PhD thesis

Epitope mapping of natural and disease associated autoantibodies against topoisomerase I in systemic sclerosis and systemic lupus erythemathosus

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SUMMARY

The main aim of this work was the epitope mapping of an autoantibody (anti-topoisomerase I) characteristic of a systemic autoimmune disease (systemic scleorsis) using the phage display technique previously developed in the Department of Immunology and Biotechnology.

In our research we demonstrated natural autoantibodies against topoisomerase I (topo I), the target antigen of the autoantibody considered to be disease associated in systemic sclerosis (SSc). To our knowledge these are the first results demonstrating that natural antibodies against topo I are present in sera of healthy individuals and patients with different systemic autoimmune diseases. Using the phage display technique we compared the epitope patterns recognized by natural and pathological autoantibodies. We identifiedd an epitope (F4), which was recognized not only by SSc patients, but also by sera of healthy individuals and patients with systemic autoimmune diseases other than SSc and SLE. This fragment F4 represents a 150 aminoacid (AA) long, evolutionarily conserved sequence of topo I.

Using the phage display technique we proved that sera of patients with SSc and SLE also show reactivity on different topo I epitopes other than the immunodominant fragment. We demonstrated that the pattern of recognized epitopes is different in dcSSc, lcSSc and SLE patients. We showed that the recognition of the majority of the topo I fragments was characteristic for individual patients, instead of being characteristic for the given disease subgroup. However fragment F1 (an evolutionarily relatively new sequence, specific for vertebrates at the N terminal part of topo I) was mainly recognized by dcSSc patients and antibodies against fragment F8 (a highly conserved sequence) could be detected in SLE patients, indicating that these fragments could represent characteristic epitopes for dcSSc and SLE, respectively. Statistical analysis of clinical data showed no association between anti-topo I antibody epitope specificity and clinical presentation of SSc. However, there was a significant difference between F1 negative and F1 positive groups of dcSSc patients in the duration of the disease. Thus autoantibodies against fragment F1 may represent a newfound marker of late stage dcSSc, while antibodies against fragment F8 may indicate a milder presentation of SLE.

INTRODUCTION

Natural immune system

The natural immune system bridging the evolutionarily older innate and the newly evolved adaptive systems has recently been described. A distinct set of lymphocytes – both T and B cells – with characteristic phenotypes and specialized functions participates in this system. These subsets of cells exhibit common phenotypic characteristics and posses both innate and adaptive features, suggesting a transitional stage in the immune system's evolution. The most important cellular components of the natural immun system according to recent knowledge are the invariant natural killer T (iNKT) cells, mucosa associated invariant T (MAIT) cells, γδ T cells and B1 B cells. The functional character of antigen recognition by these cells (and the immunoglobulins produced by B1 B cells) are closer to the pattern recognition features than to the classical adaptive type immunological recognition, however, the recognising molecules are genuine T and B cell surface receptors.

Natural antibodies and natural autoantibodies

Natural antibodies are immunoglobulins secreted by B1 cells in the absence of deliberate immunization with antigen. These antibodies can recognize genetically conserved sequences of pathogens and may serve in the first line of immune defense during an infection. Natural autoantibodies however also recognize evolutionary conserved self structures and are present in serum of both healthy individuals and patients with systemic autoimmune diseases.

The autoreactive repertoires are predominantly selected in foetal life. Most of natural autoantibodies are encoded by near germline sequence of limited immunoglobulin gene repertoire, belong to the IgM or IgG isotype and show polyreactivity with a broad range of affinities for the recognized epitopes. Polyreactivity does not mean lack of specificity, thus each polyreactive natural autoantibody encompasses its own distinct set of epitopic specificities and therefore is unique. Several functions have been suggested for natural autoantibodies: they may participate in the selection of immune repertoires, play a role in the acceleration of primary immune responses, aid the clearance of apoptotic cells, possess anti-inflammatory effects and contribute to the maintenance of immune homeostasis. Discrimination of natural antibodies from natural autoantibodies might seem artificial since the limited B1 immunoglobulin gene repertoire driving natural antibody production, and the numerous distinct antigens recognized make it probable that specificities with self non-self cross reactivity exist.

Previous results

Since their endosymbiotic evolutionary origin, proteins compartmentalized into mitochondria represent an interesting transitional stage from prokaryotic foreign to essential self molecules. The structural and functional conservation of mitochondrial components makes them candidate antigens for detailed analysis of evolutionary connections between the innate and adaptive immune response. The inner membrane enzymes, particularly the citric acid cycle enzymes (specially citrate synthase), offer appropriate models for testing their immunoreactivity, because they are in continuous connection with both innate and adaptive components of the immune system during physiologic turnover of cells. In our previous work we demonstrated the presence of citrate synthase recognizing autoantibodies in both healthy individuals and patients with systemic autoimmune diseases. We performed epitope mapping of these autoantibodies under physiological and pathological (systemic autoimmune) conditions. We found that the fine epitope pattern recognized by autoantibodies directed against citrate synthase is different between healthy individuals and patients with systemic autoimmune diseases.

These previous experiments underlined the necessity of the epitope mapping of an autoantibody which is specific for a well defined pathological condition and has a high diagnostic value. Our aim was to decide whether the target antigen of the disease associated autoantibody is also recognized by naturally occurring autoantibodies. We assumed that comparison of the epitope patterns recognized by natural and disease associated autoantibodies would contribute to the better understanding of the differences between natural and disease associated autoantibodies.

To address these issues we chose topo I as a model antigen, since anti-topo I antibodies are important in the diagnosis of SSc.

Topo I

Topo I belongs to the type IB subfamily of topoisomerases, changes the tertiary structure of the DNA molecule by relaxing supercoiled DNA through breaking and rejoining one strand at a time and binding covalently to the 3' phosphate. Topo I is a 765 AA long enzyme which contains five differently conserved distinct regions: the N-terminal domain (AA 1-215), core subdomain I-II (AA 216-435), core subdomain III (AA 436-636), the linker domain (637-713), and the C-terminal domain (AA 714-765).

Clinical relevance of anti-topo I autoantibodies

SSc is a systemic autoimmune disorder characterized by fibrosis and generalized obliterative vasculopathy of the skin and various internal organs (lung, heart, gastrointestinal tract and kidney). Clinically patients with SSc could be classified into two distinct subsets: dcSSc is characterized by extensive fibrosis of the skin, lungs and other internal organs, while in lcSSc vascular abnormalities are dominating and fibrosis is limited predominantly to the acral regions.

Anti-topo I autoantibody is found to be associated with increased mortality, pulmonary fibrosis, musculoskeletal and cardiac involvement, and proteinuria. The level of anti-topo I autoantibodies correlates with the extent of fibrosis of the skin and internal organ involvement in dcSSc, and may serve as an activity marker of disease. Anti-topo I autoantibodies are considered to be associated with dcSSc. However, the presence of anti-topo I autoantibodies may not be entirely restricted to this subset, since a subgroup of lcSSc patients were also found to be positive. In addition to SSc presence of anti-topo I antibodies has been demonstrated in SLE and other inflammatory diseases.

Epitope specificity of anti-topo I autoantibodies

A number of research groups have studied the epitope specificity of anti-topo I antibodies in SSc patients. Using molecular biology strategies several studies have demonstrated that anti-topo I antibodies of SSc patients recognize multiple epitopes on topo I, although the recombinant topo I fragments used varied in these reports. Epitope mapping performed with synthetic peptides identified four major epitopes, three of them in core subdomains I and II, and one in core subdomain III. Since some of these studies have implicated topo I fragments located in the region of AA 484–560, these findings strongly suggest that immunodominant B cell epitopes recognized by anti–topo I antibodies could be located within this region. However, fragments used in these studies were designed either on the basis of topo I domain structure or antigenicity prediction, both of which could miss possible epitopes. Until recently there was a single report of epitope mapping on topo I with random antigen fragments. They used a size selected random antigen fragment library constructed by limited deoxyribonuclease digestion, and screened this library with one serum sample of a single SSc patient. They found a major epitope region spanning AA 653-704 of the linker domain. This region has been produced as a fusion protein and was recognized by 70% of anti-topo I positive sera used in subsequent testing. Longitudinal analysis of anti-topo I autoantibodies revealed that reactivity against these regions is stable, though a study using a

limited number of sera showed that the regions recognized by anti-topo I autoantibodies vary over time. While epitope specificity of anti-topo I autoantibodies has been studied by a number of groups there are no reports of comparative epitope mapping in patients with dcSSc, lcSSc and SLE.

Possible pathogenic roles of anti-topo I autoantibodies

The pathogenic role of anti-topo I autoantibodies in SSc is not clearly demonstrated. However several lines of indirect evidence suggest that anti-topo I autoantibodies could contribute to the pathogenesis of SSc. Anti-topo I antibodies could be detected at a very early stage of the disease. The presence of anti-topo I antibodies is suggested to be associated with a more severe form of SSc, and the level of these antibodies seems to correlate with the activity of the disease. However, these important clinical findings need to be substantiated by demonstration of the direct molecular and cellular mechanisms by which anti-topo I antibodies could play a role in pathogenesis of SSc. The missing link seems to be in the connection between immunological abnormalities and fibrosis. In theory, the presence of SSc specific autoantibodies which would be capable to bind to the surface of fibroblasts and induce myofibroblast like phenotypic changes represents an attractive mechanism to form a connection between these processes. In fact presence of stimulatory autoantibodies against the platelet derived growth factor receptor (PDGFR) in sera of SSc patients has already been reported. However, confirmation of these results in an extended set of patients is not provided to date.

In case of the pathogenic role of antibodies directed against intracellular or nuclear antigens there is a general problem, namely how these antibodies are capable to bind to the cell surface and induce or sustain cellular damage. Binding of anti-topo I autoantibodies to the surface of fibroblasts has been demonstrated, and the same group has shown that topo I is capable of binding to the surface of fibroblast cell lines, thus this fibroblast surface bound topo I provides a binding site for anti-topo I antibodies. However, the ligand which forms a binding site for topo I on the fibroblast surface is not yet identified, and topo I has been found to bind in comparable quantities to both normal and SSc derived fibroblasts. Although these findings provide the first experimental evidence for cell surface binding of anti-topo I autoantibodies, neither explain why anti-topo I antibodies are formed almost exclusively in SSc, nor demonstrate a direct or indirect link between these antibodies and fibrosis.

The role of anti-topo I antibodies in pathogenesis of SSc is not fully understood, however, immune response against topo I may differ among anti-topo I positive patients

leading to production of anti-topo I autoantibodies with different epitope specificity. Thus the presence of anti-topo I antibodies could have heterogeneous clinical consequences and reflect different pathologic conditions. Consequently identification of epitopes or epitope patterns recognized by anti-topo I antibodies could contribute to early diagnosis, help in indicating the different internal organ manifestations, evaluating prognosis and choosing the adequate therapeutic strategies.

AIMS

1. Construction of a topo I antigen fragment library displayed on bacteriophage lambda.
2. Affinity selection of topo I antigen fragment library with IgG isotyped immunoglobulins purified from sera highly positive for anti-topo I antibodies of patients with dcSSc, lcSSc and SLE.
3. On the basis of fragments selected from the phage displayed topo I antigen fragment library expression of nine maltose binding protein (MBP)-topo I fusion proteins.
4. Testing the reactivitiy of a large number of sera from healthy individuals and patients with dcSSc, lcSSc and SLE on the MBP-topo I fusion fragments with ELISA and Western blot.
5. Comparison of epitope patterns recognized by anti-topo I autoantibodies of healthy individuals and patients with dcSSc, lcSSc and SLE.
6. Longitudinal analysis of epitope patterns of autoantibodies directed against topo I.
7. Evaluation of the clinical relevance of the recognized fragments of topo I.

MATERIALS AND METHODS

Serum samples

From the 293 patients of our total SSc cohort 59 cases (34 dcSSc, 25 lcSSc), out of 265 SLE patients tested 8 were anti-topo I antibody positive on a conventional ELISA kit and selected for the present study. Three serum samples from each patient were obtained between 2004 and 2007 at 6 to 12 months intervals. To evaluate the clinical relevance of the study in a wider perspective we examined anti-topo I positive serum samples of 51 SSc (11 dcSSc, 40 lcSSc) patients from the Department of Rheumatology, University of Debrecen. For controls 63 sera from Hungarian, 44 sera from Finnish and 44 sera from British blood donors and 65 sera from Hungarian elderly healthy individuals were used. Furthermore 110 sera from Hungarian elderly anti-topo I antibody negative (measured by a conventional ELISA test)

patients with inflammatory rheumatic diseases other than SSc and SLE (8 vasculitis, 40 seronegative spondylarthritis, 11 myositis, 11 Sjögren syndrome, 10 psoriatic arthritis, 20 rheumatoid arthritis, 10 polymyalgia rheumatica) were also investigated. The study has been approved by the Ethical Committee of the Medical Center of the University of Pécs. Informed consent has been obtained from all patients and healthy individuals.

Construction of topo I antigen fragment library

The coding region of full length human topo I was amplified by PCR from cDNA reverse transcribed from total RNA. The PCR product was cloned into a T/A vector and the identity of insert was verified by sequencing the entire coding region of topo I. Fragments with random starting point and length were produced by tagged random primed elongation and amplification using SpeI and NotI tagged random primers and topo I cDNA as template excised with BamHI and EcoRI digestion from the T/A plasmid mentioned above and cloned into lambdaD-bio phage display vector. Following in vitro packaging and E. coli amplification phage were concentrated with polyethylene glycol precipitation.

Affinity selection of topo I antigen fragment library

Microtiter plates were coated with IgG purified on protein G sepharose from sera of 5 dcSSc, 6 lcSSc and 4 SLE patients. Following blocking the plates were incubated with topo I-lambda phages Wells were washed five times and bound phages were recovered by in well infection of E. coli BB4 cells. The infected bacteria were plated on LB agar plates and phage were eluted then concentrated. After the third round of selection individual clones were picked up for further propagation and DNA sequencing.

Expression of MBP-topo I fusion proteins

Selected fragments of topo I were expressed as recombinant MBP fusion proteins. The cDNAs coding for selected fragments **[**AA 5-30 (F1), 69-92 (F2), 87-145 (F3), 450-600 (F4), 640-705 (F5), 170-290 (F6), 295-350 (F7), 350-400 (F8), 295-400 (F9)] were amplified with PCR primers containing EcoRI and BamHI restriction sites and cloned into the pMal-c2 vector. The reading frame and sequence of inserts were verified by sequencing. Fusion proteins were expressed in E. coli TB1 and were purified from bacterial lysates with affinity chromatography on amylose resin. Integrity of purified proteins was verified by SDS-polyacrylamide gelelectrophoresis (PAGE) on a 10% gel followed by Coomassie brilliant blue staining.

Testing the reactivity of sera from healthy individuals and patients with dcSSc, lcSSc and SLE on MBP-topo I fusion fragments with ELISA and Western blot

96-well polystyrene plates (Nunc, Roskilde, Denmark) were coated with recombinant topo I fragments or with MBP in PBS at a concentration of 10 μg/ml. Plates were washed with wash buffer (PBS, 0.05% Tween-20) and blocked with 3% bovine serum albumin (BSA) in wash buffer for 1 hour. Serum samples were incubated in triplicates at 1:250 dilutions in was buffer containing 2% BSA for 1 hour. Finally, the plate was incubated with horseradish peroxidise (HRP)-conjugated anti-human IgG or IgM secondary antibody for 60 min. The reaction was developed with o-phenylenediamine and optical density was measured at 492 nm.

The results of ELISA were confirmed by Western blot. Purified MBP fusion proteins or MBP (40 μg/ml) diluted 1:1 with SDS sample buffer were boiled for 10 minutes, separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk in wash buffer (100mM NaCl, 10mM Tris-base pH 7.4, 0.1% Tween 20) for 1 hour, membranes were incubated for 1 hour with sera diluted 1:500 in 2% non-fat dry milk in wash buffer. After washing, HRP-conjugated anti-human IgG diluted at 1:2000 was added for 1 hour. For detection of MBP fusion proteins membrane strips were first incubated with rabbit anti-MBP antibody (1:5000), followed by incubation with HRP-conjugated goat anti-rabbit antibody (1:2000). Membranes were developed with chemiluminescent substrate and exposed to x-ray films.

Longitudinal analysis of the reactivity of antibodies directed against topo I fragments

Three serum samples from each 34 dcSSc, 25 lcSSc and 8 SLE patient were tested by ELISA. The results were confirmed by testing 10 F1 positive, 10 randomly chosen F4 positive and 4 F8 positive patients' serum samples with Western blot. The serum samples were also tested with a conventional anti-topo I ELISA kit.

Evaluation of clinical relevance of the recognized topo I fragmets

To evaluate clinical relevance of the recognized fragments of topo I clinical data were statistically analysed. Categorical data were analyzed by Yates' corrected chi-square test. Frequency and mean values of continuous variables were tested by Student's t test. A p value less than 0.05 was considered statistically significant. Statistical analyses were conducted using SPSS statistical software package.

RESULTS

1. The results obtained by affinity selection of the topo I antigen fragment library demonstrated that the pattern of recognized epitopes is different in dcSSc, lcSSc and SLE patients. Sera of dcSSc patients recognize epitopes localized in the N-terminal domain, epitopes recognized by SLE patients' sera are found in core subdomain I-II, while epitopes recognized by lcSSc patients are scattered throughout the molecule.
2. On the basis of fragments identified by library selection nine MBP-topo I fusion proteins (F1-F9) were constructed and expressed. Recognition of these fusion proteins were first tested with sera of healthy individuals which were previously shown to be negative for anti-topo I antibody by a conventional ELISA test. Autoantibodies against fragment F4 (AA 450-600) could be detected in sera of healthy individuals. The other fragments (F1-F3, F5-F9) were not recognized by sera of healthy individuals.
3. Fragment F4 of topo I were recognized by all sera of patients with dcSSc, lcSSc and SLE previously tested anti-topo I positive on a conventional ELISA kit.
4. Antibodies against fragment F4 could also be detected in sera of patients with systemic autoimmune diseases other than SSc and SLE.
5. The presence of antibodies with both IgM and IgG isotype against fragment F4 of topo I was not restricted to SSc, but could be detected in healthy individuals and in sera of patients with inflammatory rheumatic diseases other than SSc and SLE, and the presence of antibodies against fragment F4 was essentially independent of the age and geographical origin.Collectively these data raise the possibility that antibodies against fragment F4 detected in healthy individuals belong to the pool of naturally occurring antibodies.
6. Fragment F1 (AA 5-30) was recognized by 26% of anti-topo I positive (by a conventional ELISA test) dcSSc patients.
7. Antibodies against fragment F8 (AA 350-400) could be detected in 50% of anti-topo I positive (by a conventional ELISA test) SLE patients.
8. Recognition of the remaining fragments (F2, F3, F5-F7, F9) was characteristic for individual patients, instead of being characteristic for the given disease subgroup.
9. Longitudinal analysis showed that reactivity to fragment F4 was stable, while the reactivity to F1 and F8 fragments varied over time.
10. Statistical analysis of clinical data (extent of skin involvement, hand contractures, azotemia and/or malignant hypertension, cardiac involvement, pulmonary artery

hypertension, dysmotility and stricture/dilatation of esophagus, extent of lung fibrosis, forced vital capacity) showed no association between anti-topo I antibody epitope specificity and clinical presentation of SSc. However, there was a significant difference between F1 negative and F1 positive groups of dcSSc patients in average age and the duration of the disease. Thus autoantibodies against fragment F1 may represent a new marker of late stage dcSSc. Comparison of clinical data of anti-F8 positive and anti-F8 negative SLE patients suggested that SLE patients with antibody against fragment F8 have Raynaud's phenomenon and a milder presentation of the disease (lack of arthritis, central nervous system and kidney involvement).

DISCUSSION

As a connection bridging the evolutionarily older innate and the newly evolved adaptive systems the natural immune system has recently been described. Similarly to the initiation of immune response the maintenance of tolerance involves all three compartments of the immune system. Disturbances in co-operation among innate, natural, and adaptive immune system components may result in the impairment of both targeting type immune response and self tolerance, thus paving the way for development of immunodeficiencies and pathological autoimmune phenomena.

Majority of natural antibodies are self reactive, and recognize genetically conserved structures. A large number of serum antibodies directed against functional structures of the cell (nucleic acid, nuclear molecules, receptors, or other functional cell components) can be detected in systemic autoimmune diseases. Their presence plays a central role in the diagnosis and classification of this kind of disorders. Moreover, several longitudinal cohort studies have shown that patients may carry autoantibodies many years before they manifest clinical symptoms and detecting these antibodies in serum has been shown to have strong predictive value.

The onset of the disease may correlate with a switch from production of IgM to IgG isotyped antibodies. Nevertheless, the exact role of autoreactive IgM in the autoantibody response and the switch to other isotypes is not known. It has to be mentioned that IgM autoantibodies can have a role in protection from autoimmunity by facilitating the removal of apoptotic cells and increasing the tolerance of B cells to self antigen. Since one of the essential functions of the immune system is the prevention of self antigens to stimulate an inflammatory reaction, the presence of autoantibodies is the consequence of a breakdown or

failure of B cell tolerance toward the corresponding autoantigens. The timing of exposure, the level of affinity of the autoreactive IgM autoantibodies and their local concentration may determine which scenario applies, i.e., autoimmunity or tolerance.

The presence of natural autoantibodies alone is not enough for the development of autoimmunity, the most components of natural immunity are reported to be in some way involved in autoimmune disorders. Some of them act mainly in the pathogenesis of the disease while others can have a role in the development of chronic disease or in some of its clinical features. The complex role played by natural immunity in autoimmune phenomena is underlined by the fact that the same component can have a protective or a triggering role according to the microenvironmental, genetic and anatomic conditions that apply.

In this study we performed the epitope mapping of anti-topo I autoantibodies specific for SSc, a systemic autoimmun disease and examined whether the target antigen of the disease associated autoantibody is also recognized by naturally occurring autoantibodies. Epitope mapping with overlapping synthetic peptides is a widely used technique, but its constraints include the uncertainties linked to *in silico* B-cell epitope prediction used for selection of antigenic regions, the partial coverage of primary sequence by synthetic peptides and the possible loss of all unpredicted or conformational epitopes. To eliminate these effects that could have influenced our results, we sought to perform the epitope mapping using a basically different technique and chose phage display, which most resembles the physiological antigen conformation and does not require prior epitope prediction. The technology is based on the expression of recombinant peptides or proteins fused to a phage coat protein. Its key advantage is in the physical coupling of the displayed protein to the nucleic acid coding it, making the repeated affinity selection and amplification possible. Bacteriophage surface display of peptides is an extensively used technique for a variety of applications. The most commonly used systems are based on fusion to a filamentous phage coat protein. However, the life cycle of these phages limits the size of the displayed peptide, therefore we have chosen phage lambda for construction of a topo I antigen fragment library.

The library contains fragments of topo I with random starting point and length; consequently it overcomes the theoretical and technical limitations associated with the overlapping synthetic peptide approach. With this phage display based approach we compared the epitope patterns recognized by anti-topo I autoantibodies found in sera of patients with dcSSc, lcSSc, and SLE. The results showed that the pattern of recognized epitopes is different between dcSSc, lcSSc and SLE patients. A common fragment recognized by all patients' sera was located in the region of AA 450-600, which is in agreement with previously published

results. In addition to this, sera of dcSSc patients recognized several short fragments at the N-terminal part of the molecule. Previous studies performed with fusion proteins covering the N-terminal domain starting from AA 70 reported that this part of the molecule is recognized by anti-topo I antibodies. However, the opposite has also been reported by using a fusion protein covering the entire length of the N-terminal domain and showing that this part of the molecule is not targeted by anti-topo I antibodies. These seemingly contradictory results may be explained by the different methods and antigen constructs used, and most importantly by possible conformational factors which could influence the accessibility of short epitopes buried in the tertiary structure. It is important to note that the majority of new epitope containing fragments we identified at the N-terminal part spans only 20-30 AA. Fragment F1 contains an experimentally proven granzyme B cleavage site, thus it is possible that *in vivo* cleavage of topo I by granzyme B released during T cell mediated cytotoxic responses results in the formation of a neo-antigenic determinant represented by fragment F1. *In vitro* assays using the full length antigen or the full length N-terminal domain may fail to detect antibodies recognizing these short epitopes suggesting strong conformational sensitivity.

On the basis of fragments identified by library selection nine MBP-topo I fusion proteins were constructed and expressed. First we tested recognition of these fusion proteins with sera of healthy individuals and found that a significant portion of healthy individuals posses antibodies with IgM and IgG isotype against fragment F4. Fragment F4 represent a 150 AA long, genetically conserved sequence of topo I. Using a large number of sera we showed that the presence of antibodies against fragment F4 is essentially independent of the age and geographical origin of healthy individuals. In addition antibodies against fragment F4 could also be detected in sera of patients with inflammatory rheumatic diseases other than SSc and SLE. Anti-F4 antibodies with IgM isotype are present in the highest titer in sera of anti-topo I antibody positive SSc or SLE patients. It is important to note that all 67 sera from anti-topo I antibody positive SSc or SLE patients were found to be positive for anti-F4 antibodies with IgG isotype, and the titer of these antibodies was the highest in this group among all groups tested. The fact that these sera were shown to be negative for anti-topo I antibody by a conventional ELISA test using the full length antigen could indicate that the sequence represented by fragment F4 could be hidden in the three dimensional structure of the full length molecule. These findings raise the possibility that antibodies against fragment F4 present in sera of healthy individuals and patients with systemic autoimmune diseases could belong to the pool of naturally occurring antibodies. To our knowledge these are the first results demonstrating that natural antibodies against topo I are present in human sera.

The phenomena that natural autoantibodies could recognize self antigens which are also targeted by antibodies in autoimmune diseases is not unprecedented. Several lines of evidence indicate that antibodies recognizing factor VIII, thyreoglobulin, DNA, endothelial cell membrane components are present in sera of both healthy individuals and patients with autoimmune diseases. Nevertheless anti-topo I antibodies were detected in sera of patients with glomerulonephritis, chronic graft versus host disease, primer biliary cirrhosis and in some cases of chronic hepatitis C virus infection induced liver diseases. These findings raise the question whether these detected antibodies are pathological autoantibodies or belong to the pool of natural antibodies. Since fragment F4 represents a 150 AA long sequence of topo I, it is possible that the fine epitope pattern recognized by natural antibodies and disease associated autoantibodies within this part of topo I is different.

After insidious onset of SSc the development of internal organ manifestations can lead to death of the patient in a few years. The prognosis is mostly determined by the activity of the disease and the extent of the developed irreversible lesions. Hence, early diagnosis and initiation of adequate therapy as soon as possible is crucial in SSc. Since anti-topo I autoantibody is found to be associated with increased mortality, pulmonary fibrosis, musculoskeletal and cardiac involvement, proteinuria and the level of anti-topo I autoantibody correlates with the extent of fibrosis of the skin and internal organ involvement in dcSSc, it may serve as an activity marker of disease. Anti-topo I autoantibodies are considered to be associated with dcSSc. However, the presence of anti-topo I autoantibodies is not entirely restricted to this subset, since anti-topo I antibodies have been demonstrated in lcSSc, SLE and other inflammatory diseases. The fact that anti-F4 antibodies were detected in sera which were tested negative for anti-topo I antibody by a conventional ELISA kit using the full length antigen indicates that an ELISA test using recombinant F4 fragment might be a more sensitive way to determine anti-topo I positivity and could contribute to early diagnosis and monitoring the activity of SSc.

With the use of sera of 67 anti-topo I antibody positive patients we showed that recognition of the majority of fragments (F2, F3, F5-7, F9) is characteristic for the individual patient sera used for library screening, instead of being characteristic for the given disease subgroup. This is in agreement with result of Henry et al., who found both individual and longitudinal differences in the recognized topo I epitopes. However, antibodies recognizing the common F4 fragment were detected in all patients' sera tested. In addition to this immunodominant part of topo I, we identified two new regions (F1 and F8) which were previously not shown to be targeted by anti-topo I antibodies. Fragment F1 (an evolutionarily

relatively new sequence, specific for vertebrates) was recognized by 26% of dcSSc patients and antibodies against fragment F8 (a highly conserved sequence) could be detected in 50% of SLE patients, indicating that these fragments could represent characteristic epitopes for dcSSc and SLE, respectively. Longitudinal analysis showed that reactivity to fragment F4 was stable, while the reactivity to F1 and F8 fragments varied over time.

Possible associations between epitope specificity of anti-topo I antibodies and involvements of skin and internal organs in SSc were also analyzed. Analysis of clinical data failed to demonstrate associations between anti-topo I antibody epitope specificity and clinical presentation of the disease. This is in agreement with results of Henry et al., who also reported lack of clear association between changes in the anti–topo I antibody response and clinical parameters. The difference in the duration of disease between anti-F1 antibody positive and negative dcSSc patients, together with findings of our longitudinal analysis, may indicate that the anti-topo I immune response could be explained by a general recognition of the immunodominant part on the molecule (fragment F4), and the disease associated autoantibodies may target the N-terminal part later during the course of the disease. Thus autoantibodies against fragment F1 may represent a new marker of late stage dcSSc.

Comparison of clinical data of anti-F8 positive and anti-F8 negative SLE patients suggested that SLE patients with antibody against fragment F8 have Raynaud's phenomenon and a milder presentation of the disease (lack of arthritis, central nervous system and kidney involvement).

The pathogenesis of SSc seems to be highly complex. Some initiator events could lead to vascular injury, inflammation, activation of both innate and adaptive immune system and fibrosis. The hierarchical order of these events, if there is such an order at all, is not yet been clearly established, however vascular injury seems to be an early and primary process. Anti-topo I autoantibodies are certainly among the most frequently detected autoantibodies in SSc. However, antibodies against several other nuclear or cytoplasmic antigens are also present in SSc. A number of groups, including ours, examined the epitope specificity of anti-topo I autoantibodies. Though the methods used and patient populations examined vary among these studies, it seems that there is an immunodominant part of topo I in core subdomain III (between AA 450-600). The association of anti-topo I autoantibodies with dcSSc, which is dominated by fibrosis could implicate either that anti-topo I autoantibodies play a causative role in fibrosis, or these antibodies are produced as a consequence of other events (vascular or cellular injury) and play no pathogenic role in SSc, but reflect only the activity of these

events. The former scenario is supported but not unequivocally proven by the demonstration of cell surface binding of anti-topo I antibodies on fibroblasts.

It is possible that the presence of natural antibodies is essential for the appearance of disease associated autoantibodies, because natural autoantibodies can, under appropriate conditions, provide the templates for the emergence of higher affinity and class-switched pathogenic autoantibodies. IgG isotyped disease associated autoantibodies may reognize genetically determined epitopes (epitope patterns) and can be detected in genetically predisposed individuals, which was also suggested by a study examining monozygotic twins suffering from SLE. Thus tolerance against conservative antigens might mostly be genetically determined. The permanent impairment of the development and maintenance of tolerance can lead to autoimmune disorders. Pattern recognition mechanisms were thought to be specific for innate immunity and considered to be the defense mechanisms of evolutionarily ancient species. According to our results natural autoantibodies, in terms of their antigen recognizing characteristics, resemble the pattern recognition receptors and recognize epitope patterns. Pathologic autoantibodies detected in autoimmune diseases however are directed mainly against a well defined, disease associated sequence (epitope).

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PUBLICATIONS

Publications related to the thesis

Diána Simon, Tamás Czömpöly, Timea Berki, Tünde Minier, Attila Peti, Eszter Tóth, László Czirják, Péter Németh:
Naturally occurring and disease associated autoantibodies against topoisomerase I: a fine epitope mapping study in systemic sclerosis and systemic lupus erythematosus
Int Immunol. 2009;21(4):415-22. IF:3.403

Tamás Czömpöly, **Diána Simon**, László Czirják, Péter Németh:
Anti-topoisomerase I autoantibodies in systemic sclerosis
Autoimmun Rev. 2009;8(8):692-6. IF:6.368

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