Investigation of pathogenetic factors (smoking, citrullination, and microvesicles) in autoimmune rheumatic diseases

PhD thesis

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Budapest, 2012

INTRODUCTION

Pathomechanism of autoimmun diseases

Autoimmun diseases affect 1.5-2.5% of the population, and often lead to severe organ damage, thus, they place a significant burden for the society. Despite the several new drugs, their treatment is challenging. Therefore, it is essential to better understand their pathogenesis and risk factors.

Both genetic and environmental factors play a role in their development. Environmental factors such as smoking or infections may evoke immunoregulatory disturbance in individuals with genetic susceptibility (specific HLA-allele carriers). This phase is considered as the preclinical stage of the autoimmune diseases. Laboratory abnormalities such as the appearance of autoantibodies (rheumatoid factor [RF], anti-citrullinated protein antibody [ACPA], and anti-Jo-1) in the blood are seen without clinical symptoms. Due to further triggering factors, the immunoregulatory disturbance may manifest in autoimmun disease. The pathological process is regulated by several effector mechanisms: inflammatory cells (T- and B-cells, macrophages, and fibroblasts) and their secreted mediators such as cytokines (tumor necrosis factor [TNF], interleukin 1, 6, 17, and interferons), prostaglandins, reactive oxygen intermediates, and extracellular vesicles. Chronic inflammation may bring on definitive organ damage (e.g. joint and bone destruction or lung fibrosis), which varies according to the specific disease. In this work certain factors from this pathogenetic chain were investigated.

Microvesicles

The role of MVs has been suggested in several autoimmune rheumatic diseases. MVs belong to the extracellular vesicles, which participate in intercellular communication. According to the size, three types of extracellular vesicles are distinguished: exosomes (50-100 nm), MVs (100-1000 nm), and apoptotic bodies (1-5 μm). Compared to healthy controls, elevated levels of MVs have been confirmed in rheumatoid arthritis (RA), systemic lupus erythematosus, systemic sclerosis, and vasculitides. According to these results, the pathogenetic role of MVs and their application as biomarkers have been suggested.

Rheumatoid arthritis

RA affects mainly the small joints of the hands and feet, and usually accompanied by symmetric polyarthritis. Epidemiological studies have confirmed an important gene-environment interaction, i.e. carrying specific HLA-DRB1 alleles and smoking confer a high risk for the development of ACPA positive RA. According to recent research, ACPAs may have pathogenetic role in the disease, however, the mechanism of ACPA production and its association with smoking is not clear.

ACPAs are produced against citrullinated proteins. Citrullination is a posttranslational modification. The peptidyl arginine deiminase (PAD) enzyme converts peptidyl arginine into peptidyl citrulline. Increased expression of the PAD2 and 4 isoenzymes is seen in RA synovitis. Smoking may promote citrullination in the lung.

TNF has an essential role in the maintenance of inflammation in RA. TNF-blockers are efficient treatment options. However, it is unclear how smoking influences the various effects and production of TNF.
It is unknown on the cellular level why smoking confers a risk for RA or how its association with genetic susceptibility contributes to the pathogenesis of the disease.

Polymyositis/dermatomyositis

Polymyositis/dermatomyositis (PM/DM) is an autoimmune disease accompanied by progressive muscle weakness and skin lesions. Both genetic (DR3 association) and environmental (e.g. infections, UV radiation) factors play a role in its development. Myositis specific autoantibodies are found in the blood of patients. They include anti-synthetase autoantibodies (e.g. anti-Jo-1), which are more frequent in antisynthetase syndrome.

AIMS

1. To investigate the mechanism of ACPA production in a non-arthritic patient group:
   A. whether smoking leads to increased citrullination and,
   B. whether increased citrullination induces autoantibody formation.
2. To evaluate the effects of cigarette smoke in human T lymphocytes:
   A. To develop a standardized method for the absorption of smoke.
   B. To investigate the effects of smoking on TNFR1 and 2 expression, and apoptosis.
3. To analyze the role of MVs in PM/DM:
   A. To measure plasma MVs (lymphocyte, monocyte, and muscle derived MVs).
   B. To evaluate the morphology of MVs.
   C. To search for biomarkers by correlating MVs with the pathogenesis, therapy, clinical picture and diagnostic laboratory parameters of the disease.

PATIENTS AND METHODS

Study subjects

Patients in the study of smoking, citrullination, and ACPA production

Samples of patients and healthy controls (n=109) were collected in the Department of Pulmonology, Semmelweis University. Ten patient groups were distinguished according to the smoking history and pulmonology diagnosis: non-smoker (never smoker) and smoker (ever smoker) healthy controls, patients suffering from bronchial asthma, sarcoidosis, chronic obstructive pulmonary disease, and lung cancer. Sera were collected for ELISA (Enzyme Linked Immunosorbent Assay) measurements (RF, PAD4 and ACPAs). Bronchoscopic samples were taken from 18 patients for tissue microarrays (TMAs), which were immunostained for CK7, PAD4, and citrullinated proteins. Further 100 TMAs of lung cancer patients were available in the Department I of Pathology, Semmelweis University, and were immunostained for the 3 tissue antigens. All patients filled in a questionnaire about their smoking habits. The intensity of smoking was quantified by packyears (= average number of cigarettes smoked a day * years of smoking / 20; e.g. 5 packyears means an average of one pack of cigarettes smoked for 5 years).

Patients in the MV study

The blood samples of twenty PM/DM patients and twenty healthy controls were collected in the Department of Rheumatology, Charles University, Prague. Muscle strength was evaluated with manual

muscle test, and organ specific and global disease activities were determined by visual analogue scales. C-reactive protein levels, creatine kinase (CK) and lactate dehydrogenase enzyme activity, myoglobin and creatinine blood levels, lymphocyte and thrombocyte counts, anti-Jo-1 positivity and lung involvement were also recorded.

Absorption of smoke

Smoke was absorbed in a sterile box with the help of a retort system worked out by our research team. The brand of cigarettes was 'red Symphonia'. Jurkat cells and a healthy individual's PBMCs were treated with the various concentrations of smoke containing cell medium.

Serum PAD4, ACPA and IgA RF ELISA

The PAD4 and IgA RF serum levels of pulmonology patients were measured with ELISA method. Serum ACPA levels were determined in two ways: with 1) a commercial ELISA kit (anti-CCP titer) and 2) citrulline or arginine containing filaggrin peptides (anti-filaggrin antibody) synthetized by the Peptide Chemistry Research Group, Eotvos Lorand University. The levels of antibodies reactive to citrulline or arginine containing filaggrin peptides were measured, and their ratio was calculated. The ratio below 1.5 means there is no autoantibody response against citrullinated proteins (filaggrin) while a value close to 1.5-2 (seen in RA) suggests ACPA production.

Measurement of CK enzyme activity

CK enzyme activity was measured with kinetic spectrophotometric method in the ultracentrifuge fraction of blood plasma of 6 PM/DM and 6 healthy controls.

Flow cytometry

TNFR1 and 2 levels, and annexing V binding (characteristic of apoptosis) were measured with the help of fluorochrome conjugated monoclonal antibodies in the cells treated with smoke.

T cell (CD3 positive), B cell (CD19) and monocyte (CD14) derived plasma MV levels of 20 PM/DM and 20 healthy controls were measured with the help of fluorochrome conjugated monoclonal antibodies for the same time in the same conditions.

Immunohistochemistry

The consecutive sections of TMAs were immunostained for CK7, PAD4 and citrullinated proteins in the pulmonology study. The expression level was quantified by immunoreactive score calculated by the staining intensity and the percentage of positive cells.

Electron microscopy

The morphology of MVs derived from PM/DM patients and healthy controls were analyzed by transmission electron microscopy.

Statistics

Mann-Whitney U-test, Kruskal-Wallis one-way variance (then Dunn's posthoc test), Fischer's exact test, Spearman's rank correlation test and linear regression. PAD4 values above the mean plus double of standard deviation of non-smoker healthy controls were regarded elevated. P values below 0.05 were considered significant.

RESULTS

The study of smoking, citrullination, and ACPA production

Tissue expression of citrullinated proteins, CK7, and PAD4

Citrullinated proteins distinguished the tumor with a strong staining. Only a mild basal staining was seen in the surrounding, healthy tissues. Citrullinated proteins and CK7 (known tumor marker) colocalized in the tumor samples, and their immunoreactive score correlated.

Furthermore, the expression of PAD4 enzyme and CK7 colocalized. Tumor cells specifically immunostained for PAD4. The surrounding tissues were negative. The immunoreactive score of PAD4 and CK7 correlated.

There was no difference between smoker and non-smoker lung cancer patients regarding the expression of citrullinated proteins, CK7, and PAD4.

Serum PAD4, ACPA, and IgA RF

After investigating the expression of citrullinated proteins and PAD4, the serum levels of the enzyme were also measured.

Increased PAD4 levels were seen in a high ratio (46%) of smoker lung cancer patients, compared to controls, however, PAD4 levels of non-smoker lung cancer patients were in the range seen in non-smoker healthy controls. The PAD4 levels of smoker lung cancer patients were significantly higher than non-smoker healthy controls.

Most patients and healthy controls were anti-CCP negative except two smoker lung cancer patients. Anti-filaggrin ACPAs were not found in the pulmonology patients.

Increased IgA RF levels were seen in a high portion (23.5%) of smoker lung cancer patients, and they significantly differed from the RF levels of non-smoker lung cancer patients, whose samples were RF negative.

Effects of smoke on TNFR expression

Absorbed cigarette smoke lead to apoptosis. The level of apoptosis was below 5% in cells treated with 5-10-fold dilution of smoke containing medium.

TNFR1 expression increased as a function of smoke concentration. 5.5-fold increase was seen in the medium containing 10-fold diluted smoke, while 50-fold dilution resulted in an appr. 2-fold increase of TNFR1 expression. TNFR2 expression did not change by such a magnitude. About 3.5-fold increase was seen upon 10-fold smoke dilution, and TNFR2 expression was similar to that of untreated cells upon 50-fold smoke dilution.

Microvesicles

The amount of CD3, CD14 and CD19 positive MVs (measured for the same time in the same conditions) were significantly higher in PM/DM patients, compared to healthy controls. Therefore, significantly more T- and B-cell, and monocyte derived MVs may be present in the blood plasma of PM/DM patients, compared to healthy individuals. CK enzyme activity was not detected in the MV rich ultracentrifuged pellet.

Monocyte and B-cell derived MVs positively correlated to muscle strength. Significantly more T- and B-cell, and monocyte derived MVs were seen in the patients with anti-Jo-1 positivity and/or lung involvement.

The electron microscopy revealed MVs in various size and density in healthy controls, while nanotube like structures were seen in PM/DM patients.

CONCLUSIONS

1. It was found that increased PAD4 expression and citrullination were specific for lung cancer, therefore, they may serve as tumor markers in lung cancer.
2. Increased serum PAD4 and IgA RF levels were seen in smoker lung cancer patients, but tissue citrullination and PAD4 expression were similar in smokers and non-smokers.
3. It was confirmed in a non-arthritic patient group (lung cancer patients) that smoking and increased citrullination in the lung might not lead to autoantibody (ACPA) production.
4. An *in vitro* system was developed to investigate the effects of smoking on cell function in a standardized way.
5. It was shown that smoke induced apoptosis, and might increase TNFR1 and 2 levels in human T-lymphocytes as a function of smoke concentration.
6. Significantly higher amounts of T and B cell, and monocyte derived MVs were detected in the blood plasma of PM/DM patients, compared to healthy controls.
7. Immune cell derived MVs correlated to muscle strength, anti-Jo-1 positivity and lung involvement. Thus, they may be used as biomarkers in the disease.
8. CK enzyme activity (suggesting the muscle origin of MVs) was not detected in the plasma MVs of PM/DM patients.

LIST OF PUBLICATIONS

Publications related to the thesis

Baka Z, György B, Géher P, Buzás EI, Falus A, Nagy G. (2012) Citrullination under physiological and pathological conditions. Joint Bone Spine, in press. **IF: 2.460**

Baka Z, Barta P, Losonczy G, Krenács T, Pápay J, Szarka E, Sármay G, Babos F, Magyar A, Géher P, Buzás EI, A, Nagy G. (2011) Specific expression of PAD4 and citrullinated proteins in lung cancer is not associated with anti-CCP antibody production. Int Immunol, 23: 405-414. **IF: 3.301**

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Baka Z, Buzás E, Nagy G (2009) Smoking and rheumatoid arthritis: putting the pieces together. Arthritis Res Ther, 11: 238. **IF: 4.271**

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Cumulative impact factor (research articles only): **11.384**

Cumulative impact factor (with review articles): **18.115**