal) ($p<0.05$). Memory B cells expressing CD19 or CD22 were significantly decreased in SSc patients compared to NC ($p<0.05$). Similar findings but to a lesser extent were also found in pathological controls. CD27–CD19+ (naïve B cells) were more frequent in SSc patients compared to controls (<0.01). The memory CD19+/naïve CD19+ and the memory CD22+/naïve CD22+ ratios were lower in SSc patients compared to controls. Significant loss of CD27+ expressing CD19+ and CD22+ B cells was a characteristic feature of SSc patients with lung involvement compared to SSc patients without lung involvement (pulmonary fibrosis) ($p<0.01$).
Conclusions. Our data demonstrate that patients with SSc, and in particular those with lung fibrosis, are characterized by a significant loss of memory B cells.

PS111

IRF8 GENE CONTRIBUTES TO DISEASE SUSCEPTIBILITY AND INTERACTS WITH NF-KB BY MODULATING INTERFERON SIGNATURE IN PATIENTS WITH SYSTEMIC SCLEROSIS

M.I Arismendi[1,2], M. Giraud[1], N. Ruzehaji[1], P. Dieudé[3], B. Ruiz[1], P. Airo[1], M. Matucci-Cerinic[5], G. Cuomo[6], E. Hachulla[7], P. Caramaschi[8], V. Riccieri[9], J. Avouac[1,10], C. Kayser[2], Y. Allanore[1,10]
1 Paris Descartes University, INSERM U1016, Institut Cochin, Sorbonne Paris Cité, Paris, FRANCE, 2 Federal University of Sao Paulo, Rheumatology Departament, Sao Paulo, BRAZIL, 3 Paris Diderot University, INSERM U699, Rheumatology department, Hôpital Bichat Claude Bernard, APHP, Paris, FRANCE, 4 Rheumatology and Clinical Immunology, Spedali Civili and University of Brescia, Brescia, ITALY, 5 Department of Biomedicine & Division of Rheumatology AOUC, University of Florence, Florence, ITALY, 6 Department of Clinical and Experimental Medicine, Rheumatology Unit, Second University of Naples, Naples, ITALY, 7 Université Lille II, Médecine Interne, Lille, FRANCE, 8 Rheumatology Unit, Azienda Ospedaliera Universitaria Integrata di Verona, Verona, ITALY 9 Division of Rheumatology, Dept. of Medical Clinic and Therapy, University Sapienza of Rome, Rome, ITALY, 10 Paris Descartes University, Rheumatology A department, Cochin Hospital, APHP, Paris, FRANCE.

Introduction. Systemic Sclerosis (SSc) is a polygenic autoimmune disease (AID) characterized by fibroblast dysregulation. It shares some genetic bases with other AIDs, as evidenced by autoimmune gene pleiotropism. Fibroblast dysregulation can be also observed in Primary Biliary Cirrhosis (PBC), another polygenic AID, which can be associated with SSc in the so called Reynold's Syndrome.

Objective. The present study was undertaken to investigate whether single nucleotide polymorphisms (SNPs) identified by a large GWAS in PBC might contribute to SSc susceptibility by a cross-disease approach.
Methods. Sixteen PBC susceptibility SNPs were genotyped in a total of 1,616 SSc patients and 3,621 healthy controls all of whom were of European Caucasian origin.
Results. We observed an association between PLCL2 rs1372072 (OR=1.23 [95% CI 1.12-1.33] Padj=7.22x10-5, NF-kB rs7665090 OR=1.16 [95% CI 1.06-1.25], Padj=0.01, and IRF8 rs11117432, OR=0.75 [95% CI 0.67-0.86], Padj=2.50x10-4 with SSc susceptibility. We subsequently queried associations according to the main subtypes and found that rs1372072 and rs11117432 were associated with the limited cutaneous subgroup (Padj=0.001 and Padj=0.003, respectively) and that rs7665090 was conversely associated with the diffuse cutaneous subset (Padj=0.007). We then looked for genotype – phenotype correlations by measuring mRNA expression of PBMC, obtained from patients (n=39) and controls (n=24), and observed that the IRF8 protective allele was associated with decreased IFIT1 expression reflecting type 1 interferon signature. We investigated gene interactions between the 3 associated SNPs that revealed an epistatic interaction between NF-kB and IRF8 SNP (OR=0.56 [95% CI 0.00-0.74], P=4x10-4). Interestingly, we observed that the effects of IRF8 and NF-kB were only observed in patients carrying the susceptibility allele from both genes.
Conclusion. By a cross disease approach querying pleiotropic genes, we identified 2 new susceptibility genes for SSc and confirmed IRF8 locus. We also identified functional effects of IRF8 variant affecting interferon signature and that an interaction between IRF8 and NF-kB genes might play a role in SSc susceptibility.