The four and a half LIM-domain protein 2 (FHL2) is an interacting partner of the beta-subunit of integrins as well as focal adhesion- and mitogen-activated protein kinases. FHL2 also carries an intrinsic activation domain and acts as a co-transcription factor with AP-1. Furthermore, FHL2 has been shown to interfere with mesenchymal cell activation. Here, we studied the expression of FHL2 in RA and OA synovial and analyzed its expression and function in RA and OA synovial fibroblasts (RASF and OASF). Using FHL2 specific antibodies, the expression of FHL2 was studied in synovial tissue samples obtained from 4 RA and 3 OA patients by immunohistochemistry. SF were isolated from these tissues and the expression of FHL2 was analyzed by Western blot analysis to compare the total amount of FHL2 in these cells. Migration assays on collagen and fibronectin matrices were performed to investigate the migratory behaviour of these cells. siRNA was used to knock down FHL2-expression in RASF, and FHL2-silencing was analysed by Western blot and real-time PCR. To investigate the influence of FHL2 on the invasive behaviour of RASF, FHL2 was silenced and RASF were stimulated with TNFalpha. We found a significant up-regulation of FHL2 in patients with the IRF5 SLE risk haplotype (T:C at the rs2004640:rs2070197 cluster) compared to OASF. Furthermore some of the cytokines associated with clinical manifestations of lupus. We found a positive association of nephritis and IL-2, negative association of arthritis with IP10 and IL-12 and a negative association of CNS lupus and MIP1α. The results obtained suggest a disease-specific up-regulation of FHL2 in patients with RA. Moreover, the data imply that FHL2 is involved in balancing the invasiveness and migratory behaviour of RASF. Further studies are needed to determine the functional role and the molecular basis of increased FHL2 expression in RASF.

**Aim:** The bone marrow has an important role in the development of B cell tolerance, the presentation of antigens to autoreactive lymphocytes and in homing the plasma cells that produce antibodies. Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the production of antibodies with specificity for a wide range of self-antigens. In view of the importance of bone marrow in B cell biology, we examined the microenvironment of the bone marrow in these patients by the use of DNA microarrays and multiplex cytokine assay.

**Patients and methods:** We isolated: a) bone marrow mononuclear cells (BMMCs) from 20 SLE patients, 12 with active (SLEDAI >8) and 8 with inactive (SLEDAI <8) disease and b) peripheral blood mononuclear cells (PBMCs) from 27 patients, 16 with active and 11 with inactive disease. We used in-house arrays representing 21,325 genes. We also performed 23-multiplex cytokine assay using serum from 50 SLE patients.

**Results:** Our analysis (unpaired student t-test) resulted in ~100 genes which are differentially expressed between bone marrow patients’ samples and controls. These genes represent several important biologic processes. 54 out of 100 differentially expressed genes are involved in 4 major networks including cell death, cancer, cell signalling and cellular growth and proliferation. Moreover, when the highly expressed genes were clustered, we identified 2 major SLE clusters in the bone marrow analysis but not in the peripheral blood. The first cluster represents patients with active disease and the second patients in remission. The upregulated genes in the bone marrow of active patients included genes involved in cell death and granulopoiesis. Comparative analysis of the bone marrow with the peripheral blood of SLE patients resulted in 88 genes; 61 out of 88 participate in various mechanisms that include cancer, cellular movement and morphology, immune response as well as functions of the hematopoietic system. In the multiplex cytokine assay, six cytokines were elevated in patients’ serum relative to controls: IL-1Rα (p = 0.0046), IP-10 (p = 0.03), IL-8 (0.0026), TNF-A (0.048), IL-15 (0.0097), and MCP-1 (0.0034). Furthermore some of the cytokines associated with clinical manifestations of lupus.

**Conclusion:** The microarray analysis of the bone marrow differentiated better the active from the inactive lupus patients and the patients from the controls. Further investigation of the bone marrow gene expression may reveal patient subgroups with different prognosis/response to therapy.
heritable SLE risk factor. There is a trend towards increased IFN-α production in the presence of the IRF5 haplotype that is associated with SLE risk, however this haplotype is found predominantly in certain ethnic backgrounds. High IFN-α expression is frequently found in SLE patients regardless of ethnic background, and it is likely that many genetic factors underlie this complex trait.

**154 GENETIC ASSOCIATION OF PROGRESSIVE SYSTEMIC SCLEROSIS (SSC) WITH PTPN22 POLYMORPHISMS**

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Recently, associations between type 1 diabetes, rheumatoid arthritis as well as several other autoimmune diseases (AIDs) and the PTPN22 single nucleotide polymorphism (SNP) 1858C→T were discovered. PTPN22 (encoding protein tyrosine phosphatase, non-receptor type 22) located on chromosome 1p13 has 21 exons spanning 58 kb. The variant 1858C→T in codon 620 results in the exchange of Arg to Trp (R620W). PTPN22 (also known as Lyp or Pep) is expressed primarily in lymphoid tissue, and most probably involved in the negative regulation of T cell activation via interaction with the protein tyrosine kinase Csk. It was suggested that the mutation 620W may interfere with the interaction between Lyp and Csk. However functional data comparing homozygous and heterozygous 620W carriers are not yet available. We collected DNA from 177 patients with SSC and 184 healthy blood donors (HD). Samples were analyzed for 13 SNPs covering PTPN22, including 1858C→T (rs2476601). SNPs were selected to represent the most common haplotypes. The analysis was performed by PCR, single base extension and MALDI-TOF mass spectrometry. Among HD, the allele frequency of 1858T was 9.0%, similar to published data. Genotype frequency of 1858T/T was 0.5%. Among SSC patients, the allele frequency of 1858T was 12.4%, genotype frequency of 1858T/T was 2.8%. The difference in allele frequencies did not reach statistical significance, however, the difference in genotype frequencies was significant (p = 0.01, genotype relative risk test: Lathrop. Tissue Antigens 1983;32:160–6). Data concerning subgroups of patients, additional polymorphisms and the other 4 major haplotypes of PTPN22, together accounting for about 98.5% of detectable variants, will also be discussed. So far, associations observed in AIDs with PTPN22 always only concerned a subpopulation of patients only a subset of the patients carried the disease associated variant. For PTPN22, maximally 20% of AID patients showed to carry the allele 1858T and even less carried the disease associated variant. For PTPN22, maximally 20% of AID patients showed to carry the allele 1858T and even less carried the allele 1858T/T was 2.8%. The difference in allele frequencies did not reach statistical significance, however, the difference in genotype frequencies was significant (p = 0.01, genotype relative risk test: Lathrop. Tissue Antigens 1983;32:160–6). Data concerning subgroups of patients, additional polymorphisms and the other 4 major haplotypes of PTPN22, together accounting for about 98.5% of detectable variants, will also be discussed. So far, associations observed in AIDs with PTPN22 always only concerned a subpopulation of patients only a subset of the patients carried the disease associated variant.

**155 ASSOCIATION OF ANTI-CCP ANTIBODIES AND YKL-40 LEVELS WITH RADIOGRAPHIC OUTCOME OF ETANERCEPT TREATED PATIENTS WITH RHEUMATOID ARTHRITIS**

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Radiographic progression is a main outcome measure in rheumatoid arthritis (RA). Anti-CCP antibodies which are highly specific to RA have been described as being a good predictive marker for distinguishing between erosive and non-erosive RA. YKL-40, the human cartilage glycoprotein 39, has been identified as a biomarker for articular cartilage degradation. We conducted this study to monitor radiographic changes in etanercept (ETN) treated RA patients in relation to the CCP antibody levels and serum concentrations of YKL-40 and to evaluate the diagnostic importance of these parameters. Clinical and radiological characteristics of 54 patients (11 male, 43 female, mean age 51.8±11.5 years) who met the ACR criteria for rheumatoid arthritis with a mean disease duration of 10.3±8.5 years and who had received between 1–4 different D-MARDs were included in the study. Patients were treated with ETN twice weekly at 25 mg. At study entry and at the one year follow up, the modified total Sharp scores (TSS), RA-associated joint space narrowing and erosions were determined on hand and foot radiographs. To compare the radiographic progression of patients with different disease duration the mean yearly progression rates (TSS/year, erosion score/year, JSN score/year) were calculated. Serum levels of anti-CCP antibodies and YKL-40 at the start of ETN treatment were analysed by ELISA (Immunoassan RA Mark 2, Euro-Diagnostika, Netherlands and METRA YKL-40 EIA Kit, Quidel, USA, respectively) according to the manufacturer’s instructions. At the start of treatment a mean TSS of 53.9±6.2, a mean erosion score of 17.7±2.9, and a mean JSN score of 36.5±4.0 were determined. In the course of ETN treatment the mean value increased slightly without reaching statistical significance. Calculated for the mean yearly progression rate (TSS/year) in 54 patients at study entry was 6.30±0.84, the erosion score/year 2.18±0.41 and JSN score/year 4.17±0.57. In 40 out of 53 patients (75.5%) anti-CCP antibodies were detected, 13 patients were negative. The group of patients with anti-CCP antibody levels >1000 U/ml (n = 20) had a higher mean yearly progression rate (TSS/year) in 54 patients (13 patients) at study entry was 6.30±0.84, the erosion score/year 2.18±0.41 and JSN score/year 4.17±0.57. In 40 out of 53 patients (75.5%) anti-CCP antibodies were detected, 13 patients were negative. The group of patients with anti-CCP antibody levels >1000 U/ml (n = 20) had a higher mean yearly progression rate (TSS/year) in 54 patients (13 patients) at study entry was 6.30±0.84, the erosion score/year 2.18±0.41 and JSN score/year 4.17±0.57.
EVALUATION OF OXIDATIVE STRESS MARKERS AFTER SIMVASTATIN SHORT-TERM THERAPY IN SCLERODERMA

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Aim: The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used in first-line management of cardiovascular diseases because of their efficacy in improving lipid profiles and direct vascular actions. Recent studies demonstrated that statins also possess pleiotropic immunomodulatory and anti-inflammatory capabilities, such as downregulation of the expression of adhesion molecules, of monocyte chemotactic protein-1 (MAC-1) on leucocytes and endothelial cells, and the ingress of leucocytes into inflammation sites. Statins also scavenge free radicals and block the induction of nitric oxide synthesis. They are thought to be beneficial against autoimmune disorders, especially those characterized by ingress of activated leucocytes into the skin such as SSc. The aim of the present study was the evaluation of endothelial response of a 2 months treatment with simvastatin in patients affected by SSc.

Materials and methods: 15 patients (all female; median age: 59) affected by SSc according to ACR criteria (1980) were enrolled at the division of Rheumatology of the University of Pisa. They were treated with simvastatin (10–20 mg/day PO according to their cholesterol LDL serum level) for 2 months. Fasting blood samples were drawn in the morning at baseline and after 2 months treatment. Lipid profile markers were determined: total cholesterol and HDL-cholesterol with an enzymatic colorimetric assay, triglycerides, apolipoprotein A1 and apolipoprotein B with a nephelometric assay, homocysteine with a polarized light immunofluorescent assay (ELISA). The markers were determined in the lipid peroxide, the malondialdehyde (MDA) with a colorimetric assay for lipid peroxidation, the ferric reducing ability of plasma (FRAP). We also evaluated basal biochemical markers to assess therapy toleranceability (hemoglobin, plaques, CPK, transaminases, nitrogen, creatinine). Clinical parameters (total skin thickness score (modified Rodnan TSS), activity score, severity score, nail capillaroscopy) were measured in SSc patients.

Results: At baseline SSc patients showed altered serum levels of the following parameters: total cholesterol, LDL cholesterol, HDL cholesterol, LPO, MDA, FRAP. The following correlations were found at baseline: severity score vs MDA (r = 0.0014), activity score vs LPO (r = 0.0049) and vs MDA (r = 0.017). After two months treatment we observed significantly reduced levels of total cholesterol (r = 0.0012). After therapy we also observed slightly decreased levels of LPO (3.299 ± 0.554 with respect to 3.555 ± 1.532), MDA (4.471 ± 0.914 with respect to 5.504 ± 0.953), and increased levels FRAP (661.5 ± 30.67 with respect to 610.3 ± 25.89).

Conclusions: The statistically significant reduction of total cholesterol values has confirmed the efficacy of simvastatin therapy. The positive correlations between severity score vs MDA and between activity score vs LPO and MDA suggest that the oxidative stress is related to the development of SSc. So statins with their antioxidant properties should be useful in SSc.

SKIN ULCERS IN SYSTEMIC SCLEROSIS: POSSIBLE ROLE OF INFLAMMATORY AND ANGIOGENETIC FACTORS

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Aim: Skin ulcers represent one of the disabling organ manifestation in systemic sclerosis patients (SSc). In this study we addressed the issue of some possible factors that may have a role in the development of skin ulcers in a large cohort of patients with SSc.

Patients and methods: 119 patients with SSc (mean age 56.9 SD 12.0 years, mean disease duration 8.5 SD 8.0 years, 14 males and 105 females), 29 with diffuse (dcSSc) and 90 with limited (lcSSc) skin involvement were enrolled in a cross-sectional study. 31 (26%) patients had major ulcers (MLED > 15) at the moment of their admission at the rheumatology unit. The same therapeutic approach was used for all the patients with skin ulcers. The plasma levels of sTNFRII, IL-6, HGF, IL-13, VEGF, VEGF-R1 and VEGF-R2 were determined by an ELISA procedure in all SSc patients at baseline.

Results: Patients with ulcers differed from those without for having diffuse skin involvement (p = 0.0012) and having higher systemic inflammation as shown by CRP (p = 0.03), fibrinogen (p = 0.02), sTNFRII (p = 0.0013) and IL-6 (p = 0.013). Patients with ulcers did not differ from patients without ulcers for VEGF, VEGF-R1 and VEGF-R2 plasma levels. Similarly, no difference was seen between patients with diffuse and limited skin involvement.

Analyzing the subgroup of patients with ulcers, we found higher plasma levels of VEGF in patients with major lesions (156.8 SD 51.2 pg/ml) versus patients with minor ulcers (76.7 SD 68.7 pg/ml, p = 0.02) and without (101.1 SD 75.9 pg/ml, p = 0.01 vs major ulcers). There was no difference in plasma levels of VEGF-R1 and VEGF-R2 between patients with major and minor ulcers. Patients with major lesions differed from patients with minor ulcers for higher VEGF/VEGF-R1 (52.6 SD 62.3 vs 3.5 6.8, p = 0.001) and VEGF/VEGF-R2 (0.03 SD 0.006 vs 0.03 SD 0.011, p = 0.005) ratios. Patients without skin ulcers had similar VEGF/VEGF-R1 and VEGF/VEGF-R2 (10.4 SD 20.0 and 0.017 SD 0.014, respectively) ratios compared to patients with minor ones. Concerning IL-6 and sTNFRII, there was no difference between their plasma levels in patients with major or minor ulcers. Considering the inflammatory parameters, we found a positive correlation between VEGF-R1 and IL-6 plasma levels (r = 0.32, p = 0.01) and a negative correlation between VEGF-R1 and both IL-6 and TNF-R1 plasma levels (r = -0.44, p = 0.0005 and r = -0.27, p = 0.04, respectively). There were no differences in the plasma levels of HGF and IL-13 between the two groups of patients with and without ulcers and in the two subgroups with limited and diffuse skin involvement.

Conclusions: The aetiology of skin ulcers in SSc is multifactorial. Inflammatory status and deficiency in angiogenetic factors may both contribute to skin ulcerative lesions appearance. Higher ulcerative process seems to be characterized by a defective angiogenetic stimulus rather than an inflammatory stress.

LYMPHANGIO-GENESIS IN RHEUMATOID SYNOVITIS: HISTOPATHOLOGICAL AND CLINICAL CORRELATES

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Background: Lymphatic vessels are involved in the regulation of inflammatory responses by transporting leukocytes from peripheral tissues to draining lymph-nodes. Lymphatic vessels can proliferate in response to inflammation, conditioning the degree of tissue flogosis and the magnitude of T-cell responses. Yet, whether lymphatic vessel amplification occurs within the inflamed synovium in rheumatoid arthritis (RA) and the functional consequences of this phenomenon are unknown.

Materials and methods: Synovial samples were obtained from 60 RA patients undergoing synovial biopsy, synovectomy or joint replacement. Clinical and laboratory parameters were recorded. Control materials consisted of 2 normal and 14 osteoarthritic synovial samples. Lymphatic vessels were investigated by immunohistochemistry and immunofluorescence for the specific markers LYVE-1 and podoplanin, and the median lymphatic vessel density (MLVD) was quantified as number of LYVE-1+ podoplanin+ structures per medium-power field. Ki-67 served as proliferation marker. Expression of the lymphatic decay receptor D6 was explored. Endothelial expression of lymphoid chemokines, characterization of infiltrating immune-cells and lymphoid neogenic features were assessed by flow-cytometry. The immunohistochemical classification was follicular/diffuse and quantified with a semiquantitative score (0–3).

Results: In the majority of RA tissues, the MLVD was very low (MLVD 0.1 ± 0.2), and lymphatic vessels were predominantly located in the fibrous synovium of the joint capsule. Control tissues exhibited this same lymphatic density and distribution. By contrast, significant lymphatic amplification was observed in the superficial and deep sublining in 25% of RA samples (MLVD 3.1 ± 1.5), where numerous lymphatics were proliferating (Ki-67+). In samples exhibiting lymphangio-genesis, most of LYVE-1+podoplanin+ lymphatics were also D6+, whilst D6 immunoreactivity was weak/absent in tissues with low MLVD. Lymphatic vessels constitutively expressed CCL12 and CXCL12, and CCL21 appeared up-regulated in samples with lymphangio-genesis. In areas of dense cellular infiltration, some lymphatics contained mature dendritic cells, T and B lymphocytes. High MLVD tissues were characterized by higher inflammatory scores (2.5 ± 0.8 vs 1.7 ± 0.8; p = 0.002). Lymphoid aggregates in tissue areas with densely distributed lymphatics tended to be less organized and lacked typical lymphoid-neogenic features. Patients exhibiting synovial lymphangio-genesis presented longer disease duration (185 ± 88.5 vs 107.1 ± 90.1 months; p = 0.02) and higher median values of C-reactive protein (3.6 vs 1.1 mg/ dl; p = 0.019).

Conclusions: Our results demonstrate that: 1) the lymphatic network can proliferate in the inflammatory environment of RA synovitis, at least in disease subsets/stages or tissue areas; 2) through up-regulation of the scavenger receptor D6, synovial lymphatics may contribute to the control of local inflammation; 3) synovial lymphatics can mediate immune-cell recirculation through the expression of specific chemokines; 4) the
development of a synovial lymphatic network may condition the topographical arrangement of immune-infiltrating cells. Lymphangiogenesis regulation thus appears an attractive target of future pharmacological research in RA.

**REDUCTION OF HAEMOSTATIC AND INFLAMMATORY BiomARKERS BY TUMOR NECROSIS FACTOR-ALPHA BLOCKADE IN PATIENTS WITH RHEUMATOID ARTHRITIS**


**Objective:** It has long been recognized that rheumatoid arthritis (RA) is associated with increased cardiovascular risk, activation of coagulation and inflammatory pathways. Treatment with Infliximab, a chimeric monoclonal antibody (mAb) to tumor necrosis factor-alpha (TNF-α) has been shown to reduce inflammation and improve clinical and laboratory measures of disease activity in RA. To date during this treatment the relationship between coagulation and inflammation has not been explored. The aim of this study was to investigate whether the haemostatic and inflammatory biomarkers were modified by administration of Infliximab in RA patients.

**Methods:** Eleven patients with active RA and 50 healthy controls (HC) were included. RA patients had been taking a stable dose of methotrexate of at least 10 mg/week and Infliximab (3 mg/kg) at week 0, 2, 6 and 14. At baseline and prior to the infusion at week 14 the following parameters were determined: disease activity score (DAS28), General Health Questionnaire (GHQ), visual analogue scale (VAS) pain, hemoglobin concentration, erythrocyte sedimentation rate (ESR), plasma levels of C-reactive protein (CRP), TNF-α, IL-6, prothrombin fragment 1+2 (F1+2) and D-dimer.

**Results:** At baseline, ESR, levels of CRP and IL-6 were significantly higher in RA patients than in HC (p = 0.0001), and levels of TNF-α tended to be increased; also F1+2 and D-dimer levels were significantly higher (p = 0.0001). After 14 weeks of Infliximab treatment, there was a significant clinical and laboratory improvement as judged by a decrease in the DAS28 (p = 0.0001), GHQ (p = 0.001), VAS pain (p = 0.002), number of swollen (p = 0.003) and tender joints (p = 0.001). Similarly, an increase of hemoglobin concentration (p = 0.02), and a decrease of ESR (p = 0.03), CRP (p = 0.02) and IL-6 (p = 0.05) were detected. F1+2 and D-dimer levels significantly decreased during the course of the study (p = 0.05).

**Conclusions:** We show that administration of anti-TNF-α mAb in RA patients results in a rapid clinical improvement and a decrease of both acute-phase proteins and haemostatic biomarkers. The reduction of prothrombotic biomarkers indicates that Infliximab treatment may reduce the thrombotic risk in RA patients.

**INFLAMMATION-INDUCIBLE ANTI-TNF GENE EXPRESSION BY INTRA-ARTICULAR INJECTION OF SEROTYPE 5 ADENO-ASSOCIATED VIRUS REDUCES ARTHRITIS**

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**Aim:** Tumor necrosis factor (TNF)-α plays a central role in rheumatoid arthritis (RA) and current biotherapies targeting TNF-α have a major impact on RA treatment. The long-term safety concerns associated with the sustained systemic immunosuppression due to the TNF-blockade prompted us to optimize therapeutic anti-TNF approaches. We recently demonstrated that intrarticular gene transfer using a recombinant adeno-associated virus serotype 5 (rAAV5) is far more efficient than the other serotypes for transgene expression in arthritic joints. In the present study we aimed at combining our optimized vector for local delivery to arthritic joints and regulated therapeutic gene expression to address the concern of safe and local gene therapy in RA.

**Methods:** A recombinant adeno-associated virus serotype 5 (rAAV5) encoding either the TNFR1-mlgG1 fusion protein (TR1) or a dimeric sTNFR2 (TR2) under a CMV or a NF-κB-responsive promoter was used to neutralize TNF-α in both human primary RA synovial fibroblasts and collagen-induced arthritis (CIA) model. DBA/1 mice were immunized with collagen type II, boosted 21 days after and 1.5-10⁷ particles of rAAV5 were injected in both knee joints 2 days after. Clinical course of the disease was assessed by paw thickness measuring over time, radiological and histological scores were obtained at euthanasia. The cytokine profiles were measured by ELISA in sera and knee-joint conditioned media. The immunological balance was also assessed using anti-type II collagen assays.

**Results:** The intra-articular gene transfer of both rAAV5-CMV-TR1 and rAAV5-CMV-TR2 vectors resulted in delayed disease onset (46.5 ± 0.7 and 48 ± 0 vs 38.2 ± 2.7), decreased incidence (44.5 and 12.5 vs 100%) and severity of joint damage compared with control group, the TR1-expressing vector being therapeutically more efficient (p = 0.05). Importantly, when TR1 was expressed under the NF-κB-responsive promoter, the therapeutic effect was associated with a transient expression of the anti-TNF molecule, only detectable during disease flares, while the antagonist was rapidly, highly and stably expressed for nine weeks when delivered by a CMV-driven vector.

**Conclusion:** These findings strongly support the feasibility of improving the anti-TNF approach for the treatment of arthritis by local rAAV5-mediated gene expression under an inflammation-responsive promoter, able to provide a regulated, transient and therapeutically efficient dose of an immunomodulatory molecule.

**ANTIBODIES AGAINST TYPE II COLLAGEN (CII) ARE ASSOCIATED WITH DESTRUCTION AT RA ONSET, WHEREAS RF AND ANT-CCP RELATE TO LATE ACCELERATION OF THE DESTRUCTIVE PROCESS**

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**Aims:** We have recently shown that anti-CII are associated with accelerated destruction during the first two years of RA, as well as with late appearance of extended inflammation. Anti-CII antibodies on the other hand associate with acute inflammation at disease onset (Mullazehi et al Ann Rheum Dis in press), and this is probably due to cytokine stimulation by solid phase immune complexes at the cartilage surface (Mullazehi et al, Arthritis Rheum 2006;54:1759). Both anti-CII and anti-CCP define phenotypically distinct populations of RA patients that are inversely related. We therefore hypothesized that these two autoantibodies might characterize patient groups with divergent radiological outcome.

**Materials and methods:** 265 early RA patients (189 women and 76 men; mean age 56 years, range 18-85) were enrolled. Identical radiographs of the hands and feet from inclusion, 1 and 2 years were scored blindly according to Larsen. Rate of change was expressed as Larsen score between the investigated time points. RF was measured with nephelometry, and anti-CCP and anti-CII with ELISA.

**Results:** As we have earlier shown, anti-CCP positive patients had significantly higher Larsen score between baseline and 2 years compared to anti-CII negative patients (p = 0.0080). The difference was even stronger during the second year (p = 0.0001). Figures for RF were comparable but somewhat weaker (p = 0.0192 and 0.0010, respectively). Anti-CII positive patients showed increased baseline Larsen score (p = 0.0472) when only the very high anti-CII positive sera associated with immune complex-induced cytokine production in vitro were considered.

**Conclusions:** Serologically defined groups of early RA patients have divergent prognosis concerning future and present radiological destruction. Patients with very high anti-CII levels present both with acute inflammation and more radiological destruction at the time of diagnosis, probably due to local immune complex stimulation at the cartilage surface.

**P38MAPK ISOFORM EXPRESSION AND ACTIVATION IN ANCA-ASSOCIATED GLOMERULONEPHRITIS**

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Introduction: Anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitits (AAVS) is a life-threatening autoimmune disease and renal involvement presenting as crescentic glomerulonephritis is a major predictor for poor outcome in patients with AAVS. Activation of pro-inflammatory cytokines such as TNF potentially contributes to renal damage by activation of pro-inflammatory signal transduction pathways and perpetuation of inflammation. Indeed, TNF-blockade by anti-TNF antibody can be effective in inducing remission in patients with ANCA-associated glomerulonephritis. p38MAPKs and its downstream mediators play a major role in the regulation of inflammation and are activated by pro-inflammatory cytokines such as TNF. Additionally, p38MAPK inhibitors have been shown to inhibit renal inflammation in animal models of crescentic glomerulonephritis. However, it is unclear which of the four known p38MAPK isoforms (alpha, beta, gamma and delta) are expressed, activated and hence of major importance in ANCA-associated glomerulonephritis.

Methods: Histological sections of renal biopsy specimens of patients with ANCA-associated glomerulonephritis and healthy controls (living kidney donors) were investigated for the glomerular expression and phosphorylation of p38MAPK isoforms and MAPKAPK2 protein by immunohistochemistry. Further, we analysed the mRNA and protein expression of p38MAPK isoforms in a human podocyte cell line and determined p38MAPK isoform activation in podocytes upon activation with TNF.

Results: Immunohistochemical stainings revealed expression of p38 alpha, beta and gamma isoforms in glomerular podocytes, whereas glomerular epithelial cells predominantly expressed p38 gamma isoform. Activation of both p38MAPK and its downstream mediator MAPKAPK2 were seen in AAVS but not control kidneys. Phosphorylation was localized to glomerular podocytes, inflammatory infiltrates and glomerular crescents but not glomerular epithelial cells. Interestingly, kidney biopsies of pre-treated vs. untreated AAVS patients showed significantly less p38MAPK activation. Densitometry of the histostainings showed glomerular expression localization of phosphorylated p38 protein with p38 alpha, beta and gamma isoforms. Next, we determined p38MAPK expression in a podocyte cell line and found expression of all 4 p38MAPK isoforms by RT-PCR and immunoblotting. However, upon activation with TNF, we found predominant activation of p38 alpha, weak activation of p38 gamma and delta and no activation of the beta isoform in vitro.

Discussion: This study shows, that p38 alpha, beta and gamma isoforms are predominantly expressed in vivo in glomeruli of patients with ANCA-associated glomerulonephritis and co-localize with phosphorylated p38MAPK protein. In vitro, activated podocytes predominantly show phosphorylation of p38 alpha.

Comparative Analysis of FOXP3 and CD25 in Target Organ and Circulation of Patients with Chronic Arthritis

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Background: Thymus derived regulatory T cells constitute a unique lineage of immune cells that are fundamental in control of autoimmunity in normal hosts. Co-expression of CD4 and CD25 (IL-2Ra) on the cell surface has allowed for isolation and functional characterization of regulatory T cells in vitro. In humans regulatory T cells are defined as CD4+ T cells with the highest surface density of CD25 (CD4+CD25bright). However, at the site of inflammation, e.g. in the rheumatic joint, CD25 is also upregulated on activated T cells making it difficult to clearly distinguish activated from regulatory T cells. The identification of the forkhead transcription factor sSrfin (encoded by FOXP3 in humans) expressed specifically in regulatory T cells has made it possible to distinguish them from activated CD25+ T cells. We investigated the longitudinal pattern of FOXP3 expression in regulatory T cells in paired peripheral blood and synovial fluid of patients with inflammatory arthritis.

Methods: Mononuclear cells were isolated from paired peripheral blood and synovial fluid from 9 patients at several relapses. Intracellular FOXP3 staining was carried out and analyzed by flow cytometry.

Results: Our data demonstrate that FOXP3 expression is most abundant in the CD4+CD25bright T cell population independent of site of sampling (blood or synovial fluid). Interestingly, the frequency and the amount of FOXP3 among the CD4+CD25+ and CD4+CD25+ T cells was significantly elevated in the synovial fluid compared to peripheral blood (p<0.0001 and p<0.001 respectively). Our results indicate that at the site of inflammation FOXP3 is induced on CD25- T cells or CD25 is down-regulated on natural regulatory T cells, while still retaining FOXP3 expression. To support the latter hypothesis we could detect significantly increased amounts of sCD25 in the serum of patients with arthritis compared to healthy controls. A striking observation was that the frequency of CD4+CD25bright expressing FOXP3 in synovial fluid T cells varied greatly between individual patients (range 41–91%) compared to the counterparts in circulation (range 74–96%). Furthermore, low/high phenotype of FOXP3 expression among the synovial fluid mononuclear CD4+CD25bright T cells was consistent over time.

Conclusion: In summary, FOXP3+ T cells were detected at the site of inflammation of patients at each relapse. The question still remains why in spite of the presence of these potent suppressor T cells at the site of inflammation, symptomatic RA still persists in the patients. Further studies are in progress to correlate the level of FOXP3 to the disease activity in the patients at the time of sampling.

Novel Use of Rituximab in Lupus Pneumonitis

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Rationale: Rituximab is a chimeric monoclonal antibody that targets the CD20 B cell surface antigen. We report the first use of rituximab to treat a Systemic Lupus Erythematosus (SLE) and secondary Sjögren’s syndrome (SS) patient who had developed refractory lupus pneumonitis.

Methods: A 29-year-old White woman with a 5-year history of SLE/SS presented with complaint of shortness of breath. His initial chest CT was significant for pneumonitis. The patient was treated with high dose intravenous steroids and was discharged from the hospital on prednisone and hydroxychloroquine. At
follow up the patient continued to complain of moderate dyspnea on exertion and pulmonary function tests (PFTs) revealed a restrictive defect with impaired gas exchange (FEV1 49%, FVC 51%, DLCO 42%). He did not respond to the addition of mycophenolate mofetil, and refused treatment with intravenous cyclophosphamide. The patient agreed to rituximab treatment, and was infused with 1000 mg of rituximab at weeks 0 and 2.

Results: Patient returned to clinic one week after second infusion and reported increased activity with resolution of dyspnea. Clinical improvement was confirmed by PFT findings. FVC, FEV1, and DLCO all improved; 74%, 66%, and 59% respectively.

Conclusions: Rituximab may be a valid alternative in the management of autoimmune disorders, especially in cases where the adverse effect profiles of traditional immunomodulators are not acceptable or well tolerated. We report what we believe to be the first successful use of rituximab for the treatment of refractory pulmonary disease in a patient with SLE/SS.

167 PROTEOMIC ANALYSIS OF HUMAN WHOLE SALIVA IN SJÖGREN SYNDROME

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Background: Sjögren’s syndrome is a chronic autoimmune disease which involves typically the lachrymal and salivary glands with changes in gland structure and function that might be reflected in the composition of salivary fluid.

Aims: To compare the protein patterns of saliva of patients affected by Sjögren’s syndrome with healthy donors, using a proteomic approach.

Materials and methods: Saliva samples were obtained from eleven patients with SS and eleven healthy donors with similar mean age and demographic characteristics. Saliva samples were centrifuged to remove undissolved materials. Aliquots of supernatants were mixed with rehydration solution and subjected to isoelectrofocusing in Immobiline Dry Strip, pH 3–10. Before the second dimension, dry strips were equilibrated in two steps in equilibration buffer and processed in second dimension using acrylamide gel (12%) applying a continuous buffer system. Gels were stained with silver and images were analysed with ImageMaster2D Platinum. Protein spots from each gel were detected, edited manually and matched automatically. The differentially expressed proteins in patients were identified with peptide mass fingerprinting.

Results: We obtained the resolution of 260 spots approximately. In comparison to healthy donors, the samples of patients with Sjögren’s syndrome showed a reduction of some of the typical salivary proteins. In particular, a remarkable reduction up to the total absence of carbonic anhydrase VI was seen in Sjögren’s syndrome samples with respect to control. Moreover a set of differentially expressed proteins were detected, related both to inflammation and to oxidative stress injury.

Conclusions: This study has shown many qualitative and quantitative differences in the protein patterns of saliva in Sjögren’s syndrome in comparison to normal controls which might be related both to the autoimmune inflammatory involvement of salivary glands and to the tissue damage. Nonetheless further studies are necessary to confirm our preliminary results and to better understand their potential clinical and pathogenetic implications.

168 ANTIBODIES AGAINST CITRULLINATED VIMENTIN IN RHEUMATOID ARTHRITIS: HIGHER SENSITIVITY AND EXTENDED PROGNOSTIC VALUE CONCERNING FUTURE RADIOLOGICAL PROGRESSION AS COMPARED TO ANTI-CCP

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Aims: Citrullinated vimentin can be found in inflamed RA synovium, and at least part of the humoral RA-specific anti-Sa response is directed against citrullinated vimentin. We have evaluated the sensitivity, specificity and prognostic value of determination of antibodies against citrullinated vimentin (anti-MCV) as compared to antibodies against cyclic citrullinated peptides (anti-CCP) in an early RA inception cohort.

Methods: 273 early RA patients were followed with clinical investigations, radiographs and measurement of anti-MCV and anti-CCP antibodies at baseline and after three months, 1, 2, 3 and 5 years. Autoantibodies were also analyzed in 100 healthy controls.

Results: 70.7% [119/173] of the patients were anti-MCV positive and 57.9% [118/203] were anti-CCP positive at the time of diagnosis, with equal specificities (95% and 96%, respectively). 14.7% [40/273] were anti-MCV positive only and 1.8% [5/273] were anti-CCP positive only. Anti-MCV positive and negative patients had similar disease activities at baseline, but presence of anti-MCV was predictive of subsequent high disease activity and continued radiological progression. Changes in anti-MCV showed stronger correlation to changes in clinical parameters than changes in anti-CCP. The anti-MCV-positive and anti-CCP-negative subgroup showed a higher rate of radiological destruction as compared to patients negative for both anti-MCV and anti-CCP.

Conclusions: Analysis of anti-MCV yields a greater sensitivity together with unchallenged specificity as compared to anti-CCP in early RA. As compared to anti-CCP, anti-MCV also defines an extended group of early RA patients with poor radiological prognosis.

169 OPSONIZATION OF LATE APOPTOTIC CELLS BY SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) AUTOANTIBODIES INHIBITS THEIR UPTAKE VIA AN FC\R-DEPENDENT MECHANISM

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Objective: Decreased clearance of apoptotic cells is suggested to be a major pathogenic factor in systemic lupus erythematosus (SLE). We investigated whether binding of SLE-autoantibodies to apoptotic cells influences phagocytosis of these cells by macrophages.

Methods: ApoCytosis was induced in human T-Cell (Jurkat) and keratinocyte (HaCaT) cell lines by UVB exposure. Binding of purified IgG from 26 SLE patients and 15 controls to apoptotic cells was assessed by flow cytometry and western blotting. Phagocytosis of IgG-opsonized apoptotic cells by monocyte-derived macrophages (MDM) was assessed by light microscopy. Similar experiments were performed with a monoclonal antibody against SSA/Ro and IgG fractions from patients with Sjögren’s syndrome (n = 5) and rheumatoid arthritis patients (n = 5).

Results: IgG fractions from all 26 SLE patients bound to late apoptotic (LA) but not to early apoptotic (EA) cells. IgG fractions isolated from SLE patients with different autoantibody profiles showed comparable levels of binding. Control IgG did not bind. Oposonization of apoptotic cells with IgG fractions from SLE patients resulted in a significant inhibition of phagocytosis as compared to control IgG fractions. A monoclonal antibody directed against SSA/Ro and IgG isolated from 5 ANA-positive patients with Sjögren’s syndrome were also able to elicit these effects, whereas IgG from 5 ANA-negative patients with rheumatoid arthritis did not. The inhibitory effect of patient IgG was abolished by blocking either FcR receptors (FcR) or the constant region of IgG, using a specific Fc-blocking peptide.

Conclusion: Autoantibodies from SLE patients are able to opsonize apoptotic cells and inhibit their uptake by macrophages via an FcR-dependent mechanism.
the Dutch RA families, the odds ratio for a DERAA-negative RA patient of having a DERAA-positive mother compared to a DERAA-positive father was 0.36 (p = 0.11). The DERAA frequency of the mothers (16.1%), but not of the fathers (26.2%) was significantly lower (p = 0.02) compared to that of the general Dutch population (29.3%). This difference could not be ascribed to a difference in DERAA-frequency between men and women as this was neither observed in a Dutch healthy control cohort (n = 420, OR 1.10: 95% CI 0.70–1.74, p = 0.65) nor in the largest RA patient control cohort (n = 65 “DERAA-positive” mothers, OR 0.73: 95% CI 0.35–1.55; p = 0.48). Within the English RA families, the odds of having a DERAA-positive mother was significantly lower compared to having a DERAA-positive father (OR = 0.18: 95% CI 0.04–0.70, p = 0.01). Combined analysis (i.e. Dutch and English families together) also showed a significantly lower DERAA allele frequency in mothers of DERAA-negative RA patients as compared to fathers (OR 0.25: 95% CI 0.09–0.65; p = 0.003). These data indicate that in contrast to a DERAA-positive father, a DERAA-positive mother can transfer the protective effect for RA-development to her DERAA-negative child, presumably by induction of a protective lifelong immune response in the child after being exposed to DERAA-containing antigens of the mother. Further, these data show for the first time that a NIMA may protect the child from an auto-immune disease.

171 UPREGULATED EXPRESSION OF THE NOVEL SUMO SPECIFIC PROTEASE SENP7 IN RA SYNOVIAL FIBROBLASTS

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Small ubiquitin-like modifiers (SUMOs) are key post-translational regulators of large numbers of proteins that play important roles in diverse cellular processes. It has been shown previously that SUMO-1 is overexpressed in rheumatoid arthritis (RA) synovium and contributes to the resistance of RA synovial fibroblasts (RSF) against apoptosis. The recently described SENP7 protein is a member of the family of SUMO-specific proteases (SENP), which can remove SUMO moieties from its substrates and thereby balance SUMOylation processes. Despite our knowledge about SUMO-1 in RA, the expression of SENP7 has not been analyzed in synovium so far. Synovial tissue samples were obtained from 4 RA and 3 OA patients and used for histological analysis as well as for the isolation of synovial fibroblasts. Using specific antibodies, the expression of SENP7 was analyzed by immunohistochemistry in all synovial tissue specimens. The expression levels of SENP7 in RSF and OASF were compared by PCR and Western-blot analysis. The subcellular localization of SENP7 was studied by immunocytochemical co-staining with cell-compartment specific markers and confocal laser scanning microscopy. By immunohistochemistry, we found high expression of SENP7 in RA synovial tissue. SENP7 was expressed most prominently in the superficial lining layer with strong staining of synovial fibroblasts and around blood vessels. Cultured RSF showed a marked upregulation of SENP7 mRNA as compared to OASF. These data were confirmed by Western-blot and immunocytochemistry, where RSF showed significantly higher SENP7 protein expression than OASF. In OASF, SENP7 showed nuclear and cytoplasmic staining of SENP7, although the staining was less prominent in OASF. Endogenous nuclear SENP7 protein localized to few prominent nuclear dots. In the cytoplasm SENP7 was localized in the centrofusome, which was identified by using the centrofusome marker F-actin and in Golgi-like structures as shown by co-staining with the marker TGN38.

173 ACTIVATION OF THE INTERFERON SIGNALLING PATHWAY IN PATIENTS SUFFERING FROM SLE

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Purpose: Both type I and type II interferons (IFN) are thought to play an important role in SLE. Serum IFNα is increased and associated with SLE disease activity, while murine models suggest a more causative role for IFNα. IFNα as well as IFNα signal via phosphorylating Stat1.

Methods: PBMC of 30 SLE patients and 30 healthy individuals (HC) were isolated over Ficoll-Hypaque gradients. Stat1 immunofluorescence staining was increased in SLE lymphocytes (1.64 fold mean 0.47 vs 0.37, p<0.002), but not in monocytes of both SLE patients and HC. In SLE lymphocytes, but not in monocytes, Stat1 phosphorylation was significantly correlated with disease activity (lymphocytes: r = 0.65, p<0.001). Within 24 h, IFNα increased Stat1 to levels comparable to those of SLE patients. In contrast, the effect of IFNα was limited to monocytes, while no significant change in lymphocytic Stat1 was seen. IL-10 reduced Stat1 in both lymphocytes and monocytes, and diminished IFNα-induced Stat1 phosphorylation (1.37 fold mean 0.16 vs 0.47, p<0.001, n.s.). IFNα increased Stat1 phosphorylation in SLE lymphocytes (1.16 ± 0.36 vs 1.37 ± 0.62, p = 0.0001) and monocytes (4.53 ± 1.79 vs 3.35 ± 0.92, p = 0.0005). Incubation with IFNα for 15 minutes resulted in Stat1 phosphorylation in SLE and HC PBMC. In contrast, IFNα induced Stat1 phosphorylation in SLE lymphocytes only (SLE: from 1.56 ± 0.25 to 1.72 ± 0.37, p<0.002 vs HC; from 1.39 ± 0.16 to 1.46 ± 0.16, p=n.s.). Monocytes of both SLE patients and healthy individuals increased pStat1 upon stimulation with IFNα, but this effect was much more pronounced in SLE (SLE: from 4.1 ± 1.2 to 6.9 ± 3.3, p<0.001; HC: from 3.5 ± 0.9 to 4.4 ± 1.5, p<0.005). Nevertheless, both sensitivity flow cytometry was used to follow peripheral B-cell counts in RA patients receiving RTX, in parallel with immunohistochemistry (IHC) analysis of lymphocyte infiltration of synovial tissue.

Methods: 15 RA patients were treated with 2 RTX infusions and their response monitored for 9 to 12 months. EULAR clinical responses were measured 3 monthly. Blood samples were obtained at infusion and 3 months thereafter. B-cell counts were measured using MRD analysis. Lymphocytic infiltrations were taken by knee arthroscopy and 6 months post-treatment from 10 patients. IHC and semi-quantitative scoring were used to measure B-cells (using anti-CD19). T-cells (anti-CD3) and disease activity (anti-CD68, clone EBM11). Visual analogue score (VAS) for macroscopic inflammation was recorded at arthroscopy.

Results: The results of patients are available in 10/15 patients. At 6 months, synovial B-cells were reduced in 7 patients (mean score 0.85 at baseline (BL) reduced to 0.13 at 6 months), but were unchanged or increased in 3 patients (mean score 0.44 at BL and 0.76 at 6 months). B-cell reduction was associated with a reduction in T-cell infiltration (mean score 0.58 at BL reduced to 0.40) and in disease activity (mean score 2.23 at BL reduced to 1.32). Disease activity and T-cell infiltration scores directly correlated with VAS (R = 0.67 and R = 0.73 respectively). In addition B-cell infiltration also correlated with VAS (R = 0.63). Of the 7 patients with synovial B-cell reduction, 6 had initial profound peripheral blood B-cell depletion (over 98%). One patient exhibited only partial peripheral blood depletion (60%) after the 2nd RTX infusion and reconstitution by 3 months. Good or moderate EULAR clinical responses, sustained at 9 months, were achieved in 8/15 patients. The 3 patients in whom synovial B-cell depletion was not demonstrated had either no clinical improvement or relapse following brief response by 6 months. However, 2 of these patients showed profound blood B-cell depletion lasting 2 to 6 months. At 6 months, in peripheral blood, 13/15 patients had detectable B cells (mean 0.029±107/L), and no correlation was observed with clinical responses that varied between good, moderate and non-response.

Conclusion: These results show that synovial B-cell status is not always reflected in peripheral blood and at 6 months after RTX, reduction in the former seems a more reliable indicator of better clinical outcome. We are currently extending this work to investigate further the mechanism of action of RTX, using additional synovial cell markers and studying the phenotypes of peripheral and synovial B-cells.

172 SYNOVIAL B-LYMPHOCYTE REDUCTION PREDICTS CLINICAL RESPONSE IN RHENMATOID ARTHRITIS PATIENTS TREATED WITH RITUXIMAB

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Aim: B-cell depletion therapy with rituximab (RTX) is increasingly used in rheumatoid arthritis (RA). However, depth and duration of depletion of peripheral blood B-cells does not always explain clinical response. The effect of RTX on B cells in other compartments is not yet clear. High
INHIBITION OF EGFR BY GW282974 IN RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS

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Objective: To investigate the effects of GW282974, a novel compound against epidermal growth factor receptor (EGFR) ErbB1/2, with respect to inflammation and joint destruction in RA.

Materials and methods: Rheumatoid arthritis synovial fibroblasts (RAFs) were stimulated with EGF (1 ng/ml), IL-1beta (1 ng/ml) and TNF-alpha (10 ng/ml). In addition, GW282974 was added in different concentrations (5–10 uM) to stimulated cells for 24 h. Gene expression was checked by TaqMan PCR, using 18S as housekeeping gene. Protein analysis was quantified by ELISA. Cell growth and proliferation was measured using the ‘VioLight’ proliferation assay. Cytotoxicity was analysed by FACS using Annexin-V/PI double staining. Expression and phosphorylation of ErbB1/2 receptors were checked by immunostaining with specific antibodies.

Results: EGF had no effect on the gene expression profile of RAFLs when used as single stimulatory agent. In combination with pro-inflammatory mediators such as TNF-alpha and IL-1beta, however, EGF showed a synergistic effect. Thus, the expression of matrix metalloproteinase (MMP)-1, -3, -13, as well as cyclooxygenase (COX)-2 on mRNA level was increased by up to 40000–50000-fold, respectively. Addition of the inhibitor GW282974 strongly abrogated these effects. In this regard, gene expression of MMP-1, -3, -13, and COX-2 were reduced in a dose-dependent manner. Strongest inhibitory effects were observed when GW282974 was used at a concentration of 10 uM. These data could be confirmed on protein levels analysing the supernatants of RAFLs by ELSA. Similarly, cell growth and proliferation of RAFLs were inhibited by GW282974 in a dose- and time-dependent manner. On the other hand, no cytotoxic effects were seen within the doses used.

Discussion: GW282974 appears to interfere with both the inflammatory and the destructive pathway in RA and might be used as novel therapeutic approach for the treatment of RA.

ACPA AND SHARED EPITYPE: THE BEHAVIOUR OF ANTIVIRAL CITRULLINATED PEPTIDE ANTIBODIES

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Rheumatoid arthritis (RA) sera contain antibodies specific for peptides in which arginine is substituted by the deiminated form citrulline (anti-citrullinated peptide antibodies, ACPA). A peptide corresponding to the EBNA I 35–58 sequence containing citrulline in place of arginine (Viral Citrullinated Peptide, VCP) was recently proposed as a new probe to detect ACPA. Anti-VCP antibodies for their specificity and clinical associations can be considered bona fide ACPA. Anti-VCP antibodies are detected in 50% RA sera and their level is correlated with anti-CCP antibodies; moreover, purified anti-VCP antibodies bind CCP and deaminated fibrinogen. Anti-CCP and anti-deaminated fibrinogen antibodies are strongly associated with the shared epitope. We analysed the relationship between anti-VCP antibodies and DRB1 alleles in RA patients. 172 RA patients were typed for DRB1 alleles. Among the shared epitope positive (140 pts), 64 were anti-VCP+ and 76 anti-VCP−; of the 32 share epitope negative patients, 12 were anti-VCP + and 20 anti-VCP−. The lack of association with the shared epitope was further confirmed by experiments in which EBNA I-derived deaminated peptides do not associate with shared epitope-positive alleles. These results demonstrate the great heterogeneity of ACPA family and suggest that anti-VCP antibodies represent the first ACPA not associated with shared epitope.
**Aim:** To investigate the role of the Vitamin D Receptor (VDR) in inflammatory arthritis.

**Methods:** To determine the role of Vitamin D in TNF-mediated joint damage, cartilage tissue was removed and imprints were created. The imprints were then cut into 2 main groups of FLS with distinctive gene expression profiles. FLS from low inflammatory tissue showed increased expression of IGF2 as compared to FLS from high inflammatory tissue. IGF2 and H19 are a pair of reciprocally imprinted genes. IGF2 is expressed from the paternal allele, contributing to the increased expression of IGF2 in FLS. In addition, we will investigate whether this is accompanied with epigenetic deregulation for the IGF2/H19 imprinted domain, a phenomenon that is often observed in cancer. First, we confirmed the IGF2 expression levels by quantitative RT-PCR. Next, to determine whether increased IGF2 expression is associated with IGF2 LOI, we identified a panel of FLS that were heterozygous for a SNP in exon 9, to discriminate between the allelic transcripts. LOI was observed in 7 out of 9 informative FLS, most of which showed high expression of IGF2. Second, we investigated whether the observed LOI is associated with epigenetic changes of the imprinting control regions (ICRs), which are normally differentially methylated. We analyzed the methylation of the ICR, which binds Ctcf upstream of the ICR. We used a quantitative method for assessing methylation differences at specific CpG sites based on bisulfit treatment of DNA followed by single nucleotide primer extension (MS-SnUPF). Unexpectedly, this analysis showed the normal imprint methylated status for this region. Currently, we are investigating a second ICR upstream of IG2. Taken together, these data show that a subtype of RA-FLS is characterized by high IGF2 expression, which occurs in the presence of LOI, driven by a so far unknown mechanism.

**Conclusion:** These findings suggest that deficiency of the VDR leads to an increase in inflammation and bone erosion in an animal model of inflammatory arthritis. This leads to the assumption that treatment with an active metabolite of vitamin D may have potentially positive immunomodulatory effects in arthritis.

**Introduction:** Vitamin D is an important hormone for calcium homeostasis and bone metabolism and may have immunomodulatory effects. Aim: To investigate the role of the Vitamin D Receptor (VDR) in inflammatory arthritis.

**Methods:** To determine the role of Vitamin D in TNF-mediated joint damage, cartilage tissue was removed and imprints were created. The imprints were then cut into 2 main groups of FLS with distinctive gene expression profiles. FLS from low inflammatory tissue showed increased expression of IG2 as compared to FLS from high inflammatory tissue. IGF2 and H19 are a pair of reciprocally imprinted genes. IGF2 is expressed from the paternal allele, contributing to the increased expression of IGF2 in FLS. In addition, we will investigate whether this is accompanied with epigenetic deregulation for the IGF2/H19 imprinted domain, a phenomenon that is often observed in cancer. First, we confirmed the IGF2 expression levels by quantitative RT-PCR. Next, to determine whether increased IGF2 expression is associated with IGF2 LOI, we identified a panel of FLS that were heterozygous for a SNP in exon 9, to discriminate between the allelic transcripts. LOI was observed in 7 out of 9 informative FLS, most of which showed high expression of IGF2. Second, we investigated whether the observed LOI is associated with epigenetic changes of the imprinting control regions (ICRs), which are normally differentially methylated. We analyzed the methylation of the ICR, which binds Ctcf upstream of the ICR. We used a quantitative method for assessing methylation differences at specific CpG sites based on bisulfit treatment of DNA followed by single nucleotide primer extension (MS-SnUPF). Unexpectedly, this analysis showed the normal imprint methylated status for this region. Currently, we are investigating a second ICR upstream of IG2. Taken together, these data show that a subtype of RA-FLS is characterized by high IGF2 expression, which occurs in the presence of LOI, driven by a so far unknown mechanism.

**Conclusion:** These findings suggest that deficiency of the VDR leads to an increase in inflammation and bone erosion in an animal model of inflammatory arthritis. This leads to the assumption that treatment with an active metabolite of vitamin D may have potentially positive immunomodulatory effects in arthritis.
body mass index (BMI), lipid status, and smoking habits. The IMT was evaluated on both left and right carotid arteries in the common carotid (CC), bifurcation (Bl), and internal carotid (IC) arteries. Three measurements in each site were performed, and mean and maximal (max) IMT were calculated. The presence of atherosclerotic plaque was defined as IMT ≥ 1.5 mm. Clinical work-up included determination of the disease activity score (DAS 28), physical disability score (mHAQ), patient's global assessment, as well as the estimation of the functional status. Two-dimensional and color Doppler echocardiography was performed in all patients. Laboratory assessment included measurements of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor, lipid status, and vWF. Clinical and laboratory findings were compared in RA patients with and without subclinical atherosclerosis.

**Results:** RA patients had significantly higher maximal and mean IMT (mm) than healthy controls at all measuring points (Max-CC: 0.764 ± 0.148 vs. 0.703 ± 0.100, P < 0.05; Mean-CC: 0.671 ± 0.119 vs. 0.621 ± 0.085; P < 0.05, Max-Bl: 1.055 ± 0.184 vs. 0.941 ± 0.161, P < 0.01; Mean-Bl: 0.889 ± 0.168 vs. 0.804 ± 0.124, P < 0.05; Max-IC: 0.683 ± 0.108 vs. 0.613 ± 0.093, P < 0.01; Mean IC: 0.577 ± 0.101 vs. 0.535 ± 0.076, P < 0.05). Fifteen out of 42 RA patients (35.7%) and 1/32 controls (3.1%) had subclinical atherosclerosis defined as mean IMT above mean ± 2 SD of the control group at any observation point (>0.791 at CC, >1.042 at Bl, and >0.684 at IC). Atherosclerotic plaques (IMT > 1.5 mm) were revealed in carotid bifurcation in 12/42 RA patients (28.6%) in contrast to 3/32 controls (9.4%).

**Conclusion:** Despite investigated RA population had a low incidence of traditional risk factors for atherosclerosis, both maximal and mean IMT were significantly higher than in matched healthy controls. von Willebrand factor serum levels were significantly increased in RA patients with subclinical atherosclerosis in contrast to patients with normal IMT revealing their potential role as an early marker of endothelial dysfunction and atherosclerosis in RA.

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### INTRAARTICULAR ADMINISTRATION OF ANTI-TNF ALPHA DRUGS UNDER ECHOGRAPHIC CONTRAST TECHNIQUE

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**Introduction:** Anti-TNF-alpha biological drug therapy has shown a great efficacy in the treatment of inflammatory arthropathies (rheumatoid arthritis, psoriatic arthritis and anklyosing spondylitis), if administered systemically and in accord with the specific therapeutic regimens for each drug. In the last few months sporadic cases of local therapy with anti-TNF-alpha on single joints have been published; in some cases the efficacy of therapy has been evaluated through Infliximab marked scintigraphy. Here we report the case of a female patient treated with intraarticular Infliximab is presented; drug efficacy has been evaluated through articular echography with echographic contrast agent.

**Patient and method:** The patient, C.T., aged 62, has been suffering from psoriatic arthritis for several years now. She was visited two years ago for a worsening of articular pain, while taking Cyclosporin 250 mg and Diclofenac 150 mg. She presented with swollen and sore joints, severe morning stiffness and a serious reduction in self-sufficiency, with DAS28 = 5.0. She has been on therapy for 12 months with Infliximab 5 mg/kg according to the planned therapeutic regime, then changed to methotrexate 15 mg/w and Celecoxib 200 mg twice a day. Patient response to therapy has been excellent, achieving a reduction in the number of swollen and sore joints and reaching a DAS28 = 2.6. Six months ago the swollen and tender joint was obtained in the patient’s right knee but no other joint was involved. Serological tests have shown an ESR = 30 (nv 18) and a CRP = 1.7 (nv 0.5), with DAS28 = 2.2. Considering the excellent tolerability of Infliximab, as already shown, it was decided to inject the drug in the inflamed right knee during echography.

**Results:** Intra-articular therapy showed a serious synovial thickening; with Power-Doppler technique and echographic contrast agent a high intraarticular vascularization was observed, indicating an inflammatory process. Intraarticular Infliximab 100 mg has been injected during echography. During the administration there were no problems nor complaints on part of the patient. A progressive reduction of pain and swelling has been observed in the patient’s right knee. After 10 days a control echography with Power-Doppler technique and echographic contrast agent has shown a persistent synovial thickening but no signs of inflammation. At the moment no joint swelling is present in the patient and she feels a mild pain in her right knee, which is easily controlled with Celebrex 200 mg/day.

**Discussion:** Our case report suggests the possible intraarticular administration of anti-TNF-alpha biological drugs in patients with early monoarthrities or in patients with a good response to systemic therapy but poor or no response in the individual joints. In these patients the intraarticular treatment could represent the best cost/benefit balance, considering the costs of biological drug therapy.

**183** RELATIONSHIP BETWEEN T CELL AREA LYMPHOID CHEMOKINE (CCL21, CCL19) INDUCTION AND CD4+-NAIVE T CELL LOCALIZATION IN SYNOVIAL ECTOPIC LYMPHOID TISSUE

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**Aim:** Naïve T-cell recirculation through secondary lymphoid organs (SLO) is a critical step for the genesis of adaptive immune responses, and is physiologically driven by lymphoid chemokines (CK), constitutively expressed in the T-cell area, such as CCL21 and CCL19. In chronic inflammatory states associated with persistent antigenic stimulation, such as rheumatoid arthritis (RA), the inflammatory infiltrate can acquire some morphological and functional features of SLO, including the expression of lymphoid CK. An unresolved issue remains whether the ectopic expression of lymphoid CK can mediate the misdirection of naïve T cells from their homeostatic route to SLO to inflammatory ectopic lymphoid tissues (ELT) in humans. In this study we analyzed, by using specific combinations of cell surface markers, the in situ relationships between T-cell area lymphoid CK ectopic expression and naïve T-cell localization in rheumatoid synovial ELT.

**Methods:** Twelve synovial tissue (ST) samples from RA patients were selected for the study. Paired ST, synovial fluid (SF) samples were also collected in 5 cases. Expression of CCL21 and CCL19 was examined by immunohistochemistry and quantified by digital image analysis. Naïve TCD4+ cells (CD3/CD4/CD45RA) were detected by triple immunofluorescence and confocal microscopy in the ST and by FACS in the SF.

**Results:** Comparative analysis in the inflamed sublining demonstrated a significant association between CCL21 and CCL19 expression with the formation of synovial ELT (Mann-Whitney test p<0.001). Both these CK showed also a strong association with the dimensional enlargement of the lymphoid aggregates, and a significant reciprocal association within the same aggregates both in terms of global prevalence (Fisher exact test p<0.001) and quantitative expression (Spearman Rho = 0.78, p<0.001). Microanatomical studies revealed the possible cognate perivascular expression of CCL21/CCL19/PNAd around the CXCL12/PNAd positive HEVs as physiologically required in SLO for naïve T-cell recruitment. Notably, phenotypic tissue analysis did not reveal the expected co-localization of CD3/CD4/CD45RA cells inside synovial ELT both in small perivascular aggregates and in large organized lymphoid clusters. FACS analysis of SF samples from RA patients confirmed the rare presence of CD3/CD4/CD45RA cells. Similar findings were obtained with paired ST and SF samples that were collected and analysed in parallel for CK expression and T cell subset distribution. As expected, CD3/CD4/CD45RA cells were found both in peripheral blood and lymph nodes used as positive control.
Conclusions: Our data support the concept that a T-cell area lymphoid CK system can associate with lymphoid neogenesis in chronic inflamed synovium, leading to highly organized structures with qualitative features of SLO. Nevertheless, this expression within the synovial lymphoid tissue does not appear sufficient to allow constitutive naïve T cell misdirection in ELT, at least in specific disease stages. Altogether, our findings open new perspectives for future studies aimed to address the molecular rationale and physio-pathological consequences of naïve-cell exclusion from RA ELTs.

184 MESENCHYMAL CELLS ARE SUFFICIENT TARGETS FOR TNF IN MODELLED JOINT AND GUT PATHOLOGIES

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TNF plays an essential role in the pathogenesis of rheumatoid arthritis and Crohn’s inflammatory bowel disease. Anti-TNF therapies have proved successful in their clinical treatment. The cellular mechanisms of TNF/TNF-R function in these diseases have remained poorly characterized. In general it is thought that TNF delivers mostly innate activation and pro-inflammatory signals through its action on myeloid/monocytic cells or other haemopoietic cell types. Using reciprocal bone marrow grafting experiments in previously established TNF transgenic animal models of arthritis in Crohn’s-like IB (tg197 and TNFAA¨RE mice), we show that development of arthritis requires the expression of TNFR1 in cells of the radiation-resistant compartment, which are also sufficient targets for TNF in the development of the Crohn’s-like IBD as previously demonstrated. Moreover, we have generated a Cre-expressing mouse line (Cre expression driven by Collagen VI (α1) promoter) that we expected, Cre activity could be detected in mesenchymal cells such as synovial fibroblasts, articular chondrocytes, skeletal myocytes, keratinocytes, dermal fibroblasts and intestinal myofibroblasts. Mesenchymal cell-specific reactivation of a mutant floxed TNFRI generated a Cre-expressing mouse line (Cre expression driven by Collagen VI<sup>−/−</sup>) in a wild type background. Finally, we show that transfer of naïve thymic-derived T cells is sufficient to induce arthritis in the absence of TNF-R1 expression in target cells. In particular, naïve CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells, can induce arthritis when transferred into recipient mice. These findings suggest that TNF-R1 is not required for the development of arthritis in these models.

185 PHOSPHATIDYLINOSITOL 3- KINASE GAMMA REGULATES MATRIX METALLOPROTEINASES PRODUCTION IN RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS

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Matrix metalloproteinases (MMPs) are proteolytic enzymes that are secreted by rheumatoid arthritis fibroblasts (RAF) and play an important role in cartilage destruction in patients with RA. Different studies have shown that the MMP expression is linked to signalling pathways in which phosphatidylinositol 3- kinase (PI3K) is also implicated. However, there is only sparse knowledge about the relevance of the PI3K isoforms for the activation of RAF. Therefore, we analyzed the expression of the gamma isoform of PI3K (PI3Kgamma) in RAF of rheumatoid arthritis synovial fibroblasts (RA-SF) and investigated its effects on epidermal growth factor (EGF) induced phosphorylation of Akt and on the EGF-mediated expression of MMP-1 and MMP-3. Synovial fibroblasts from RA- and OA- patients were analyzed for the expression of mRNA for the catalytic p110α and regulatory p101α subunit of PI3K by RTQ-PCR. Expression of PI3Kgamma was confirmed by Western blot. The involvement of PI3Kgamma in Akt-phosphorylation was studied in EGF- stimulated cells (10–100 ng/ml, 5 min) using the pan-PI3K-inhibitor LY294002 (200–500 nM) and a PI3Kgamma specific inhibitor 1 (50–200 nM) together with phospho-specific antibodies. The expression of MMP-1 and MMP-3 was studied by ELISA following stimulation of synovial fibroblasts with EGF. The expression of PI3Kgamma was confirmed by PCR and Western blot revealed the expression of the p110 catalytic subunit of PI3Kgamma but not of the regulatory p101alpha subunit in RAF. OA-SF showed only negligible levels of the p110 subunit. Stimulation of RAF and OA-SF resulted in the phosphorylation of Akt with LY294002 inhibiting completely the EGF- mediated activation. The PI3Kgamma specific inhibitor 1 reduced the EGF-stimulated phosphorylation of Akt in RAF but had no effect in OA-SF. Interestingly, treatment of RAF with both LY294002 and compound 1 significantly reduced the EGF-mediated induction of MMP-1 (63% and 42%, respectively). Similar effects were seen with MMP-3 with a reduction of 20% and 40%, respectively. These data suggest a disease-specific expression of PI3Kgamma in RAF that contributes to EGF-mediated phosphorylation of Akt and subsequent induction of MMP-1 and MMP-3. Therefore specific inhibitors of PI3Kgamma may be a possibility to interfere with the activation of RAF and to reduce cartilage destruction in RA.

186 ANALYSIS OF PATHOGENIC AND PROTECTIVE T CELL SUBPOPULATIONS IN A MURINE ARTHRITIS MODEL

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Introduction: Rheumatoid arthritis is a chronic disease with an autoimmune character which affects about 1 percent of the world population. Patients mainly suffer from severe destruction of bone and cartilage. Although there is indirect evidence that CD4+ T cells are key players in rheumatoid arthritis, their exact role in disease pathogenesis is still not clear. Therefore, our aim was to define protective or pathogenic effector functions of T cells recognizing joint-specifc antigens using the ovalbumin-induced arthritis model. In this model, arthritis is induced by intra-articular (i.a.) injection of cationic ovalbumin into mice which have been previously sensitised to the same antigen either by immunisation or by adoptive transfer of Ova-specific CD4+ T cells.

Results: We show that i.a. injection of ovalbumin into immunised mice leads to a strong arthritis characterised by massive cell infiltration, hyperplasia and severe bone and cartilage destruction. Adoptive transfer of Ova-specific Th1 cells alone into naive mice which received i.a. Ova also induced arthritis. Th1-induced disease is less pronounced than after Ova-immunisation and completely lacks bone or cartilage destruction. Th2 cells, which were thought to counterbalance the pathogenic effect of Th1 cells, were only partially able to abolish Th1-induced arthritis after co-transfer and even induced mild inflammatory symptoms when transferred alone. In contrast, Ova-specific CD4+CD25+ regulatory T cells (Treg) completely suppressed Th1-induced arthritis. However, if mice were sensitized by Ova-immunisation, CD4+CD25+ antigen-specific regulatory T cells (Treg) were only partially protective. Non-specific Treg had little to no protective influence on arthritis severity.

Conclusions: Our data support the concept that a T-cell area lymphoid CK character which affects about 1 percent of the world population. Patients mainly suffer from severe destruction of bone and cartilage. Although there is indirect evidence that CD4+ T cells are key players in rheumatoid arthritis, their exact role in disease pathogenesis is still not clear. Therefore, our aim was to define protective or pathogenic effector functions of T cells recognizing joint-specifc antigens using the ovalbumin-induced arthritis model. In this model, arthritis is induced by intra-articular (i.a.) injection of cationic ovalbumin into mice which have been previously sensitised to the same antigen either by immunisation or by adoptive transfer of Ova-specific CD4+ T cells.

187 QUANTIFICATION OF THE INVASIVE POTENTIAL OF RA SYNOVIAL FIBROBLASTS BY A NOVEL INVASION ASSAY USING TRANSEPITHELIAL ELECTRICAL RESISTANCE MEASUREMENT (TEER)

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RA synovial fibroblasts (RAFs) are key effector cells in the rheumatoid synovium and have been associated strongly with the destruction of articular structures. RAFs exhibit features of stable cellular activation that result in their attachment to the articular cartilage and deep invasion of extracellular matrix. However, quantification of the invasive potential of RAFs has been a major challenge, and the question of whether these cells can transmigrate actively from one joint to another has been a matter of debate. Here, we established a novel in vitro assay to exactly determine the invasive potential of SFs from patients with RA and study their transmigration through epithelial cell layers. Synovial tissue samples were obtained from three RA patients and RAFs were isolated and cultured under standard conditions. For the measurement of their invasiveness, we used a transwell system with an electrochemical technique that is based on the measurement of the electrical resistance of a monolayer of the C7 subclone of Madin-Darby canine kidney cells (MDCK-C7). This high resistance clone was grown to confluency in a filter cup and the transepithelial electrical resistance (TEER) was measured. The resistance across the monolayer was measured with an electrode. The monolayer was grown to confluency in a filter cup and the transepithelial electrical resistance (TEER) was measured using an electrode. Following full establishment of the MDCK-C7 monolayer, RAFs were added to the donor side of the MDCK-C7 cells, and their invasion was assessed four times of every patient through measurement of electrical resistance breakdown in the assay. Using this assay, RAFs exhibited a strong invasive through the monolayer. Resistance decreased to 60% of
SYNDECAN-4 DEFICIENCY RESULTS IN DELAYED ENCHONDRAL OSSIFICATION AND REDUCED AGGREGAN CLEAVAGE IN OSTEARTHRITIC CARTILAGE

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During limb development chondrocyte differentiation into hypertrophic chondrocytes is essential for enchondral ossification of long bones. In pathologic situations such as osteoarthritic chondrocytes differentiate again into hypertrophic-like chondrocytes in affected cartilage areas. However, the mechanisms that link chondrocyte hypertrophy to cartilage remodelling are poorly understood. Based on recent data that bone-invasive transmembrane heparan sulfate proteoglycans in matrix turnover and cell differentiation, we analyzed the distribution and functional role of syndecan-4 during limb development in mice and studied its expression and function in osteoarthritis (OA). Syndecan-4 promoter activity was detected in whole embryos by staining for beta-Galactosidase in syndecan-4/- LacZ knock-in mice. For cellular localization of syndecan-4 expression within cartilage immunohistochemistry with antibodies against syndecan-4 and type X collagen was performed. Alizarin red S staining was carried out to analyze the mineralization of bones in wild type and syndecan-4/- littersmates at days E13.5 and E14.5. To study syndecan-4 in OA, we compared its expression in normal and OA articular cartilage by Northern blot analysis and immunohistochemistry. For functional analysis, osteoarthritis changes were induced in syndecan-4/- mice and their wild-type controls by surgically achieved joint instability, and safranin-orange staining assessed the loss of proteoglycans. Staining for syndecan-4 and ADAMTS generated aggrecan neo-epitopes was performed in the knees of these mice. Beta-Gal-staining of syndecan-4/- mice at E12.0 showed a strong expression of syndecan-4 promoter at sites of cartilage condensations. In later stages syndecan-4 was detected in the growth plates of long bones. In wild-type embryos, syndecan-4 protein was also found mainly in chondrocytes of the hypertrophic zone, where it co-localized with type X collagen. The loss of syndecan-4 was associated with a significant retardation in the mineralization of axial and appendicular bones. There is an upregulation of syndecan-4 in human OA cartilage both at the mRNA and the protein level. Analysis of osteoarthritic changes in mice revealed a strong and early induction of syndecan-4, and there was a significant reduction of proteoglycan loss in the syndecan-4/- mice compared to their wild-type controls. This was accompanied by a significantly reduced staining for ADAMTS generated aggrecan neo-epitopes in syndecan-4/- mice. Our data demonstrate that syndecan-4 is induced in hypertrophic chondrocytes both during embryogenesis and in OA cartilage. By promoting ADAMTS mediated cleavage of aggrecans, syndecan-4 facilitates enchondral ossification but is involved also in cartilage degradation by hypertrophic chondrocytes in OA. Inhibition of syndecan-4 may, therefore, constitute a promising strategy to interfere with osteoarthritic cartilage damage.

REGULATION OF CYTOKINE-INDUCED HIF-1\alpha EXPRESSION IN RHEUMATOID SYNOVIAL FIBROBLASTS

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Aim: The transcription factor hypoxia-inducible factor (HIF)-1 plays a central physiological role in oxygen and energy homeostasis, and is activated during hypoxia by stabilisation of the subunit HIF-1\alpha. Activation can also occur by pro-inflammatory cytokines during inflammation. Hypoxia as well as proinflammatory cytokines play an important role in the synovial inflammation in rheumatoid arthritis (RA) patients. Expression of HIF-1\alpha has been demonstrated in RA synovial lining layer. The aim of the study was to investigate the regulation of the intracellular signal transduction pathways, involved in the expression of HIF-1\alpha, and the expression of genes regulated by HIF-1\alpha in rheumatoid synovial fibroblasts.

Materials and methods: Rheumatoid synovial fibroblasts (RSF) were cultured under proinflammatory conditions (IL-1beta and TNF-alpha stimulation) and under chemical hypoxia (CoCl2 treatment). Expression of HIF-1\alpha was analyzed in nuclear extracts by Western blotting. The effects of inhibitors of the phospho-inoositide 3 kinase (PI3K) pathway, the extracellular signal-regulated kinase (ERK) pathway, and the Ca2+/ Calmodulin kinase II (CAMKII) pathway on HIF-1\alpha expression was measured. mRNA expression of HIF-1\alpha, COX-2, vascular endothelial growth factor (VEGF) and stromal cell-derived factor (SDF)-1 was determined by real-time RT-PCR and protein production of VEGF and SDF-1 was determined by ELISA.

Results: Treatment of the synovial fibroblasts with 150 mM CoCl2 as well as stimulation with 10 ng/ml IL-1beta or TNF-alpha resulted in strong protein expression of HIF-1\alpha, measured with Western blotting. Pretreatment with the MEK1/2 inhibitor PD98059, the PI3K inhibitor LY294002 and the CAMKII inhibitor KN93 resulted in inhibition of the cytokine-induced HIF-1\alpha. Furthermore it was shown that cytokine-induced mRNA expression of HIF-1\alpha was inhibited by LY294002. IL-1beta and TNF-alpha stimulation was able to induce mRNA expression of VEGF and COX-2, which was inhibited by the MEK1/2 inhibitor. VEGF but not SDF-1 protein production was induced after cytokine stimulation, but no significant effect of kinase inhibitors was found.

Conclusions: Expression of cytokine-induced HIF-1\alpha at the mRNA level in rheumatoid synovial fibroblasts is regulated by the PI3K pathway, whereas HIF-1\alpha protein expression is also influenced by other pathways. We found that cytokine stimulation induced VEGF mRNA and protein production, but no significant effect of kinase inhibition was found on VEGF production in cytokine-stimulated rheumatoid synovial fibroblasts.

ROLE OF CAVEOLIN-1 IN THE PATHOGENESIS OF TISSUE FIBROSIS IN SYSTEMIC SCLEROSIS

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Systemic sclerosis (SSc) is a multi-systemic disease associated with autoimmun activation and widespread fibroproliferative vascular damage, causing progressive tissue fibrosis and subsequent multi-organ dysfunction. The fibroproliferative process occurs essentially in all organs, but is often particularly evident and aggressive in the kidneys and lungs, causing scleroderma renal crisis or pulmonary arterial hypertension (PAH) respectively, which combined represent the major cause of mortality in SSc patients. Recent studies on idiopathic pulmonary hypertension and idiopathic pulmonary fibrosis independently identified caveolins as important inhibitors of progression of both diseases. Caveolins comprise a family of membrane proteins involved in the regulation of TGF-\beta, VEGF, and endothelin pathways, three pathways universally accepted to play a crucial role in the SSc progression. Similarly, recent data indicate that caveolin-1 (Cav-1) is down regulated in SSc lung and dermal fibroblasts in vitro. In this study, we analyzed the expression of Cav-1 in vivo in SSc patients and the role of Cav-1 protein in the regulation of collagen production by human normal and scleroderma fibroblasts in vitro.

Materials and methods: Lung biopsies from SSc patients affected by pulmonary hypertension and pulmonary fibrosis were analyzed by confocal laser microscopy for Cav-1 expression. Cav-1 knock out mice lungs were analyzed by histology/histochemistry studies and by hydroxyproline content to assess pulmonary fibrosis. Normal and scleroderma derived fibroblasts were stimulated with TGF-\beta and treated with recombinant caveolin. Cellular mRNA, intracellular proteins and tissue supernatants were analyzed by real time PCR and immunoblotting. Cell viability was assessed by WST-1 assay.

Results: We observed that Cav-1 expression was markedly reduced in affected SSc tissues as demonstrated by quantitative confocal microscopy analysis of lung biopsies of SSc patients affected by pulmonary hypertension and/or pulmonary fibrosis. Consistent with these findings, lungs from Cav-1 knockout mice showed pulmonary fibrosis remarkably similar to that seen in SSc patients, as evidenced by histological analysis and hydroxyproline content of the lungs. Additionally, TGF-\beta, the main pro-fibrotic cytokine in SSc pathogenesis, down regulated Cav-1 expression in normal human fibroblasts in vitro. Moreover, Cav-1 markedly reduced collagen production of SSc fibroblasts in vitro and suppressed TGF-\beta induced ECM gene unregulation without affecting cell viability.
Conclusions: This study indicates that Cav-1 expression is reduced in SSc in vivo and that Cav-1 plays a key role in TGF-β-induced tissue fibrosis and in the development of the sclerodermatric fibroblast phenotype of fibroblasts. Furthermore, Cav-1 can suppress collagen production of SSc fibroblasts and of TGF-β-stimulated fibroblasts in vitro without displaying evidence of cell toxicity. These results suggest that Cav-1 is a novel therapeutic target for the treatment of SSc patients.

191 SYSTEMIC SCLEROSIS (SSC) LYMPHOCYTES INDUCE ANGIOGENESIS IN HEALTHY MICROVASCULAR ENDOThelial CELLS

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Background: SSC pathogenesis and progression may involve three cellular types: endothelial cells (leading to vascular damage), fibroblasts (inducing fibrosis) and lymphocytes (producing autoimmune reaction). Endothelial damage and defective neoangiogenesis are hallmarks of SSc and often precede the other disease features. We previously showed that the conditioned medium from SSc-fibroblasts and SSc-microvascular endothelial cells (SSc-MVEC) inhibits angiogenesis in healthy MVEC (H-MVEC).

Aim: To evaluate the effects of SSc peripheral blood lymphocytes on H-MVEC angiogenic potential.

Methods: H-MVEC were incubated with the conditioned medium (CM) from healthy lymphocytes (H-CM), from limited (lSSc-CM) and diffuse SSc (dSSc-CM) lymphocytes. 5 CM for each subset were evaluated. Angiogenesis was assayed by chemoinvasion (Boyden chamber invasion assay, using Matrigel as invasion matrix) and in vitro angiogenesis (capillary morphogenesis assay). Induction by vascular endothelial growth factor (VEGF) 50 ng/ml was considered as positive control. CM from SSc-MVEC (SSc-MVEC-CM) or from SSc-fibroblasts (SSc-Fb-MC) was used to inhibit angiogenesis. All CM were obtained by incubating cells in MVEC basal medium (MCD1B13) plus 0.2% FCS, without other growth factors, for 48 hours. Basal chemoinvasion and capillary morphogenesis (0.2% FCS) were considered as 100%.

Results: H-CM had slight but significant stimulatory effects on basal chemoinvasion (138+8.7%, p<0.05) and capillary morphogenesis (123±4.8%, p<0.05). Furthermore, both lSSc-CM and dSSc-CM significantly increased basal migration (303±14.2%, p<0.001 and 247±19.8% p<0.01, respectively) and tubular-like structure formation (141.7±5.2 p<0.005 and 129.0±6.4 p<0.05, respectively). Chemoinvasion was significantly reduced by SSc-MVEC-CM or SSc-Fb-MC (55±8.1% and 64±9.2% respectively, p<0.05 vs basal), and also capillary morphogenesis was inhibited (27±5.1% and 38±6.2% respectively, p<0.001). Co-incubation with SSc-CM or dSSc-CM was able to contrast the anti-angiogenic potential of SSc-MVEC-CM and SSc-Fb-CM, maintaining the capacity of H-MVEC to form capillaries (64±3.3% and 85±4.8%, p<0.001) and to migrate (72±8.8% and 88±5.0%, p<0.05), even if reduced (p<0.05) in comparison to basal unstimulated cells.

Conclusion: These data show that SSc lymphocytes may influence the H-MVEC microenvironment, probably secreting pro-angiogenic molecules that stimulate their angiogenic properties and partially counteracting the response to anti-angiogenic factors. For this reason, the role of lymphocytes in SSc breakdown of capillary formation might be reconsidered. This may suggest that the early impairment of MVEC may depend on factors secreted by fibroblasts.

192 EXPRESSION AND REGULATION OF ST2 ISOFORMS IN SYNOVIAL MEMBRANE CELLS FROM PATIENTS WITH ARTHRITIS

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ST2 is a member of the IL-1 receptor family widely reported to have a negative regulatory function on IL-1R and TLR signalling. However, recent reports have identified a novel cytokine (IL-33) that binds ST2 (the membrane bound isoform) and induces Th2-type cytokine release from Th2 T cells. We previously reported that ST2 is significantly elevated in synovial fluids from patients with inflammatory synovitis and have now investigated the presence of both ST2 and the soluble isoform sST2 in synovial tissue from OA and RA patients. Our aim was to determine the source of ST2 present in inflammatory synovitis and factors regulating ST2 expression. Synovial membranes were obtained from consenting OA and RA patients and prepared as whole tissue digests or passaged fibroblast cultures. The isolates were stimulated as described and probed for isoform expression by RT-PCR and Western blot. Membrane bound ST2 was detected as mRNA and protein in synovial tissue extracts and in fibroblast cultures. The cognate ST2 co-receptor (IL-1RAcP) was also identified in tissue and fibroblast isolates by Western blot. Functionality of the ST2 receptor was confirmed by detection of p38 MAPK phosphorylation in response to IL-33 stimulation of fibroblasts. Expression of ST2 was regulated by pro-inflammatory factors such as TNF-alpha and IL-1 beta, which markedly increased ST2 mRNA expression. The soluble variant (sST2) was also detected by RT-PCR, and could be upregulated in isolates by stimulation with IL-33. Th2 T cells are known to produce sST2 upon activation, but the low level of Th2 cells associated with RA suggests that the stromal environment contributes the majority of ST2 detected in RA synovial fluid. Increased sST2 expression provides a mechanism whereby stromal cells could regulate synovial T cell maturation.

193 EVALUATION OF ANGIOGENESIS AND FIBROBLASIS IN PLACENTA FROM SYSTEMIC SCLEROSIS

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Aim: Systemic sclerosis (SSc) is an autoimmune disease affecting the skin and internal organs, characterized by impaired angiogenesis and abnormal fibrosis. Several angiogenic and connective tissue remodelling mediators are known to be involved in SSc pathogenesis. Angiogenesis and vascular transformation are important processes in the normal development of the placenta during pregnancy. Therefore, a tight regulation of angiogenic factors is essential for reproductive success. The aim of this study was to evaluate the expression of angiogenesis and fibrosis markers in placenta from a 29-year-old woman affected by diffuse SSc.

Materials and methods: Placenta biopsies were obtained from one SSc patient after caesarean delivery at 32 weeks and from three normal uncomplicated pregnancies (32–35 weeks), as controls. The samples were routinely processed and embedded in paraffin. Immunohistochemistry was performed on serial sections (5 μm). We analysed the expression of VEGF, PLGF, VEGFR-1 and VEGFR-2, as angiogenic factors. Moreover, Masson’s stain and immunohistochemistry for CTGF, collagen type I and α-smooth muscle actin (α-SMA) were performed to analyse tissue fibrosis and stromal fibroblast transdifferentiation to myofibroblasts.

Results: The trophoblast, decidual cells, endothelial cells (ECs), fibroblasts and Hoflober cells showed immunoreactivity for VEGF. In SSc placenta, ECs displayed a stronger immunopositivity for VEGF, whereas the trophoblast showed a disomogeneous and weaker immunostaining than in controls. VEGFR-1 was detected in SSc trophoblastic layer and ECs similarly to control placentas. VEGFR-2 expression was evident in ECs of the villi, but the staining intensity and the number of positive vessels were higher in SSc compared with controls. PLGF immunopositivity was weaker in decidual cells, ECs and fibroblasts in SSc than in healthy placentas, while it was similar to controls in trophoblastic cells. Masson’s stain revealed a diffuse fibrosis in SSc placenta, mostly around the vessels. Similarly, the perivascular immunoreactivity for collagen type I was stronger in SSc placenta compared with controls. In SSc, a very strong immunopositivity for CTGF was detected in decidual and Hofbauer cells and, in particular, in the larger vessel wall, in ECs and fibroblasts compared with healthy placentas. α-SMA was evident around the larger vessels in both healthy and SSc placentas, whereas it was disomogeneously distributed around SSc microvessels. Stromal cells showed a strong immunositivity for α-SMA only in SSc placenta.

Conclusion: This is the first study on morphologic expression of angiogenesis and fibrosis markers in placental tissue from a SSc patient. Our preliminary results suggest that the dysregulation of angiogenic factors, as well as the tissue fibrosis and the transdifferentiation of stromal fibroblast to myofibroblasts could lead to functional abnormalities in SSc placenta.

194 FUNCTIONAL CHANGES IN SCLERODERMA FIBROBLASTS CO-CULTURED WITH AUTOLOGOUS PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs)

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Introduction: Increasing evidence suggest that an interplay between T cells and fibroblasts plays a pivotal role in promoting matrix accumulation in systemic sclerosis (SSc).

Aim: We investigated if the co-culturing of fibroblasts derived from SSc patients with autologous PBMCs could induce peculiar modifications in any cell types.

Materials and methods: Fibroblasts were obtained from the skin of patients undergoing biopsy for diagnostic purposes. PBMCs were obtained from the same patients. All the patients were fully informed of the meaning of the study and accepted to donate blood. Cells were taken in culture at 37°C 5% CO2 for ten days, then cells were stained with monoclonal antibodies for HLA-DR, CD3, CD4, CD56, CD95, TCRαβ, TCRγδ. After the staining, the cells were analyzed by flow-cytometry using a FACScalibur.

Results: We found that T cells bearing an αβ receptor were expanded in the co-cultures. Moreover, these cells were positive for the expression of HLA-DR, suggesting an activation of T cells induced by co-culturing with autologous fibroblasts. No expansion of DR, suggesting an activation of T cells induced by co-culturing with Conclusions: We recently showed that peripheral blood T cells from SSc patients expand when co-cultured with autologous fibroblasts and acquire the same T cell clonotypes that are increased in the affected skin. The results presented here further support that such expansion may be due to a specific antigenic activity of fibroblasts on T cells. In addition, the up-regulation of FAS expression on SSc fibroblasts may be related to phenotypic changes that indicate an increased ability of these cells to escape normal pathways leading to cell death. The meaning of these findings in the pathogenesis of SSc remains to be further investigated.

EVIDENCE FOR REDUCED ANGIOGENESIS IN BONE MARROW (BM) IN SYSTEMIC SCLEROSIS (SSC)

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Background: Dysfunctional angiogenesis is a pathogenetic marker of SSC. Microvascular endothelial cells have a reduced capacity to form capillaries, reduced circulating levels of endothelial progenitor cells have been found and mesenchymal stromal cell differentiation into endothelial cells has shown a defective capacity to form capillaries. This suggests that pathophysiologically relevant changes may already exist in SSc-BM stromal cells.

Aim: To study in SSc BM angiogenesis, the cellular immune system and fibrosis.

Methods: 8 SSc patients affected by a severe diffuse cutaneous SSc and screened for autologous hematopoietic stem cells transplantation, underwent a BM biopsy to assess cellularity and morphology. BM biopsies were compared with 5 healthy controls.

To evaluate angiogenesis, cellular immunity and fibrosis the following antibodies were used: VEGF, KDR/flk-1, MMP-9, CD34/QBEND10, vWF, CD20, CD3, CD4, CD8, CD38, K, λ, CD68/PGM-1, CD61. To evaluate fibrosis silver impregnation for reticulum was used. The number of vessels, the mean area of vascularisation (μm²/ %), the perimeter and microvessel area (MVA) were measured with a multiparametric computerized imagine analysis.

Results: The morphology of BM was similar in SSc and controls. Also the B cell population was similar but the T cells showed a reduction of CD4/CD8 ratio in SSc. A significant reduction in BM vascularity was found: both microvessel density and number of vessels were lower, while VEGF expression (observed in myocardial cells and in megakaryocytes and histioocyte-macrophage system) was much higher in SSc BM samples than in controls. In seven patients, a weak expression of KDR/flk-1 was observed and MMP-9 expression was low in all cases. Out of 8 patients 2 had a maximal while another 2 had a moderate degree of BM fibrosis.

Conclusion: In SSc, BM is characterised by a reduction of angiogenesis that may induce the increase of VEGF.


EVALUATION FOR MARKERS OF FIBROSIS IN THE UPPER GASTROINTESTINAL TRACT OF PATIENTS WITH SYSTEMIC SCLEROSIS

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Aim: Systemic sclerosis (SSc) is a chronic connective tissue disease characterized by fibrosis and destruction of the microvasculature. Increased deposition of collagen and other extracellular matrix components affect not only the skin but most of the internal organs including the gastrointestinal (GI) tract. Few reports assessed the morphology of the stomach in SSc. We studied a case of a 52-year-old female with SSc, showing an important involvement of upper digestive system. She underwent distal esophageal myotomy and subsequently total gastrectomy. Moreover, we analysed gastric biopsies obtained during gastroscopical examination from SSc patients to evaluate the expression of markers for fibrosis in the gastric wall of SSc patients.

Materials and methods: Full-thickness gastric wall samples were obtained from one SSc patient and biopsies were taken from 10 SSc patients, which underwent esophagogastrectomy. The full-thickness gastric samples were fixed in formalin and embedded in paraffin. The endoscopic samples were embedded in OCT, snap frozen in liquid nitrogen and stored at ~80°C. In order to investigate tissue fibrosis, the sections were stained by Masson’s trichromic method and analysed by immunohistochemistry for the expression of connective tissue growth factor (CTGF), collagen type I, collagen type IV, α-smooth muscle actin (α-SMA) and endothelin-1 (ET-1). Full-thickness gastric wall biopsies, free from inflammatory responses and neoplastic infiltration, from 3 patients who underwent total gastrectomy due to other disease entities, and endoscopic biopsies from 5 controls were also examined.

Results: In SSc gastric wall, Masson’s stain revealed a mild fibrosis in the lamina propria that became more severe in the muscularis mucosae. In the muscle layers, wide areas of focal fibrosis surrounded smooth muscle cells (SMCs) increasing intercellular spaces. A marked fibrosis was evident also around the vessels. Collagen type I expression mirrored the fibrosis areas revealed by Masson’s stain. The immunopositivity for collagen type IV was well detectable in the basal lamina around the glands and the vessels in SSc compared with the control gastric wall. In SSc, a strong CTGF expression was evident in the muscularis mucosae, and it was focally distributed in the muscle layers when compared with the control. Moreover, most of the vessels showed a strong immunopositivity for CTGF in SSc gastric wall, while they were weakly positive in the controls. In SSc the α-SMA reactivity was stronger in the lamina propria, muscularis mucosae, muscle layers and in the vessel wall than in controls, whereas ET-1 positive cells were evident mostly in SSc muscle layers.

Conclusion: Our results show that the expression of markers for fibrosis is relevant in SSc stomach. The involvement of gastric wall components in fibrosis process most likely is involved in the decreased mobility and atrophy of the stomach in SSc patients.

COMPLEX METABOLIC COMPOSITION OF SYNOVIAL FLUID IN SCLERODERMA DETECTED BY PROTON MAGNETIC RESONANCE SPECTROSCOPY

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Objectives: Because of the difficulties of synovial fluid elevation in scleroderma, previous studies have given little information about possible correlation between the disease’s pathogenesis and the complex composition of joint effusions.

Study methods: We tried to characterize the complex metabolic composition of the joint fluids affected by diffuse scleroderma in a 35 year old patient. Our previous study used proton magnetic resonance spectroscopy (MRS) for the simultaneous detection and measurement of the metabolic components of synovial fluid in different pathologies. In the
Abstract 195.

present study, we focused on simultaneous detection of synovial potential markers for sclerodema. Our patient met criteria for scleroderma diagnosis and underwent determination of synovial fluid composition after 3 h, 10 h and 24 h refrigeration. The patient was treated with vasodilators, non-steroid anti-inflammatory drugs and antiaggregant medication, having Raynaud, esophageal symptoms and arthritis. Rodnan score was 15. Spectra were recorded on a Bruker apparatus operating at 400 MHz.

Results: This method led to the possibility to attribute the signals for glutamine, threonine, lactate, hydroxybutyrate, glycine, dimethylamine and liprotein-associated fatty acids, ceramide and citrulline. We didn’t detect a highly intense signal for ceramide as in RA samples, showing that apoptosis is not an important process in sclerodermic joints. We have shown extremely weak signal intensity for citrulline at 3.15 ppm (highly specific for RA synovial fluid—sensitivity 80%, specificity 100% in our previous study). Increased synovial fluid levels for chylomicron- and VLDL-associated triacylglycerals was found, the same observation being valid for lactate levels as well.

Conclusion: MRS investigation of synovial fluid provides valuable information consisting of simultaneous detection of different metabolites in sclerodermic synovial fluid. We can correlate the presence of lipid components with the endothelial dysfunction and possible early progression of atherosclerosis in sclerodermic patients.

198 CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN SYSTEMIC SCLEROSIS ARE RELATED TO IMPAIRED ANGIOGENESIS AND VASCULAR DISEASE MANIFESTATIONS

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Background: Endothelial damage is one of the earliest events in the pathogenesis of vascular injury in systemic sclerosis (SSc). Given the essential role of endothelial progenitor cells (EPCs) in ongoing endothelial repair and neovascularization, it is likely that insufficient angiogenesis seen in SSc is related to EPC alterations.

Material and methods: We measured EPC numbers in blood of 40 SSc patients (all female, mean age 44 ± 10 yr, 15 dSSc and 25 lSSc) and 24 controls by flow cytometry analysis and studied their relation to disease activity, severity of internal organ damage and vascular manifestations. Endothelium dependent and endothelium-independent vasodilatation was assessed by high-resolution ultrasonography of the brachial artery. Structural capillary changes were studied quantitatively using nailfold video capillaroscopy.

Results: Compared to control subjects, SSc patients showed higher levels of both early CD133+/VEGF-R2+ (0.0159 ± 0.0026%, p < 0.05) and more mature CD34+/VEGF-R2+ positive (0.0041 ± 0.0027%, p < 0.01) EPC populations in association with increased membrane expression of Fab4 (CD95) on CD34+/VEGF-R2+ progenitor cells (0.0076 ± 0.0025, p < 0.005). Early stage of SSc and high disease activity were accompanied by a rise in EPC numbers in blood that correlated positively with severity of peripheral vascular manifestations (r = 0.34, p < 0.05). EPC reduction was attributed to the late stage of disease and had a strong relation to the development of severe internal organ (predominantly cardiac) involvement and pulmonary hypertension. There was a lack of EPC mobilization from BM in response to ischemia and tissue damage late in disease course. A decrease in circulating EPC numbers was closely associated with endothelial dysfunction and morphological signs of destructive microangiopathy.

Conclusions: At an early stage of systemic sclerosis EPC mobilization in response to tissue ischemia was preserved, but the levels of these cells dramatically dropped along with disease progression. EPC reduction strongly contributed to endothelial dysfunction and impaired angiogenesis leading to the development of severe cardiac disease and pulmonary hypertension—life-threatening complications of SSc.

199 CULTURAL AND HIGHLY SENSITIVE MOLECULAR TECHNIQUES FOR DETECTION OF CHLAMYDIA PNEUMONIAE IN SYNOVIAL FLUID AND BLOOD SPECIMENS FROM PATIENTS WITH CHRONIC SYNOVITIS

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Background and aim: Chlamydia pneumoniae (C pneumoniae) is a ubiquitous pathogen associated with upper respiratory tract entities and, although controversial, with a number of illness such as atherosclerosis, multiple sclerosis and joint diseases. While Chlamydia trachomatis-triggered reactive arthritis (ReA) is a well-documented entity extensively described, the role of C pneumoniae in the inflammatory oligoarthritis is less known. C pneumoniae is difficult to grow from synovial fluid (SF), and serology is not always effective for final diagnosis. We report a case of ankylosing spondylitis in whom C pneumoniae was repeatedly isolated from the SF and blood specimens by cultural and molecular methods.

Methods: SF and PBMC specimens were collected from 6 patients with chronic synovitis, one of them with relapsing joint swelling of the knee affected by ankylosing spondylitis. Serum anti-Chlamydia immunoglobulins were searched by ELISA (Cp Quant®, Eurospital, Italy). Specimens were inoculated on HEP-2 cells in duplicate wells, and incubated in CO2 for 3 h at 35°C. Cells grown in the half wells were harvested and fixed onto immunofluorescence (IF) slides using monoclonal antibodies FITC-conjugate specific for C pneumoniae (Institute Pasteur, Paris, Unit of Biological Cellular Interactions). To increase the number of bacterial inclusions, the resting wells were centrifuged and incubated for other 72 h with additional centrifugations on 3rd and then on 4th and 5th culture day, with medium refreshment on 72 h only. Culture supernatants underwent DNA extraction. PCR, Reverse Transcriptase (RT) PCR targeting the 16S rRNA, the Momp gene and the Heat shock Protein 60 of C pneumoniae were employed. Real-time PCR was also performed.

Results: Of the 6 patients, 5 had a positive PCR result which was confirmed by RT-PCR. When these samples were put into culture, only three did grow Chlamydia after culture. For the patient with ankylosing spondylitis, the anti-Chlamydia antibodies in the serum were high and negative by IF. The patient became PCR positive after 144 h. This indicates that C pneumoniae is difficult to grow from synovial fluid (SF), and serology is not always effective for final diagnosis. We report a case of ankylosing spondylitis in whom C pneumoniae was repeatedly isolated from the SF and blood specimens by cultural and molecular methods.

Conclusions: Our culture method proved efficacious; the sensitivity was improved by additional centrifugation associated with extension of culture time. C pneumoniae reaches the articular cavity within monocytes surviving in a vegetative state, and triggers joint inflammation by mechanisms which are still unknown. The PBMC specimens, negative for DNA when directly extracted, became PCR positive after 144 h. This indicates that C pneumoniae is able to survive in PBMC in an infective stage, as demonstrated by their passage on HEP-2 cells. The high expression of C pneumoniae Hsp60 in the PBMC and synovial fluid confirms the ability of C pneumoniae to survive inside these compartments in vital and metabolically active forms. With concern with real-time PCR, the number of chlamydial DNA copies found with Hsp60 gene was higher before than after culture. By contrast, the selective decrease of 16S rRNA before culture in SF and PBMCs leads to hypothesize, as shown by RT-PCR, a different expression of Chlamydophila genes during the different phases of infection.
ENDOTHELIAL PROGENITOR CELLS IN SYSTEMIC SCLEROSIS: QUANTIFICATION, COLONY FORMING AND PROLIFERATION ASSAYS

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Background: Contradictory results have been reported regarding the number of circulating endothelial progenitor cells (EPCs) participating in vasculogenesis in systemic sclerosis (SSc).

Objective: To determine the number of circulating EPCs, assessed by flow cytometry and count of EPCs colony forming unit (CFU) in SSc patients and healthy controls.

Patients and methods: 30 SSc patients and 18 controls matched for age and sex were included. SSc patients had a mean disease duration of 9 ± 10 years, 66% had the limited cutaneous subtype, 12% pulmonary arterial hypertension and 20% digital ulcers.

Patients and methods: 30 SSc patients and 18 controls matched for age and sex were included. SSc patients had a mean disease duration of 9 ± 10 years, 66% had the limited cutaneous subtype, 12% pulmonary arterial hypertension and 20% digital ulcers.

Quantification by flow cytometry: the negative lineage mononuclear cells were enriched from peripheral-blood mononuclear cells using a human progenitor cell enrichment cocktail and collected on a Ficoll density gradient centrifugation. This negative lineage population was then subjected to a triple labelling with VEGFR2-APC, CD133-PE and CD34-FITC antibodies associated with 7AAD labelling (identification of dead cells). EPC population was defined as the Lin-CD34+/CD133+/KDR+/7AAD-.

Evaluation of the number of EPCs colonies: blood mononuclear cell fraction was collected on a Ficoll density gradient centrifugation and cultured on collagen-coated chamber slides in EGM2 medium for at least four weeks. The number of CFU-EPCs and their delay of appearance in patients and controls were compared. Population doublings: The proliferation curves of patients and controls CFU-EPCs were compared to those of human umbilical vein endothelial cells (HUVECs).

Results: SSc patients had significantly higher circulating EPC levels than controls (median 92 [11-248] vs 54 [7-275] EPCs for 1 million lin-mononuclear cells; p = 0.002). There was no association between the number of EPCs and patients' phenotype.

The colonies' formation and delay of appearance did not differ between SSc patients and controls, but the proportion of subjects having more than 5 EPC-CFU was significantly higher in the SSc group than in controls (8/15 versus 1/7; p<0.05). In SSc patients, the formation of colonies was associated with a high number of EPC detected by flow cytometry (p=0.002). EPCs isolated from SSc patients and controls had a significantly higher proliferation capacity than HUVECs.

Conclusion: The number of circulating EPC is increased in SSc. This high level may have a multifactorial origin as we found no association between the number of these cells and disease phenotype. These cells have the ability to form colonies in vitro and have high proliferation capacity. Further analyses are now warranted to study the differentiation ability toward mature endothelial cells and the functional characteristics of EPCs in SSc.
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