The investigation of the role of regulator and effector factors in the pathomechanism of autoimmune diseases

Ph.D thesis

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Introduction

In nature, not only mammalian, but fungal and bacterial surfaces also wear high amounts of complex carbohydrate structures. The primary autoimmune target tissue of rheumatoid arthritis (RA) is cartilage that has two major components: a dense network of glycosylated collagen fibers, and the proteoglycan aggrecan. With its negatively charged carbohydrate side chains aggrecan can draw water into the tissue thus supports a tough and resilient load-bearing surface. This complex cartilage matrix can be degraded during RA. While numerous studies have characterized the role of fibroblast-derived proteases in cartilage destruction, during the past decades surprisingly little attention has been paid to the activity of glycosidases in rheumatology. We have demonstrated earlier the ability of exoglycosidases to deplete hyaline cartilage in glycosaminoglycans (GAGs). This study is hardly followed by reports on the role of glycosidases in RA. The synovial glycosidase gene expression pattern has not yet been described. Thus, we decided to characterize for the first time the glycosidase expression profile of synovial membrane (SM) and synovial fibroblasts (SF) isolated from SM samples and to analyze whether the gene expression of glycosidases in SFs is regulated by inflammatory cytokines.

B-cells play an important role in RA pathogenesis. Depletion by the anti-CD20 monoclonal antibody rituximab decreases disease activity and provides prolonged remission. B cells produce a variety of autoantibodies including rheumatoid factor, anti-type II collagen and anti-cyclic citrullinated peptide antibodies. Surprisingly, carbohydrate-specific antibodies have not yet been investigated in RA in spite of the fact that hyaline cartilage and synovial fluid contain large amounts of GAGs including hyaluronic acid, chondroitin sulphate and keratan sulphate. These carbohydrates are degraded and released from cartilage during the course of joint inflammation. Thus, we studied anti-GAG antibodies in the serum of healthy and RA patients. We also tried to find GAG-specific antibodies in serum with the potential to serve as disease or disease-state markers in RA.

It is well known that the RA-associated HLA-DRB1 alleles encode the shared epitope (SE) pentapeptide motif. This SE could either contribute to disease development by preferential binding of a given RA-associated peptide or by molecular mimicry. The C-

terminal G3 domain of proteoglycan aggrecan contains a sequence highly similar to the SE (peptide 135, abbreviated P135: TTYKRRL**QKRSS**RHP). It proved to be a dominant and arthritogenic T-cell epitope in mice. It has been shown earlier that the difference between the QKRAA SE in the HLA sequences and the QKRSS pentapeptide in aggrecan allows cross-recognition of the above peptides by cells isolated from peptide primed mice. Moreover, the arginine(s) within the SE and its flanking region renders them to be potentially susceptible to citrullination. This may be of interest because of the known significance of anti-citrullinated protein-specific autoantibodies in RA and reports describing stronger binding of citrullinated peptides to HLA-DRB1*0401 haplotype than of the noncitrullinated ones. Given the suggested important role of CD4 positive IL-17 secreting cells in the pathomechanism of autoimmune diseases including RA, in the present study we tested the IL-17 response to citrullinated and non-citrullinated SE-carrying peptides, including P135 of cartilage PG.

Finally, we investigated the role of nitrogen monoxide (NO) in the pathogenesis of RA. Several studies in patients with RA have documented evidence of increased endogenous NO synthesis, supporting the hypothesis that overproduction of NO may be important in the pathogenesis of RA. NO, produced in the inflamed joints, may contribute to the peri-articular bone loss observed in RA. Furthermore, NO is known to provoke T lymphocyte dysfunction in systemic lupus erythematosus. During physiological conditions NO was found to regulate physiological T cell activation, mitochondrial biogenesis and increase cytoplasmic calcium level. Thus, we decided to investigate the connection between RA T-cells and the free radical NO.

Aims

The aims of our work were as follows:

1. **The determination of the glycosidase gene expression profile in synovial membrane and synovial fibroblasts of patients with RA and OA.**

a. To characterize the expression of Hex, Gus, Hyal-1, Spam-1, klotho and Hc-gp 39 genes.
b. To investigate the enzyme activities of the most abundantly expressed glycosidases (HEX, GUS) in RA and OA synovial membrane, synovial fibroblast and synovial fluid samples.
c. To study the effect of key cytokines (TNF-α, IL-1β, IL-17, TGF-β1) in the pathomechanism of RA and the effect of NO on the gene expression and enzyme activity of glycosidases.
d. To isolate synovial fibroblast-derived microvesicles and to analyze the microvesicle-associated glycosidase activity.

2. **The detection of anti-glycosaminoglycan antibodies in RA serum and synovial fluid samples.**

a. To identify anti-GAG antibodies, which may serve as disease-state marker in RA.
b. To investigate the cross-reactivity of anti-GAG antibodies with other kind of GAGs, peptidoglycans and fungal polysaccharides.
c. To analyze the antibody recognition of glycosidase digested human cartilage aggrecan.
d. To determine RA-specific carbohydrate-recognition patterns.
e. To support the binding of carbohydrate-specific antibodies to the matrix of hyaline cartilage.

3. The investigation of the effect of peptide epitope citrullination on the IL-17 response of peripheral blood cells.

a. To analyze the IL-17 response of RA and control peripheral blood cells against citrullinated / non-citrullinated, shared epitope carrying / non-carrying peptides (HLA DRB1, cartilage aggrecan peptides: P135 and ATE).
b. To try to find correlation between the P135 citrullination-related break of tolerance and the HLA DRB1 genotype.
d. To investigate the serum antibody reactivity against citrullinated peptides.

4. The determination of intracellular NO level, cytoplasmic calcium concentration and mitochondrial mass in RA and control T-lymphocytes and the investigation of the effect of TNFα on the NO production of RA T-cells.

Methods

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 and in the Imperial College Healthcare National Health Service Trust, London.

Cell culture

Isolation of synovial fibroblast cell strains

SFs were obtained by Dispase II (Roche) enzymatic digestion and were cultured for four to nine passages. These repeated passages ensured the purity of fibroblast cell populations without contaminating macrophages.

Isolation of peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma) gradient separation.

Real Time PCR

Total RNA was extracted from SFs and SMs using the RNeasy® Mini Kit (Qiagen). Relative quantification of hexosaminidase A subunit (HexA), hexosaminidase B subunit (HexB), hexosaminidase D (HexDC), β-D-glucuronidase (GusB), hyaluronidase 1 (Hyal1), 39 kDa human cartilage-glycoprotein (Hc-gp 39), klotho, sperm adhesion molecule 1 (Spam1), matrix metalloproteinase 1 (MMP1) and MMP3 mRNAs was performed with TaqMan quantitative-PCR assays on an ABI PRISM 7000 Sequence Detector (Applied Biosystems).

Enzyme assays

The enzyme activities of SM, SF and SFl samples were determined using chromogenic substrates (GusB: p-nitrophenil-β-D-glucuronide, β-D-N-acetyl-glucosaminidase: p-nitrophenil-N-acetyl-β-D-glucosaminide, β-D-N-acetyl-galactosaminidase: p-nitrophenil-N-acetyl-β-D-galactosaminide (Sigma)). Enzyme activities were normalized to protein content (50 μg) determined by Bradford protein assay.

Effect of cytokines on expression and secretion of glycosidases by synovial fibroblasts

SFs were cultured in the presence of human TNFα (BD Biosciences), IL-1β and TGF-β_1 in 0, 1, 10 and 50 ng/ml concentrations, and of IL-17 (ImmunoTools) in 0, 1, 10 and 100 ng/ml concentrations for 24 hours. The nitric oxide donor (NOC-18) (Molecular Probes) was used in 0, 100 and 1000 μM concentrations.

Histochemical investigations

Enzyme histochemistry of glycosidases in SFs

Fibroblasts were incubated with either 50 μM ImaGene Green C_{12}FDGlcU β-D-glucuronidase or ELF® 97 *N*-acetylglucosaminide substrates (Molecular Probes).

Binding of anti-GAG antibodies to the matrix of hyaline cartilage

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-cytometric analyses

FACS analysis of synovial fibroblast-derived microvesicles

Microvesicles (MVs) were isolated from 24 hours supernatant of RA and OA SFs by ultracentrifugation (500g 10 min, 100000g 1 h). Their enzyme activity was determined using chromogenic substrates and was standardized to 5 μg total protein content.

Determination of immunophenotype and cytokine production of PBMCs

Immunophenotyping of peripheral blood cells has been performed by anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD20, anti-CD56 and anti-CD25 antibodies. Before intracellular cytokine staining (anti-humán-IL-17A-PE) the cells were stimulated *in vitro* for 24 hours in the presence or absence of synthetic peptides (25μg/ml). Flow cytometric bead-based multiplexed assays were used to measure the soluble cytokines.

FACS analysis of peripheral blood lymphocytes

The cell viability was determined by using Annexin V.-FITC (R&D Systems) and propidium-iodide. The mitochondrial mass was monitored by staining with MitoTracker Green-FM, and nonyl acridine orange. Cytoplasmic calcium levels were assessed by loading the cells with Fluo-3-acetoxymethyl ester. Production of NO was assessed using DAF-FM (Molecular Probes).

Electron microscopy of synovial fibroblast-derived MVs

The MV preparations of SF supernatants were examined in a Hitachi 7100 transmission electron microscope.

Detection of carbohydrate-specific autoantibodies

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.

Detection of anti-peptide antibodies

Synthetic peptides were covalently bound to the surface of the CovaLink plates (Nunc) at 1 μg/100μl/well, sera were applied onto the plates using a final 1:100 dilution and finally plates were incubated with peroxidase-conjugated anti-human polyvalent IgGAM (Sigma) in 1:30,000 dilution.

Glycochip

IgGs of serum and SFl 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.

Enzyme-linked immunosorbent spot assay (ELISPOT)

Human IL-17 ELISPOT Ready-Set-Go system (eBioscience) and sterile clear MultiSceenTM-IP plates (Millipore) were used to our ELISPOT measurements. Freshly isolated PBMCs ($2x10^5$) were plated into each well in the presence or absence of 25μg/ml synthetic peptide.

Synthesis of linear peptides

Solid phase methodology using Fmoc/tBu strategy on Rink Amide MBHA resin was applied for the synthesis of linear peptides by our colleagues from the Department of Organic Chemistry, Lóránd Eötvös University, Budapest.

HLA genotyping

The HLA genotyping was carried out in the National Institute of Haematology and Immunology, Budapest.

Anti-cyclic citrullinated peptide (anti-CCP) antibodies

The anti-CCP antibody levels were determined by using Immunoscan RA anti-CCP test kit (EURO-Diagnostica). A cutoff value of 5.5 U/ml was used as a criterion for anti-CCP positivity.

Statistical analysis

Statistical analysis was performed using STATISTICA 7.1 software. The Mann–Whitney rank sum test and paired *t* test was performed for analysis of glycosidases. A 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. Results of T-cell NO production were analyzed by Student's *t* test or Mann–Whitney *U* rank sum test for non-parametric data. Changes were considered significant at $p < 0.05$.

Results

First, we have investigated the glycosidase expression profile and its regulation by inflammatory cytokines in the RA and OA joint. We found a robust gene expression of the glycosidase-like Hc-gp 39 in the SMs, and in particular in SFs. According to our data, hexosaminidase is the glycosidase with the highest expression and activity in the joints. We showed for the first time that SFs appear to be major sources of this enzyme in the SMs as they are characterized by strong expression of both HexA and HexB genes. We have also detected for the first time the expression of HexDC gene in SMs and SFs that had been described in an expression vector only several months ago. We hypothesize that even though SFs show relatively low expression of GusB, they might accumulate significant amounts of this lysosomal enzyme – some of which might be released by cell-derived MVs. This concept is supported by the GusB activity detected in cell lysates that was comparable with that detected in SM homogenates, and also by its association with cell-derived MVs.

Numerous cytokines exert complex regulatory mechanisms in RA. MMPs and other proteases have been reported to be highly inducible by proinflammatory cytokines. In sharp contrast, we found that glycosidases were moderately downregulated by proinflammatory cytokines IL-1β, IL-17 and TNFα. The most pronounced cytokine effect was seen in the case of TGF-β_1, which profoundly downregulated glycosidase expression in both RA and OA fibroblasts. Our observation that glycosidases appear to be under negative control and are downregulated rather than stimulated by inflammatory cytokines, may suggest that an enhanced expression of these enzymes could lead to severe and unforeseeable consequences. The extracellular matrix has been reported to serve as a repository of transforming growth factor beta and other growth factors of which the release is regulated via degradation of proteoglycans. It can be hypothesized that a stringent control of the gene expression of glycosidases may prevent the synchronized release of the plethora of tissue-bound proteins. However, we should not neglect that synovial membrane-infiltrating inflammatory cells (such as immigrant neutrophil granulocytes, macrophages) may carry glycosidase enzymes to the joints that may also considerably contribute to cartilage degradation.

In the study of anti-GAG natural antibodies, we found that while GAG-specific antibodies were absent in neonates, they were present in high amounts in sera of adults. We have shown that all IgM anti-GAG antibodies and certain IgG-type anti-GAG antibodies were significantly elevated in sera of patients with RA compared with adult controls. The next important step was to test if the levels of anti-GAG antibodies showed any correlation with the disease activity in RA. Intriguingly, using a multistep approach, our work has demonstrated that chondroitin sulphate C (CSC)-specific IgM antibody levels show a clear inverse correlation with the activity of RA (DAS28 score) and with the level of CRP, thus we suggest that CSC-specific IgM is a disease-state biomarker in RA. The absence of GAG-specific antibodies in neonates and the abundance of them in adults, as well as their highly cross-reactive nature, raise the possibility that anti-GAG antibodies are produced on exposure to environmental microbial carbohydrates or glycosylated antigens. We next assessed the cross-reactivity of GAG-specific antibodies with microbial antigens. We found that circulating anti-GAG antibodies were capable of binding to bacterial peptidoglycans and fungal polysaccharide. Further, we found significantly increased antibody recognition of aggrecan modified in its GAG chains by bacterial glycosidase (β-galactosidase or hyaluronidase) digestion.

Therefore it is very likely that the increased level of anti-GAG antibodies in RA is the result of the increased efflux of GAGs from the inflamed joint. We found that the higher anti-GAG level is associated with less severe disease. The mechanism of the "protective" role of anti-GAG antibodies is not known. Both peptidoglycans and HA and HS fragments have been shown to bind to certain toll-like receptors (TLRs). Our hypothesis is that one of the roles of the natural anti-GAG antibodies is to bind to the degrading matrix molecules and thus, prevent the excessive ligation of danger receptors like TLRs. Hence, the NAbs may reduce the chance of priming of naive T-cells to self molecules. Therefore, by masking GAG epitopes and facilitating the elimination of immunogenic glycopeptides (possibly in the form of immune complexes), anti-GAG antibodies may have an important function in preventing or decelerating the autoimmune processes.

Next, we investigated the peripheral blood cell response of control and RA patients against *in vitro* SE-containing P135 peptide stimulation. We have found that patients with RA but not controls showed a significantly elevated in vitro TH-17 response

to P135. In contrast, the IL-17 responses were profoundly depressed to another human aggrecan epitope suggesting tolerance to the ATE sequence, and we could not detect significant in vitro IL-17 response to the SE-containing HLA DRB1 peptide. As a group, neither RA patients nor controls responded differentially to citrullinated altered peptide ligands compared to the arginine-containing wild type peptides. Unexpectedly, however, in select subgroups of RA patients and controls IL-17 unresponsiveness to P135 was broken by citrullination. The 3-4 fold stimulation of IL-17 responses by citrullinated P135 was found comparable in the above subgroups of both patients and controls. The similar peripheral blood IL-17 responsiveness in both healthy controls and patients might be due to the immunosuppressed state of patients with RA. It remains to be elucidated what factors dictate IL-17 responsiveness towards citrullinated P135 in approximately 30% of the controls and patients with RA. According to our data, this citrullination-responsiveness was not associated neither with cigarette smoking, nor with HLA DRB1 genotype, nor with any other clinical/laboratory parameters of the individuals enrolled in this study.

Therefore, these are the first data that show that peripheral IL-17 response can be induced by a SE-containing human cartilage aggrecan epitope. This peptide may be of particular interest in RA due to its structural characteristics. On the one hand, the presence of the SE in this cartilage proteoglycan peptide raises the intriguing possibility of molecular mimicry with both HLA DRB1 and several microbial heat shock proteins. On the other hand, the presence of arginine residues renders the peptide (and its homologues) susceptible to citrullination, a posttranslational protein modification strongly implicated in RA.

Finally, we studied NO production by T-cells in RA. We observed that unstimulated T cells from RA patients express approximately two-fold more NO than healthy donor T cells. Although NO is an important physiological mediator of mitochondrial biogenesis, the mitochondrial mass is similar in RA and control T cells. In contrast, increased NO production is associated with increased cytoplasmic Ca^{2+} concentrations in RA T cells. *In vitro* treatment of human peripheral blood lymphocytes or Jurkat cells with TNF increases NO production, whilst infliximab treatment in RA patients decreases T cell derived NO production within 6 weeks of the first infusion.

Together, these data indicate that TNF-induced NO production in T lymphocytes may contribute to perturbations of immune homeostasis in RA. The functional outcome of increased amounts of T cell derived NO in patients with RA is likely associated with the direct effect of NO on signal transduction pathways, the effect of increased cytosolic Ca^{2+} on gene expression, in particular that of inflammatory cytokine genes and the potential for enhanced migratory capacity of NO expressing T cells.

Conclusions

1. Our data drive attention to the dominant negative regulation of a functional group of genes – glycosidases – by paramount cytokines in SFs that differs remarkably from regulation of proteases. The fact that we did not find significant differences between patients with RA and OA with respect to their glycosidase gene, suggests a similar role and regulation for exoglycosidases in the two diseases. This hypothesis does not contradict that these enzymes (if carried into the joints by immigrant inflammatory cells) may contribute significantly to cartilage degradation in both joint diseases if acting in concert with MMPs to deplete cartilage in glycosaminoglycans. Our data suggest that the earlier reported elevated glycosidase activities in RA joints were probably not due to enhanced gene expression of resident SFs, but rather resulted from enzyme release by cells (including infiltrating inflammatory cells) within the joints.

2. It has been proposed that changes in the global NAb network (referred to as "immunculus distortions") may serve as predictors or early disease markers. In the current work we found that anti-GAG antibody levels sharply discriminate between adult controls and RA patients with low disease activity (DAS 28 $\leq$ 3.2), and thus, anti-GAG antibodies may be the key to an inexpensive early disease activity biomarker. Thus, they might have the justification to be incorporated into autoantigen microarrays.

3. Given the suggested role of IL-17 in the pathomechanism of the autoimmune inflammation, the IL-17 response that we detected upon *in vitro* stimulation by the P135 human aggrecan peptide and/or to its citrullinated variants, may reflect a novel mechanism by which SE and citrullination, coinciding with several other genetic and environmental factors, may contribute to disease development in susceptible individuals.

4. Given the results of NO production in RA T cells, NO is an important regulator of T-cell function and it's production is regulated by TNF-α not only *in vitro* but *in vivo*. We suggest that increased levels of NO in T cells may contribute to perturbations of cytokine production and T cell signal transduction characteristic of patients with RA. These data

lend further support for NO-inhibiting therapeutic strategies for the treatment of chronic inflammatory diseases such as RA. Because of the number of important physiological functions of NO, local inhibition of NO synthesis at the site of synovial inflammation may provide better therapeutic tool than systemic inhibition, and provide a rationale for therapeutic targeting of NOS activity in these patients.

List of own publications

Publications related to the subject of the thesis:

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