Biomarkers (autoantibodies, T cells, extracellular vesicles) in rheumatoid arthritis

Ph.D thesis

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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease with joint inflammation affecting 1% of the adult population. Without treatment it results in the development of severe joint deformities and disabilities. Therefore the early therapy for which the assessment of biomarkers and pathogenetic factors in RA is essential, has a striking importance. In our work we investigated the role of three different factors, the natural antibodies, T cell epitope citrullination and the exracellular vesicles (EVs) in the pathomechanism of RA.

Natural autoantibodies are polyreactive, bind with low-affinity, and they are mostly IgM, IgG or IgA immunoglobulins in the serum. They are derived from B1 B cells predominantly. The role of carbohydrate - specific natural autoantibodies in RA has not been fully investigated in spite of the presence of various carbohydrate types (such as glucoseaminoglycans, GAGs) in the joints. Previous studies demonstrated that in a mouse model of arthritis (PGIA, also referred to as aggrecan arthritis) human aggrecan, partially depleted in its chondroitin sulfate (CSC) GAG chains, can provoke a chronic, progressive polyarthritis inducing a strong B cell response. In our experiments we investigated the natural glucosaminoglycan-specific natural autoantibodies in RA.

The role of citrullination in the pathogenesis of RA is shown by the enhanced levels of anti-cyclic citrullinated protein(anti-CCP)-specific antibodies associated with RA. These autoantibodies may be present in the serum decades before the onset of the disease. Citrulline is a non essential amino acid derived from postranslationally modified arginine. The posttranslational modification is catalysed by the Ca2+ dependent peptidyl-arginine deiminase enzyme (PAD), which has five isoforms characterised by different tissue distributions. The presence of two isoforms of the PAD enzyme, PAD2 and PAD4 in the RA synovial fluid (SF) and cells has been already confirmed. Although, the production of citrullinated protein-specific autoantibodies is associated with B cells, several data derived from humans and animal model systems support the hypothesis that besides B cells, T cells have also an

important role in the pathogenesis of RA. Thus, in the second part of our work we assessed the impact of the citrullination of an immunodominant T cells epitope on the T cell recognition, in a well-characterised animal model of RA (PGIA or aggrecan arthritis).

Finally, in the third part of our work, I introduce our results related to extracellular vesicles (EVs) as a newly discovered type of disease biomarkers. EVs that have important role in the intercellular communication are surrounded by phospholipid membrane bilayer, and released by resting, apoptotic cells or by cells upon activation. Several types of EVs have been described recently including exosomes, microvesicles and apoptotic bodies. Exosomes having a diameter of approximately 50-100nm are released from cells by the exocytosis of multivesicular bodies. Microvesicles (MVs) are larger structures with the diameter between 100 and 1000nm and they are formed by blebbing of the plasma membrane. Apoptotic bodies (ABs) are 1-5µm in diameter released as blebs of cells undergoing apoptosis. EVs are present in several biological fluids including synovial fluid, blood and urine. Their use as potential future biomarkers is supported by the fact that their number is elevated in some cancerous and autoimmune diseases. In our work we focused on the comparison of the biophysical parameters of MVs (derived from RA and osteoarthritic synovial fluids and sera) and protein complexes which are often present in biological fluids, and also on the proteomic analysis of BALB/c thymus derived MVs and ABs.

Goals of the studies

We aimed at the assessment of autoantibodies, T cells and microvesicles as potential biomarkers and pathogenetic factors in RA.

1. Assessement of natural, glucosaminoglycan (GAG)-specific autoantibodies in rheumatoid arthritis (RA)

1.1 To investigate the anti-GAG antibodies as potential disease markers

1.2 To assess the cross-reactivity of GAG antibodies

1.3 To determine the carbohydrate recognition pattern of RA specific anti-GAG antibodies.

2. Investigation of T cell antigen recognition in BALB/c mice

2.1 To analyse the effect of the citrullination of an immunodominant T cell epitope on the T cell response

2.2 Gene expression analysis of the PAD enzymes in the thymus

2.3 To detect the polyclonal T cell response of the epitope-specific T cell receptor transgenic mice

3. Assessment of the extracellular vesicles

3.1 To investigate the biophysical parameters of microvesicles and protein complexes

3.2 To analyse the proteomic content of the microvesicles and apoptotic bodies derived from BALB/c thymus

Methods

Mice and immunisation protocols

To assess T cell response in mice we used 12-16 week old BALB/c female mice and ATEGRVRVNSAYQDK (P70-84, ATE peptide) specific TCR transgene mice.

Peptide immunisation

Female BALB/c mice were subcutaneously injected in their hind paws with 5-25µg synthetic peptide and complete Freud adjuvant (Sigma-Aldrich). PBS in CFA served as control. The lymph node cells were isolated on the 9. day followed by the assessment of T cell response in ELISPOT experiments.

Induction of aggrecan arthritis

BALB/c female mice were intraperitoneally immunized for three times with glycosidase digested human proteoglycan with the adjuvant of dimethyl-dioctadecyl-ammonium bromide. We isolated the lymph nodes 4 months after the first immunisation.

Antigens

Glycosidase digested aggrecan

Bovine and human aggrecan were digested with 100 U/mg β-galactosidase (Sigma) in 0,15M citric acid-phosphate buffer (pH=4.3) and with 240 U/mg hyaluronidase in 0,2M NaCl-acetic acid buffer (pH=5,0) for 24 hours at 37°C in the presence of protease inhibitors.

Synthetic peptides

The aggrecan derived T cell epitope peptides were sythetised by our collegues from the Department of Organic Chemistry, Lóránd Eötvös University, Budapest.

Patients

Human samples were obtained from patients treated in the Hospital of Hospitaller Brothers of St John of God, Budapest; in the Department of Orthopedics, University Medical School of Szeged; in the Department of Rheumatology, Semmelweis University, in the National Traumatology Hospital, Budapest.

In vitro assessement of immune cells

Proliferation assay

Speen cells derived from ATE peptide specific TCR-tg mice were placed in $33x10^5$/200µl/well density in 96-well plates. Stimulating antigen concentrations were as follows: 1 and 5 µg/ml huATE-RR, 1, 5 and 25 µg/ml huATE-RX, -XR, -XX or 10 and 40 µg/ml mATE-R, -X. Incubation for 96 hours were followed by the addition of [^{3}H]timidine. Radioactivity was measured 14 hours later.

ELISPOT

We detected IFNγ secreting lymph node cells with the usage of Mouse IFNγ ELISPOT Ready-Set-Go Systems (eBioscience), and steril MultiSceen^{TM}-IP plates (Millipore Corporation) according to the instructions of the manufacturers.

Gene expression analysis

To isolate total RNA from BALB/c derived thymus, bone marrow and spleen samples we used RNeasy® Mini Kit (Qiagen). Relative quantification of Padi2, Padi4 was performed with TaqMan quantitative PCR assays on an AbiPrism® 7000 Sequence Detector (Applied Biosystems).

Experiments based on antigen-antigen interactions

Detection of anti-GAG antibodies with CovaLink ELISA

Carbohydrate antigens (chondroitin sulphate A, B and C, keratan sulphate, low molecular weight heparin sulphate, hyaluronic acid and native and glycosidase-digested human and bovine aggrecan) were covalently bound to the surface of the CovaLink plates (Nunc) at 1 µg/well concentration. Sera and SFl samples were used at 1:100 dilution, HRP-conjugated anti-human IgM and IgG (Sigma) were used as secondary antibodies at 1:50000 and 1:30000 dilutions.

IL-2 ELISA

To test the specificity of 5/4 T cell hybridomas bearing P70-84 peptide epitope-specific TCR we cultured $2x10^4$ hybridoma cells in the presence of $2x10^5$ A20 myeloma cells and synthetic peptide variants. After 24 hours IL-2 levels of the supernatans were analysed by ELISA.

Anti-complement component 3 (C3) ELSIA

Polystyrene plates were coated by 0.2 µg/well anti-human C3 antibody (Sigma). Synovial fluid samples were applied at 1:100 dilution. For detection antibody we used anti IgM-HRPO and anti-human IgG-HRPO (Sigma).

Anti-cyclic citrullinated peptide (anti-CCP) antibodies

The anti-CCP antibody levels were determined by using Immunoscan RA anti-CCP test kit (EURO-Diagnostica).

Rheuma factor (RF)

To determine the serum concentration levels of IgM and IgG RF derived from controls and RA patients we applied AUTOSTATTMII RF IgM and IgG kit (Hycor Biomedical GmbH).

C-reactive protein (CRP) level

The CRP concentrations of human samples were determined with full spectral turbidimetric assay (Randox Laboratoires Ltd.). Data analysis were occured by Olympus AU600 biochemical analysator (Olympus Medical Systems).

Glycochip

IgGs of serum and synovial fluid samples were labeled with Alexa Fluor 350-conjugated antihuman IgG antibody (Molecular Probes) and were applied to the Glycochip. The fluorescence was measured on a Perkin Elmer Victor II spectrofluorimeter.

Immunhisochemistry

Normal adult human cartilage specimens were first incubated with RA serum (1:25), then with anti-human Ig-FITC antibodies (Sigma) and finally were analyzed in a Bio-Rad MRC 1024 confocal laser scanning microscope.

Flow cytomety

For flow cytometry and data analysis we used a FACSCalibur fow cytometer (BD Bioscience) and CellQuest sofware (3.2 version, BD Bioscience).

MHC II binding

To compare the MHC binding of the wild-type and citrullinated peptides, $2x10^5$ A20 cells were pre-incubated with 2, 10 and 50 μg/ml unconjugated human peptide ligands (hATE-RR, hATE-RX, hATE-XR, hATE-XX) or 2, 10 or 50 μg/ml murine peptides (mATE-R and mATE-X) for 1 hour at 37°C. After incubation, 1.5 μg/ml biotinylated hATE-RR (wild-type) peptide was added to the cells for 2 hours at 37°C. After incubation, blocking and washing steps samples were stained with streptavidin-phycoerythrin (1:20, PE) (R&D Systems, Minneapolis, MN, USA)

Detection of immune complexes (IC) and microvesicles (MV)

To determine the event number of MV and IC samples within the MV gate we used the following antibodies: annexinV (AnnV)- FITC, AnnV-PE, anti-CD41a-FITC, anti-CD42a-peridinin-chlorophyll, anti-CD68-FITC and anti-CD45-peridinin-chlorophyll protein-cyanin 5.5 (BD Biosciences), anti-human IgM-FITC (1:150), anti-human IgG-FITC (1:300, Sigma).

Vesicular AnnV

MVs and apoptotic bodies containing samples were stained at 1:300 dilution with AnnV antibody (Sigma). Then vesicles were sedimented (20 500g 20min), and samples were stained with anti-rabbit antibody conjugated with FITC (Becton Dickinson, Franklin Lakes, NJ, USA) for 30 minutes.

Other experiments with EVs

Isolation of MVs and ABs from BALB/c thymus

For the mass spectrometry of MVs and ABs derived from the thymus we isolated the thymuses of 2-week old BALB/c mice. The cells ($1,4x10^9$) were cultured for 24 hours in a CELLine bioreactor (Integra Biosciences). After that, cells were pelleted at 300g for 20 minutes. This was followed by filtration through a 5μm pore size filter by gravity. Apoptotic bodies were pelleted at 2000g for 20 minutes. After the gravity-driven filtration through 0,8μm pore size filter (Millipore) the filtrate were centrifugated at 12 200g for 40 minutes. The MV and AB preparations were diluted in deionized water.

Isolation of human synovial fluid and plasma derived MVs

SF samples were digested with 10 U/ml hyaluronidase (Sigma) at 37°C. Platelets, cell debris and apoptotic bodies were pelleted by centrifugation at 3000g for 10 minutes. The supernatant was filtrated through a 800nm pore size filter (Millipore). MVs were pelleted at 20 500g for 60 minutes using ultracentrifuge.

Transmission electronmicroscopy

MV and apoptotic body preparations were analysed by using HITACHI 7100 electronmicroscope.

Immune electronmicroscopy

For IC detection we incubated the samples with anti-human IgG and anti-human IgM. Samples were analysed by HITACHI 7100 electronmicroscope.

Atomic force microscopy

The surface of the sample structures was performed with tapping mode AFM (PSIA serial no. XE.100). Topography images were processed with XE1 1.6 (PSIA).

Fluorescence microscopy

ICs isolated from RA and OA samples were analysed by Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss).

Dynamic Light Scattering

Sample analysis was performed by our colleges at the Department of Biophysics and Radiation Biology of the Semmelweis University.

Natural immune complex isolation

ICs were isolated from cell-free samples of RA SFs using anti-human IgM and IgG agarose column.

Arteficial ICs

Artificial ICs were generated by mixing antigens (lactoferrine, ovalbumin, mouse IgM) with specific antibodies (anti-human lacoferrine, anti-human ovalbumin and anti mouse IgM) using different ratios.

Detergent lysis of MVs and ICs

Triton X-100, Tween 20, sodium dodecyl sulfate, Igepal-CA630 (Sigma) and a commercially available lysis buffer (BD Biosciences) were used to lyse MVs and to disassemble ICs.

Mass spectrometry

The mass spectrometric analysis of our samples was performed by our collegues in the Chemical Research Center of the Hungarian Academy of Sciences.

Statistical analysis

Statistical analysis was performed using STATISTICA 7.1 software. Three-factor one-way analysis of variance (ANOVA) model was used for analysis of anti-GAG antibody ELISA results. We followed the screening strategy advised by Harrell to find which of the anti-GAG antibodies would be the best disease specific marker(s).

ANOVA was performed for analysis of the effect of citrullinated peptides. Changes were considered significant at $p < 0.05$.

Results

In our work we investigated the GAG-specific immunoglobulins. The anti-GAG antibody concentrations were almost undetectable and uniformly extremely low in the umbilical cord samples, whereas they were present in the sera of adult healthy and RA patients. Interestingly, the anti-GAG antibody concentrations were significantly higher in the sera of RA patients as compared with adult controls. These autoantibodies bind to the extracellular matrix of hyaline cartilage. We next assessed whether the anti-GAG concentrations correlates to the disease activity. Using a multistep approach we found that the anti-chondroitin sulfate C (anti-CSC) IgM antibody concentrations show an inverse correlation with the disease activity marker DAS 28 scores and the CRP concentrations. Thus, we suggest that the anti-CSC IgM is a disease-state biomarker. We hypothesize that during the active phase of the disease, the levels of anti-GAG antibodies are reduced because of the binding of these antibodies to GAGs released from the degrading cartilage. This could explain the significantly reduced level of RA synovial fluid anti-GAG antibodies as compared with anti-GAG levels measured in the serum of RA patients.

In RA unexpectedly few study assess the role of T cells. Following the analysis of the B cell derived natural autoantibodies we investigated the role of peptide epitope citrullination in the T cell recognition in PGIA (aggrecan arthritis), in an animal model of RA. To confirm that citrullination of self-antigens within the thymus was a relevant post-translational protein modification, we tested the expression of genes encoding for Pad2 and Pad4 in various lymphatic organs of BALB/c mice. Interestingly, we found differential expression of *Padi* genes in different lymphatic organs of the mice. Whether the different Pad isoenzymes have preferences for peptide substrates, remain to be established. However, if so, it would raise the interesting possibility that disparate citrullination of self-antigens within the thymus and/or the bone marrow may lead to substantial differences in the negative selection processes of self-reactive T and B cells, which, in turn, may result in mismatched T

cell function and altered antibody repertoire. Thus, post-translational modifications of self-proteins in the thymus may shape the selected peripheral T cell repertoire.
In an effort to characterize citrullinated peptide-recognition by T cells, we used the aggrecan arthritis model with a well-characterized, disease-relevant (both RA and PGIA) T cell epitope (huATE-RR or P70-84 epitope) that contains two arginines (prone to citrullination). We also tested TCR-Tg mice, a model in which T cells are reactive to the P70-84 epitope. We found evidence that the circulating T cell repertoire includes cells with marked affinities for peripherally presented citrullinated epitopes. We next assessed the immunogenicity of the wild type self-epitope (mATE-R) and its citrullinated variant (mATE-X). Interestingly, immunization with the mouse mATE-R (wild-type) peptide alone elicited a very low response, possibly reflecting the elimination of the T cells that recognize this epitope by central tolerance. In contrast, immunization with the citrullinated version of the mATE-X self epitope resulted in a significantly increased primary immune response to this peptide. This suggests that certain citrullinated self-epitope-specific T cells may escape central tolerance induction.

Next, we investigated the role of extracellular vesicles (EVs) as potential disease biomarkers. In our experiments we assessed the potential overlap between the parameters of MVs and protein complexes both present in biological fluids. We cannot differentiate the two populations based on their light scattering properties by DLS and flow cytometry. These findings prompted us to develop a simple novel method to discriminate MVs from ICs. Using detergent lysis, our data show that, in biologic samples, in which ICs are abundant, total even counts within the MV gate probably predict IC rather than MV content. Of importance, the overlapping parameters of ICs and MVs may also affect the purity of isolated MV preparations.

Finally, we determined the proteomic composition of two populations of BALB/c thymus derived membrane bound vesicles (MVs and apoptotic bodies) surprisingly underrepresentated in the literature. In our study we have identified several proteins that suggest a previously unidentified roles of the membrane vesicles in regulating intrathymic processes. We have detected several proteins that have been implicated as autoantigens in human autoimmune diseases. Both thymic Abs and MVs contained

alpha enolase, a glycolytic enzyme that has been recently suggested to play role in rheumatoid arthritis and other autoimmune diseases. Furthermore, we detected the presence of heat shock proteins implicated as autoantigens in atherosclerosis. Our work has also revealed the presence of histones (H1-H4) that considered a major autoantigenic targets in SLE in both Abs and MVs.

The presence and abundance of histone proteins in ABs were not surprising given the mechanism by which the apoptotic blebs are generated. However, unexpectedly we have identified strikingly high number of histone proteins also in MVs. This suggests that a major proportion of thymus derived MVs is possibly generated during apoptosis. Another exiting finding of our study was the identification of key regulatory and signaling molecules in membrane vesicles. Thus, these subcellular structures may mediate significant modulating functions in the thymic microenvironment. Our data suggest that not only MVs, but also ABs are likely to have complex functions in regulation of thymus, and that makes them interesting targets for the future research.

Conclusions

1. Our work suggests that the natural glucosaminoglycan (GAG)-specific autoantibodies have an important role in the pathomechanism of RA. Our results indicated that the serum and synovial fluid levels of anti-GAG antibodies are increased in RA. We found that the anti-chondroitin sulfate C (anti-CSC) IgM antibody concentrations show an inverse correlation with the disease activity. Thus, we suggest that the anti-CSC IgM is a disease-state biomarker.

2. We also investigate the role of peptide epitope citrullination in the T cell recognition in PGIA (aggrecan arthritis), in an animal model of RA. We have found that an immunodominant T cell epitope (P70-80 or huATE-RR) and its citrullinated variants provoke a significant IFNγ response in peptide immunized BALB/c mice and in epitope specific TCR-transgene mice. We showed the expression of the *Padi2* and *Padi4* genes in different lymphatic organs in mice. Assessing the peripheral T cell

response we found that the immunization with the citrullinated version of the mATE-R self epitope resulted in a significantly increased primary immune response to this peptide compared to that of the wild type peptide. Thus, our data provide evidence that citrullination may significantly effect epitope recognition by T cells.

3. Analysing the biophysical parameters of MVs and protein complexes in biological samples we have found that protein complexes can falsify both the detection results and functional assays of MVs. We developed a new method to discriminate ICs from MVs. Our results may contribute to correct the clinical laboratory assessment of the presence and biological functions of MVs in health and disease.

4. Assessing the protein composition of MVs and apoptotic bodies we have found a strikingly high number of proteins shared by ABs and MVs. We identified several proteins suggesting that a major proportion of thymus derived MVs is possibly generated during apoptosis. Based on our results, we hypothesize that both MVs and ABs may have a complex role in the intrathymic regulating processes.

Lists of own publications

Publications that are related to the subject of the thesis:

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*: contributed equally

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