FINAL REPORT

RESEARCH PROGRAM: “LA DIAGNOSI PRECOCE DELLA SCLERODERMIA”

RESEARCH UNIT: Rheumatologic and Autoimmune Diseases Unit, Department of Biomedical Sciences and Human Oncology, University of Bari.

TITLE: CORRELATION OF HLA-G EXPRESSION WITH PRE-CLINICAL MANIFESTATIONS OF SYSTEMIC SCLEROSIS.

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INTRODUCTION

In the research project funded by GILS, we have evaluated the serum levels of Human leukocyte antigen-G (HLA-G) in patients with systemic sclerosis (SSc) in relation to the clinical activity of the disease, along with HLA-G genetic polymorphism, namely the presence (Ins) or absence (Del) of a 14bp polymorphism in exon 8 at the 3′UTR of the HLA-G gene.

In addition, we have analyzed the distribution and expression of the transcription factor forkhead BOX E3 (FOXE3), which has never been implicated in the pathogenesis of SSc and which is the target of some anti-centromeric-associated protein A (CENP-A) antibodies (Ab). Patients with Ab to FOXE-3 appear to have a more favorable prognosis as compared to those which are anti- FOXE3 negative (Peroza et al., Arthritis Research and Therapy, 2004).

Finally, we have evaluated clinical correlates of Ab defining subsets of anti-CENP-A Ab.

HLA-G IN SYSTEMIC SCLEROSIS

HLA-G has a tolerogenic function and could play a role in the pathogenesis of immune-mediated diseases, including SSc. Aim of the study was to evaluate HLA-G serum expression and the HLA-G gene 14bp insertion/deletion (del/del+) polymorphism in patients with SSc to search for possible associations with clinical and laboratory variables. Soluble (s) HLA-G was measured by ELISA in sera from 77 patients with SSc and 32 healthy donors (HD); the 14bp del/del+ polymorphism was evaluated by PCR of PBMC genomic DNA. Receiver operating characteristics (ROC) analysis was used to identify the HLA-G cutoff that best discriminated dichotomized clinical and serological variables. This cutoff was employed to subdivide SSc patients into HLA-G high (HLA-G+) and low (HLA-G−) profile groups. Levels of sHLA-G were not statistically different between SSc patients and HD, nor between distinct autoantibody subsets of SSc. When SSc patients were subdivided by HLA-G positivity and negativity, significantly different scores were obtained for mRss, the general and kidney Medsger severity scores and disease activity index, and especially the Δ heart/lung. Fisher’s exact test showed an increased frequency of renal involvement, active disease (defined as a disease activity index≥3), and Δ heart/lung in the low sHLA-G group. Moreover, a higher frequency of scleredema was detected in the del+/del+ than in the del-/del- group.

The inverse relationships of HLA-G with mRss, renal involvement and deterioration of heart/lung function indicate that this molecule can have modulatory effects on the disease, thus behaving as an anti-inflammatory molecule. Future studies will address this issue, as well as whether HLA-G can be a predictive marker of the clinical course either in very early stages of the SSc, along with auto-Ab and the capillaroscopy pattern, or in fully established disease.
EXPRESSION OF THE TRANSCRIPTION FACTOR FORKHEAD BOX E3 (FOXE3) IN MONOCYTES FROM PATIENTS WITH SYSTEMIC SCLEROSIS AND CORRELATION WITH THEIR SEROLOGICAL PROFILE.

The process of epithelial-mesenchymal transition (EMT) has been regarded in SSc as one of the possible mechanisms favoring tissue accumulation of monocyte-derived fibrocytes or myofibroblasts, which contribute to tissue fibrosis [1]. Forkhead box E3 (FOXE3) is a transcription factor involved in EMT of lens epithelial cells (LEC). Its expression progressively decreases with the migration of LEC from the anterior to the equatorial region. FOXE3 expression cessation marks initiation of fiber differentiation, suggesting that the loss of FOXE3 expression favors a pro-fibrotic phenotype [2]. No data are available on mRNA FOXE3 expression in sites other than LEC. In this study, we investigated the FOXE3 mRNA expression in unstimulated and TGF-β- or IL-4-stimulated monocytes from SSc patients and HD, to establish whether i) FOXE3 is constitutively expressed in human monocytes; ii) FOXE3 expression can be modulated in vitro by cytokines involved in SSc profibrotic process; iii) there is an association between FOXE3 expression and a particular SSc serological profile.

PBMC were isolated from heparinized peripheral blood of 9 patients with SSc (5 ScI70⁺; 4 ScI70⁻), and 3 HD by Ficoll-Hypaque density gradient centrifugation. Monocytes (CD14⁺) were isolated by positive selection using microbeads. Cells (1x10⁶ cells/ml) were stimulated TGF-β (10 ng/ml) and IL-4 (40 ng/ml) for 14 days. mRNA was extracted and semi-quantitative PCR was performed to assess FOXE3 expression. GM-CSF stimulation (50ng/ml) was used as positive control. The levels of FOXE3 mRNA were quantified by normalizing its expression against that of GAPDH. Expression was measured as mean relative expression level (MREL). Variation of expression was measured as mean fold change (MFC). Similar baseline levels of FOXE3 mRNA was observed in unstimulated CD14⁺ cells from SSc patients and HD (MREL SSc= 0.32; HD=0.26). As expected, GM-CSF stimulation of CD14⁺ cells from SSc patients and HD markedly up-regulated FOXE3 expression (SSc: MFC=3.24; HD: MFC=1.84). TGF-β and IL-4 behaved similarly to GM-CSF in enhancing FOXE3 expression in CD14⁺ cells from all HD (MFC<sub>TGF-β</sub>=1.35; MFC<sub>IL-4</sub>=1.59) and from 3 out 4 ScI70 patients (MFC<sub>TGF-β</sub>=2.36; MFC<sub>IL-4</sub>=2.9), being the expression unchanged in the remaining ScI70⁺ patient. By contrast, in the 4 ScI70⁺ patients, CD14⁺FOXE3 expression markedly decreased following these cytokines stimulation (MFC<sub>TGF-β</sub>=0.28; MFC<sub>IL-4</sub>=0.31). This is the first study to demonstrate FOXE3 mRNA expression in monocytes from HD and SSc patients, and its differential expression following TGF-β and IL-4 stimulation, correlating with the serological profile of SSc patients. The data suggest that the down-regulation of FOXE3 induced by TGF-β and IL-4 may direct monocytes toward a more profibrotic phenotype in ScI70⁺ as compared to ScI70⁻ patients. The relationship of this finding with the recently detected anti-FOXE3 Ab in SSc sera [3], remains to be determined.

References:
SUBSPECIFICITIES OF ANTI-CENTROMERIC-ASSOCIATED PROTEIN A (CENP-A) ANTIBODIES (AB) CAN IDENTIFIED A SUBSET OF PATIENTS AT HIGHER RISK OF DEVELOPING PULMONARY HYPERTENSION

In SSc, anti-CENP Ab positive patients (pts) are at higher risk of developing pulmonary hypertension (PAH) compared to pts expressing other ANA specificities. In a previous investigation (1), IgG against the immunodominant epitope of CENP-A spanning amino acid 1-17 (peptide Ap1-17), were purified from sera of patient (pt) 4 and pt14 and used to isolate specific phage clones expressing peptide (pc). Pcs specific for Pt4 IgG (pc4s) and pt14 IgG (pc14s) displayed differential reactivity with anti-Ap1-17 IgG from other SSc pts (1, 2), indicating they can distinguish different subsets within anti-CENP-A Ab population. Therefore we assess the reactivity of sera from 84 anti-CENP-A Ab positive pts with pc4.1, pc4.2, pc14.1 and pc14.2, and searched for clinical correlates.

Clinical and serological data were collected as previously described (2). Systolic pulmonary arterial pressure (sPAP) was measured using Doppler echocardiography. The reactivity and specificity of serum with pc was assessed by indirect ELISA using mouse anti-M13 mAb to capture pc to the plates and xeno-Ab to the Fc portion of human IgG to detect pc-bound sera IgG. Multivariate forward stepwise linear (or logistic) regression analysis was used to define a predictive association between a clinical variable and the levels of anti-pcs Ab.

Following a linear regression analysis, anti-pc4.2 and pc14.1 Ab were the only serological variables retained in the model able to predict sPAP and DLCO, albeit in an opposite way: an increased of anti-pc4.2 predicted a sPAP increase (p<0.001) and DLCO decrease (p=0.002), while an increase of anti-pc14.1 was associated to a sPAP decrease (p=0.015). Along the same line, anti-pc4.2 and anti-pc14.1 were associated to an increase and decrease score respectively of the Medsger severity scale item lung. Neither anti-Ap1-17 nor -Ap17-30 Ab (reflecting CENP specificity) were retained in the model for any clinical outcome variable included. To evaluate the strength of prediction of sPAP by anti-pc Ab, a forward stepwise logistic regression analysis was performed by including dichotomized sPAP (cut-off >45 mmHg) as outcome variable and anti-pc Ab as predictors, along with possible confounding factors namely gender, disease duration and age at diagnosis. Again, anti-pc4.2 and pc14.1 were retained in the model: positivity for sPAP was associated with high levels of anti-pc4.2 Ab (p=0.038), while negativity for sPAP was associated with high levels of anti-pc14.1 Ab (p=0.002).

Overall the data indicate that patients expressing high levels of Ab to the subspecificities of CENP-A defined by pc4.1 are at higher risk of developing PAH, whereas those expressing high levels of Ab to the specificity defined by pc14.1 are more protected from this complication.

This is the first study in which subsets of anti-CENP-positive patients have been defined at higher risk to develop PAH. The data presented here set the ground for future studies aiming at defining molecules involved in the pathogenesis of PAH and of Ab (anti-pcp14.8 Ab like) which can block the process leading to PAH. Also, future follow-up studies will evaluate how the early recognition of these subset of auto-Ab will influence the prognosis of these patients in the clinical settings of very early or full established SSc.
References:

RAYNAUD'S PHENOMENON AND ENVIRONMENTAL FACTORS

Increased amount of evidence indicates that environment play a role in the pathogenesis of RP and SSc. Among risk factors, benzene, vinyl-chloride monomer, crystalline silica, white spirit, solvents (chlorinated and ketones), all have been reported to be associated to an increase number of RP events or in the evolution of RP to SSc. Finally, mice submitted to subcutaneous injection of oxidative substances develop SSc-like disease and SSc-specific auto-Ab, their specificity being dependent on the type of oxidative substance injected.

To get insight the role of environment factors in RP onset or its evolution to SSc, we set up an epidemiological self-administered questionnaire for about 500 patients affected by primary RP and SSc-RP and to an equal number of healthy individuals. This would be one of the largest Italian patient’s data banks available. Currently, seven Rheumatology (or Internal Medicine) centers have joined the project. The questionnaire analyzes several characteristics of RP patients (e.g. clinical features, influence on the quality of life, disease activity), family history of disease (e.g. for autoimmune diseases or RP), socioeconomic features, aspects of social life (e.g. social status, place of residence), medical history (i.e., thyroid disorders, arthritis, dermatitis, carpal tunnel syndrome), voluptuary habits (drugs, smoke), habits of daily living (pro-oxidative or chlorinated compounds (i.e., nail polish), sport practiced (e.g. tennis, golf, cycling), exposure to implants (prosthesis, silicon implants, intrauterine device and contact lenses), work history and possible exposure to pollutants (type of work and exposure to pollutants and chemicals). When possible, sera and lymphocytes are also being collected, for evaluation of genomic and sHLA-G. Data are being included in a Microsoft Office Excel sheet. Until now, we have received data from 3 out of 7 programmed centers. When completed, they will be elaborated to assess the role of environment factors on primary Raynaud's phenomenon as compared to SSc and HD.

LIST OF PUBLICATIONS REPORTING GILS FINANCIAL SUPPORTS

MANUSCRIPTS
Favoino E, Favia I, Valentini G, Perosa F. Role of the transcription factor Forked Box E3 (FOXE3) in the pathogenesis of systemic sclerosis, Manuscript in preparation.

LIST OF ABSTRACTS REPORTING GILS FINANCIAL SUPPORTS.

Favoino E, Favia IE, Digiglio L, Perosa F. Expression of the transcription factor forkhead box E3 (FOXE3) in peripheral blood mononuclear cells of patients with systemic sclerosis. Clinical and Experimental Rheumatology 2014; 32 (Suppl81), s63.


Sincerely,

Prof. Federico PEROSA