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Abstract

OBJECTIVE: To search for novel autoantibodies in patients with rheumatoid arthritis (RA) in an effort to better understand the processes of joint destruction in this disease. METHODS: Using a modified SEREX technique and complementary DNA derived from RA synovium, serpin E2 was identified as a novel autoantigen and was analyzed by immunohistochemistry. Levels of anti-serpin E2 autoantibodies in serum and synovial fluid from patients with RA, osteoarthritis (OA), psoriatic arthritis, and ankylosing spondylitis, and/or from healthy individuals were assessed by enzyme-linked immunosorbent assay. Since serpin E2 is an inhibitor of serine proteases, we studied the inhibitory activity of serpin E2 toward its target, urokinase plasminogen activator (uPA), in vitro in the presence of isolated anti-serpin E2 autoantibodies and in vivo using the uPA activity assay. RESULTS: We identified autoantibodies against serpin E2 by the SEREX technique. Serpin E2 was overexpressed in RA synovial tissues as compared with OA synovial tissues. Significantly higher levels of anti-serpin E2 autoantibodies were present in samples of synovial fluid (28%) and serum (22%) from RA patients as compared with OA patients (0 and 6%, respectively) or with healthy individuals (6% of sera). Most importantly, anti-serpin E2 autoantibodies isolated from RA sera reversed the inhibitory activity of serpin E2 by 70%. Furthermore, the levels of anti-serpin E2 autoantibodies correlated with the uPA activity in vivo. CONCLUSION: This study characterizes a functional property of a novel autoantibody in RA. Since anti-serpin E2 autoantibodies interfere with the inhibitory activity of serpin E2 toward serine proteases, they might facilitate the joint destruction in RA.
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The study was supported by SNF 3200B0-103691, Zurich Center of Integrative Human Physiology (ZIHP), EURO-RA Marie Curie, Autocure FP6, Masterswitch FP7 and Olga Mayenfisch Foundation Zurich.
Abstract

Objective
Since the identification of autoantibodies could be important for a better understanding of the processes of joint destruction in rheumatoid arthritis (RA), we searched for novel autoantibodies in RA.

Methods
By a modified SEREX technique using cDNA derived from RA synovium, serpin E2 was identified as an autoantigen and analyzed by immunohistochemistry. The levels of anti-serpin E2 autoantibodies in sera and synovial fluids from RA, osteoarthritis (OA), psoriatic arthritis (PA) and ankylosing spondylitis (AS) patients and/or healthy individuals were assessed by ELISA. Since serpin E2 is an inhibitor of serine proteases, we studied the inhibitory activity of serpin E2 towards its target, urokinase (uPA), in vitro, in the presence of isolated anti-serpin E2 autoantibodies, and in vivo using the uPA activity assay.

Results
We identified autoantibodies against serpin E2 by the SEREX technique. Serpin E2 was overexpressed in RA compared to OA synovial tissues. Significantly higher levels of anti-serpin E2 autoantibodies were present in synovial fluids (28%) as well as in sera (22%) from RA patients compared to OA patients (0 and 6%) or healthy individuals (6% of sera). Most importantly, anti-serpin E2 autoantibodies isolated from RA sera reversed the inhibitory activity of serpin E2 by 70%. Furthermore, the levels of anti-serpin E2 autoantibodies correlated with the uPA activity in vivo.

Conclusion
This study characterizes a functional property of a novel autoantibody in RA. Since anti-serpin E2 autoantibodies interfere with the inhibitory activity of serpin E2 towards serine proteases, they might facilitate the destruction of joints in RA.
**Introduction**

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint destruction. Even though it is known that the persistent activation of the immune system in RA leads to autoimmunity, the trigger activating the immune response remains unclear. Nevertheless, increased levels of cytokines and autoantibodies might be detectable years before the first symptoms of RA (1-4). Already in 1992, Silman et al. reported autoantibodies in serum samples from healthy subjects up to 10 years before they developed RA (1). The most frequently detected autoantibodies in RA patients are rheumatoid factor (RF) and antibodies directed to citrullinated peptides (anti-CCP). Even though RF is not specific for RA, since it is present in 10% of healthy population, several other chronic inflammatory diseases and patients with chronic infection, it has been accepted as one of the American College of Rheumatology (ACR) criteria for RA (5-8). In contrast, a remarkable specificity for RA of 98% characterises the antibodies directed to citrullinated peptides (anti-CCP) (9-11). Even though anti-CCPs are present very early in the disease, predict radiographic progression and are associated with HLA-DRB1; they are not yet included in the ACR criteria (12-14). Citrullinated fibrin and citrullinated vimentin are among the antigens recognized by anti-CCP antibodies (11, 15). There are several other autoantibodies described in RA up to date, including autoantibodies reactive with type II collagen (CII), decorin, enolase and aldolase A (16-19).

Antibody producing plasma cells and B cells are key players in the inflammatory process of the disease and depletion of B cells using anti-CD20 antibodies has been approved for the therapy of RA (20). Nonetheless, it is still not clear how they contribute to the pathogenesis of RA. Therefore, the identification of autoantibodies and defining their possible pathological role is a challenging task.

Serological analysis of a recombinant human cDNA expression library (SEREX) is a valuable screening method extensively used for the identification of autoantibodies related to different diseases (21-26). The SEREX technique allows for a fast identification of all cDNA encoded proteins from a tissue that are recognized by autologous sera. The main advantage of SEREX over classical methods such as Western blot is the possibility of identification of a broad spectrum of autoantibodies reactive with proteins expressed extra- and intra-cellularly since it engages screening of a complete gene expression library.

In the present study we used a modified SEREX technique using a cDNA library obtained from RA synovial tissue as a screening method to identify novel targets for autoantibodies in RA. We report here increased levels of autoantibodies to serpin E2 in
synovial fluids and sera from patients with RA. In search of a functional role of these autoantibodies, we found that anti-serpin E2 autoantibodies interfered with the inhibitory activity of serpin E2 and thus might contribute to the destructive process in joints of RA patients.
Materials and Methods

Synovial fluids and sera
Synovial fluids were obtained from 44 RA, 18 osteoarthritis (OA), 10 psoriatic arthritis (PA) and 10 ankylosing spondylitis (AS) patients and sera from 183 RA patients, 64 healthy individuals, 34 OA, 11 PA and 14 AS patients as described in the supplementary material. All RA patients fulfilled the ACR criteria (27). All experiments were performed after obtaining an informed consent from the patients and healthy individuals.

Detection of autoantibodies in RA synovial fluid by SEREX
Modified SEREX technique using synovial fluid was utilized for identification of novel autoantibodies in RA using “Human rheumatoid arthritis synoviocyte lambda cDNA library kit” (Stratagene, Cedar Creek, Texas, USA) according to the manufacturer’s instructions and as previously described (21-25). The details are given in the supplementary material.

Immunohistochemistry for serpin E2 in RA and OA synovial tissues
Mouse anti-human serpin E2 IgG antibodies were used for immunohistochemistry as described in the supplementary material. A grading system was used to score the expression levels of serpin E2 in synovial tissues (0 = no expression, 1 = weak expression, 2 = medium strong expression and 3 = strong expression).

ELISA for anti-serpin E2 autoantibodies in synovial fluids and sera
ELISA for testing the content of anti-serpin E2 autoantibodies in synovial fluids and sera was performed using rh serpin E2 (R&D Systems, Abingdon, UK) and HRP conjugated goat anti-human IgG antibodies (Jackson Immunoresearch, Magden, Switzerland) as described in the supplementary material. One selected RA serum was used for standard curve and normalization of the results from all performed tests being set as 100 Arbitrary Units (AU). Cut-off values were calculated as mean value for OA patients + 2x SD for synovial fluids and as mean value for healthy individuals + 2x SD for sera. The values below respective cut-off levels were considered to be in the normal range.
Isolation of autoantibodies specific for serpin E2 from sera

The procedure of isolation of autoantibodies specific for serpin E2 from sera was performed as previously described (28) and is detailed in the supplementary material. The presence of isolated antibodies was confirmed by Western blot following every step of antibody purification. To test the reactivity of the isolated anti-serpin E2 autoantibodies, aliquots were used for precipitation of rh serpin E2. Briefly, 25 µl of anti-serpin E2 or irrelevant anti-pituitary tumor-transforming 1 interacting protein (PTTG1IP) autoantibodies conjugated to A/G plus agarose beads were incubated overnight with 100 ng of rh serpin E2. Next, the beads were washed 6 times with aqua dest. and the amount of precipitated serpin E2 was analysed by Western blot. Samples were suspended in Lemmli buffer, separated by SDS-PAGE and transferred to nitrocellulose membranes for immunodetection as previously described (29) using HRP conjugated goat anti-human IgG antibodies (Jackson Immunoresearch) or goat anti-serpin E2 (R&D Systems) and next HRP conjugated rabbit anti-goat IgG (Jackson Immunoresearch).

Functional analysis of serpin E2 activity by the uPA activity assay

The inhibitory activity of rh serpin E2 towards its target, uPA, was determined using the uPA activity assay kit (Chemicon, Temecula, CA) according to the manufacturer’s instruction. Briefly, 100 ng of rh serpin E2 were pre-incubated for 1 hour with A/G plus agarose beads. The beads were either not coupled or coupled to anti-serpin E2 autoantibodies isolated from sera from healthy individuals/ anti-serpin E2 autoantibodies isolated from sera from RA patients or to irrelevant anti-PTTG1IP autoantibodies isolated from sera from RA patients. To each urokinase reaction the amount of added antibodies against serpin E2/PTTG1IP was the amount in which they were isolated from 1 ml of 1:4 diluted sera. Next, 5U of uPA, a uPA substrate and assay buffer were added. In the positive control reaction uPA, uPA substrate and assay buffer were used only. Reactions were performed overnight at 37°C. uPA activity was measured in an ELISA reader at 405 nm.

Statistical analysis

Student's t-test and the Mann–Whitney U-test were used for comparison between two groups of parametric and non-parametric data respectively. For assessing correlations, $r^2$ values were quantified from the Pearson correlation coefficient. P values lower or equal to 5% were considered statistically significant ($p \leq 0.05$).
Results

Identification of novel autoantibodies in RA synovial fluid

By modifying the SEREX technique and using RA synovial fluid for the identification of novel autoantibodies in RA we identified 61 immunoreactive clones in the first screening. Positivity of nine clones was confirmed by the second set of screening (Figures 1A, B). The identified clones included previously described autoreactivities against vimentin (VIM), decorin (DCN), aldolase A (ALDOA) and the eukaryotic translation elongation factor 1 alpha 1 (EEF1A1). Moreover, we were able to identify novel autoreactive antibodies in RA, namely autoantibodies against pituitary tumor-transforming 1 interacting protein (PTTG1IP), glyoxalase I (GLOI), connective tissue growth factor (CTGF), neuregulin 1 isoform GGF2 (NRG1) and serpin E2.

Serpin E2 as a novel target for autoantibodies in RA

Among other proteins, serpin E2 was recognized by autoantibodies in RA synovial fluid in the SEREX. Serpin E2 is a natural inhibitor of serine proteases such as plasmin, uPA, thrombin and trypsin that show increased activity in RA and are implicated in the pathogenesis of joint destruction. Since serpin E2 was described to play a preventive role in cartilage degradation (30), we focused on the role of anti-serpin E2 autoantibodies. The cDNA clone in the plaques reactive with anti-serpin E2 autoantibodies included the 3’ part of the serpin E2 CDS (base pairs: 1819-1854). This region of the serpin E2 gene is in close proximity to the region coding for the reactive center loop (RCL) which is essential for the interaction of serpin E2 with serine proteases (Figure 1C).
Serpin E2 is expressed in synovial tissues from patients with RA and OA
To investigate the expression levels of serpin E2 in RA and OA synovial tissues, we performed immunohistochemical staining using anti-serpin E2 antibodies (Figures 2A, B). In both RA and OA synovial tissues serpin E2 was expressed in the cells around blood vessels (median score ±SD: 2.0±0.7 in RA vs 1.5±0.7 in OA). Most interestingly, in the synovium of patients with RA, serpin E2 was overexpressed both in the lining (2.0±0.9 in RA vs 0.5±0.5 in OA, p≤0.01) and in the sublining layer (2.0±0.5 in RA vs 1.0±0.9 in OA, p≤0.01, n=10 for each, Figure 2C). This pattern differed from synovium of patients with OA, where serpin E2 was present mostly around blood vessels. Thus, serpin E2 is significantly overexpressed in RA synovial tissue.

RA patients have high levels of anti-serpin E2 autoantibodies in synovial fluids
After serpin E2 had been identified as a novel target for autoantibodies by the SEREX technique, we evaluated the levels of autoantibodies specific for serpin E2 in synovial fluids from patients with RA (n=44), PA (n=10), AS (n=10) and OA (n=18) by ELISA. In RA, anti-serpin E2 autoantibodies were detected at significantly higher levels (mean AU ±SD: 67±66) compared to synovial fluids of OA patients (31±31, p<0.05). Serpin E2 reactive autoantibodies were present in 28% of the synovial fluids in RA in contrast to OA synovial fluids of which all were in the normal range (Figure 3A). In both PA and AS patient groups anti-serpin E2 autoantibodies were present in 20% of the synovial fluids, however, the difference from the control OA synovial fluids did not reach statistical significance (mean AU ±SD: 43±53 in PA and 58±37 in AS). In conclusion, RA patients have higher prevalence of anti-serpin E2 autoantibodies in the synovial fluids than OA patients.
RA patients have high levels of anti-serpin E2 antibodies in sera

Next, we analyzed whether autoantibodies against serpin E2 found in synovial fluid are also present in sera. Blood sera from RA (n=183), OA (n=34), PA (n=11) and AS patients (n=14) and healthy individuals (n=64) were tested for the presence of anti-serpin E2 autoantibodies. In sera of RA patients, anti-serpin E2 autoantibodies were detected at significantly higher levels (mean AU ±SD: 125±90) compared to sera of OA patients (86±51, p<0.02) and normal individuals (61±51, p<0.01). Anti-serpin E2 autoantibodies were detected in 22% of the sera of patients with RA. This significantly differed from OA patients and healthy individuals, of which 6% were positive (Figure 3B). Furthermore in the group of RF negative RA sera 16% were positive for anti-serpin E2 autoantibodies. The levels of anti-serpin E2 autoantibodies in the CCP positive and negative subgroups were not statistically different. The PA sera were positive for anti-serpin E2 autoantibodies in 9% of cases and did not differ significantly from the control OA sera (mean AU ±SD: 81±53). In contrast, anti-serpin E2 autoantibodies were present in 33% of AS sera (247±304) and this group significantly differed from healthy individuals (p<0.01), OA (p<0.02) and PA patients (p<0.05). Although autoantibodies against serpin E2 were detected by the screening procedure using synovial fluids, similarly in sera the autoantibodies against serpin E2 were detected. Thereby, RA patients show significantly higher levels of anti-serpin E2 autoantibodies than OA patients and healthy controls.

Assessment of the functional role of anti-serpin E2 autoantibodies

Due to the fact that serpin E2 has profound effects on the remodelling of ECM and that the autoantibodies bind to a region of serpin E2 in proximity of the RCL, we analysed the function of autoantibodies on serpin E2 activity. To evaluate whether the inhibitory function of serpin E2 is influenced by the presence of specific anti-serpin E2 autoantibodies, we isolated anti-serpin E2 antibodies from sera of patients with RA. As a control, we used sera from healthy individuals. Serpin E2 was immobilised on sepharose beads and incubated with diluted sera of RA patients or healthy controls. The antibodies were eluted and purified from the eluents by binding to A/G plus agarose beads. We quantified the isolated antibodies by Western blot. As expected, more anti-serpin E2 autoantibodies were isolated from sera of patients with RA (mean densospot units ±SD: 42±7) in comparison to healthy individuals (19±3, Figure 4A,B). The isolated anti-serpin E2 autoantibodies precipitated serpin E2 according to their concentration (n=8, r²=0.76, p<0.01, Figure 4C). We also tested whether control irrelevant autoantibodies (anti-PTTG1IP autoantibodies) isolated from RA sera could
precipitate serpin E2 \((n=2)\). As expected, the irrelevant anti-PTTG1IP autoantibodies did not precipitate serpin E2 (Figure 4D).

Since uPA is a serine protease that is inhibited by serpin E2, we investigated the ability of serpin E2 to inhibit the activity of uPA in the presence of anti-serpin E2 autoantibodies isolated from sera of patients with RA. We analysed the activity of uPA in the presence of either rh serpin E2 or rh serpin E2 preincubated with isolated anti-serpin E2 specific autoantibodies from patients with RA. As expected, rh serpin E2 \((500\text{ng/ml})\) inhibited the activity of uPA. Most importantly, in the presence of serpin E2 specific autoantibodies isolated from sera of patients with RA, inhibition of the uPA activity by serpin E2 was diminished by \(69\pm8\%\) \((n=4)\). Anti-serpin E2 antibodies isolated from normal sera diminished the inhibition of the uPA activity by serpin E2 by \(49\pm10\%\) \((n=4)\). Thus, the reduction of the inhibitory effect of rh serpin E2 in presence of autoantibodies isolated from RA patients was significantly higher in comparison to autoantibodies from healthy individuals \((p\leq0.05, \text{Figure 5A})\). In the presence of irrelevant autoantibodies (anti-PTTG1IP autoantibodies) isolated from RA sera \((n=2)\), the inhibitory activity of serpin E2 towards uPA was not decreased. Since isolated anti-serpin E2 autoantibodies from different sera were impairing the activity of serpin E2 to different extents we correlated the amount of isolated antibodies and the respective values for uPA activity. Increasing quantities of antibodies, as measured by densometric analysis, correlated significantly with uPA activity \((r^2=0.5, p<0.05, \text{Figure 5B})\). From these experiments, we conclude that the anti-serpin E2 autoantibodies impair the inhibitory function of rh serpin E2 in vitro in a dose dependant manner.

**The levels of anti-serpin E2 autoantibodies in sera correlate with the activity of uPA in sera and synovial fluids in vivo**

Based on the results above, where we showed in vitro the function of isolated anti-serpin E2 autoantibodies on the inhibitory activity of rh serpin E2, next we investigated the function of these autoantibodies in vivo. Thereby, we analysed the uPA activity and the concentration of anti-serpin E2 in sera and synovial fluids samples. Indeed, high levels of anti-serpin E2 autoantibodies corresponded to high activity of uPA in sera \((r^2= 0.12, p\leq0.05)\), indicating that in vivo serpin E2 is a less active inhibitor in the presence of anti-serpin E2 autoantibodies (Figure 5C). In contrast, the levels of anti-serpin E2 autoantibodies in synovial fluids did not correlate significantly with the activity of uPA in synovial fluids (data not shown). In addition, the quantities of anti-serpin E2 autoantibodies found in sera correlated significantly with the activity of uPA in synovial fluids \((r^2= 0.2, p\leq0.05, \text{Figure 5D})\). In conclusion, the
levels of anti-serpin E2 autoantibodies in sera were associated with the activity of uPA in sera and synovial fluids.
Discussion

Using the SEREX screening technique we have identified several novel targets for autoantibodies in RA. Among the identified clones we found decorin, vimentin and aldolase A, to which specific autoantibodies have already been described in RA patients (11, 17, 18). Another antigen, eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) has been previously identified to be immunoreactive in Felty syndrome and has also been related to RA (31). Moreover, we were able to identify previously unknown autoreactive antibodies in RA synovial fluids, namely autoantibodies directed to pituitary tumor-transforming 1 interacting protein (PTTG1IP), glyoxalase I (GLOI), connective tissue growth factor (CTGF), neuregulin 1 isoform GGF2 (NRG1) and serpin E2.

Serine proteases such as plasmin, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), thrombin and trypsin have increased activity in RA, and are thought to contribute to the pathogenesis of the disease (32-36). In a collagen induced mouse model of RA, plasmin was shown to be an essential component of the early phase of pathogenesis (37). Thrombin is known to induce angiogenesis, fibrin formation and inflammation (38, 39), which are also the primary events of joint destruction in RA. RA synovial tissues exhibited considerably increased activity of uPA in the lining layer and RA synoviocytes were shown to be more prone to uPA-challenged invasion and proliferation (36, 40).

Specific inhibitors of serine proteases such as antithrombin, antiplasmin and plasminogen activator inhibitor-1 (PAI-1) belong to the family of serine protease inhibitors (serpins). Serpins are classified into 16 groups according to the phylogenetic analysis, and contain members in higher animals, nematodes, insects, plants and viruses. The majority of the serpin family members are active serine protease inhibitors with the reactive centre loop (RCL) active site located in the C-terminal part. The RCL forms a bait which is targeted by a serine protease. After the protease cleaves the RCL domain, it is bound covalently on the catalytic serine residue and irreversibly inhibited. Next, the protease bound to serpin is internalized and degraded.

Serpin E2, also called protease nexin 1/glial derived nexin, is classified to clade E of the serpin family together with PAI-1 and Myxoma virus SERP-1. Serpin E2 regulates matrix accumulation and coagulation by inhibiting thrombin, plasmin, tPA and uPA. Serpin E2 was shown to be induced by proinflammatory cytokines in different cell types. IL-1β, TGF and TNFα induced serpin E2 in neurons and muscle cells (41, 42), chronic exposure to TNF-α in
rat fibroblast-like synoviocytes and mouse endothelial cells (43, 44) and stimulation of human monocytes with LPS upregulated the expression of serpin E2 (45). Recently, serpin E2 was detected at high levels in atherosclerotic plaques and suggested to play a protective role against aggression of proteases under inflammatory conditions (45). Most interestingly, serpin E2 indeed prevented IL-1β/bFGF induced articular cartilage loss through the inhibition of plasmin thus preventing subsequent activation of MMPs in rabbits (30).

Given the protective role of serpin E2 against cartilage loss by inhibiting plasmin and averting subsequent activation of MMPs (30, 46) we focused on analysing autoantibodies against serpin E2 in RA.

We have established an ELISA to measure the levels of anti-serpin E2 autoantibodies in sera and synovial fluids of patients with RA, OA and of healthy individuals. We showed that patients with RA have high levels of anti-serpin E2 autoantibodies. It might be argued that the rheumatoid factor present in the sera from RA patients could react with the anti-serpin E2 autoantibodies and thereby amplify the signal obtained in ELISA. Therefore, we analysed RF negative sera from RA patients (n=37). The detected levels of anti-serpin E2 autoantibodies in RF negative sera from RA patients were also significantly higher as compared to healthy controls (data not shown).

To investigate the functional significance of elevated levels of anti-serpin E2 in patients with RA, we tested the inhibitory activity of rh serpin E2 in the presence of anti-serpin E2 autoantibodies isolated from human serum. We could show that the activity of serpin E2 in vitro was decreased by anti-serpin E2 autoantibodies isolated from sera of patients with RA. Less anti-serpin E2 autoantibodies were isolated from sera of healthy individuals which was in accordance with the results from ELISA. Anti-serpin E2 autoantibodies isolated from sera of healthy individuals also interfered with the activity of serpin E2, but to a lesser extent according to their lower concentration. Therefore, we conclude that the pathologically elevated levels of anti-serpin E2 autoantibodies found in RA patients could lead to the increased activity of serine proteases in this disease.

Moreover, the levels of anti-serpin E2 autoantibodies in sera correlated significantly with the activity of uPA measured in sera and in synovial fluids. Higher levels of anti-serpin E2 autoantibodies were detected in blood sera than in synovial fluids of RA patients. This could be due to the infiltration and attachment of the anti-serpin E2 autoantibodies to serpin E2 that is overexpressed in the synovial tissue which would result in lowering the levels of anti-serpin
E2 in the synovial fluid. This possibility is supported by the results showing that the quantities of anti-serpin E2 autoantibodies found in sera correlated significantly with the activity of uPA in synovial fluids whereas we could not observe a correlation between uPA activity and the amounts of anti-serpin E2 detected in synovial fluids.

It still remains to be elucidated whether autoantibodies reduce the activity of serpin E2 through binding and blocking of the RCL domain essential for activity, through changing the conformation of serpin E2 or through another mechanism. Analysis of the sequence of the cDNA coding for a fragment of serpin E2 reactive with sera of patients with RA in SEREX showed that the autoantibodies bind to 10 aminoacids in the C-terminal domain of serpin E2. Since the RCL responsible for the inhibition of serine proteases is located in the C-terminus, it is therefore possible that the autoantibodies binding to the C-terminal part of serpin E2 could block the interaction of serpin E2 with the proteases.

In RA synovial tissues serpin E2 was expressed in cells around blood vessels, the lining and sublining layers of the synovium. In contrast, in OA control synovial tissues serpin E2 was expressed by cells arounded blood vessels mostly. The expression of serpin E2 in vessels was described in normal human arteries but it was never shown in the plasma or in the circulation unlike other serpins such as antithrombin III, antitrypsin and antichymotrypsin which function as serine protease inhibitors regulating coagulation cascades (45). Serpin E2 localised in blood vessels could be responsible for the regulation of thrombosis or coagulation at the tissue or cellular level. The increased expression and the special localisation of serpin E2 in synovium of patients with RA could indicate that a counter-regulatory mechanism is activated to decrease the activity of proteases and that this putative defence mechanism is at least in part inhibited by anti-serpin E2 autoantibodies.

It is not known whether the direct blocking effect of anti-serpin E2 autoantibodies on the function of serpin E2 is an isolated feature of this specific antigen-antibody complex. It is possible that also other antigens are modified in their activity or properties when bound to specific autoantibodies. In this view, the production of autoantibodies could be an important part in the pathology of RA, rather than an epiphenomenon. Recently, Pullerits et al. reported that the presence of autoantibodies specific for RAGE coincidences with ameliorated erosions in RA, suggesting thereby a protective role of anti-RAGE autoantibodies against inflammation in the synovium (47). Here, on the contrary, we show that autoantibodies specific for serpin E2 might worsen the disease, since autoantibodies isolated from sera of
patients with RA reverted the protective inhibitory activity of serpin E2. Anti-serpin E2 autoantibodies might therefore favour the proteolytic milieu in RA joints, as well as enhance inflammation, since it has been reported that increased serine protease activity intensifies inflammation (48). Therefore, it would be interesting to investigate whether RA patients with increased levels of anti-serpin E2 autoantibodies respond to B cell depletion treatment, for example with anti-CD20 antibodies, as shown with some autoantibodies in SLE.

It also has to be taken into account that serpin E2 inhibits a wide range of proteases involved in the pathogenesis of RA. Thus, it is probable that the presence of autoantibodies specific for serpin E2 could have a greater effect on the destruction of cartilage than only by increasing the activity of uPA (Figure 6). However, it is difficult to evaluate the extent to which the anti-serpin E2 antibodies have an influence on cartilage destruction through the impairment of serpin E2 function in vivo. At least, here we could show that autoantibodies isolated from sera of RA patients impair the inhibition of uPA activity by serpin E2 in vitro and that high levels of anti-serpin E2 autoantibodies correlate with high uPA activity in vivo.

Moreover, since serpin E2 is abundantly present in the atherosclerotic plaques (45) and vascular changes are common in RA patients (49) we conclude that the anti-serpin E2 autoantibodies could also play a role in the formation of the plaques. We are currently addressing this question.

To our knowledge, this is the first study showing a distinct effect of an autoantibody which might be related to the pathogenesis of RA.

Acknowledgments

We thank Maria Comazzi, Allan Ogilvie, Martin Mueller and Claudia Frei for excellent technical assistance.
Figure legends

Figure 1: Identification of novel targets for autoantibodies in RA using SEREX

Human cDNA expression library from a patient with RA was used for screening with SEREX. For identification of novel targets for autoantibodies RA synovial fluid was used. (A) Multiple plaques expressing one positive antigen clone reactive with synovial fluid of a patient with RA and one negative clone on nitrocellulose membrane after second screening round. Assays were scored positive only when test clones were clearly distinguishable from control clones. The bold arrow indicates the test clone and the blank arrow indicates the control clone (B) Targets for autoantibodies in RA identified by SEREX. References are given for identified targets previously described as an autoantigen in RA. (C) Diagram showing a CDS sequence of serpin E2 (region 661-1854 of serpin E2 cDNA, accession number BC042628). The autoantibodies from RA synovial fluid were reactive with a peptide encoded by region 1819 - 1854 of serpin E2 cDNA which is in close proximity to the reactive center loop (RCL) coding region (1711-1788).

Figure 2: Expression of serpin E2 in RA and OA synovial tissues

Immunohistochemistry showing the expression of serpin E2 in RA and OA synovial tissues. Slides were counterstained with hematoxylin. Magnification x100. Serpin E2 was expressed throughout the synovium in RA (A) and mainly around vessels in OA (B). IgG controls for RA and OA are shown in small insets. Original magnification x100. (C) Scoring of immunohistochemical staining for serpin E2 in OA (n=10, white bars) and RA synovial tissues (n=10, black bars) in the areas of the lining layer (plain bars), sublining layer (dotted bars) and vessels (striped bars). Values are given as score median ±SD. P values were calculated using Mann-Whitney U Test. P ≤ 0.05 was considered significant and indicated with *.

Figure 3: Levels of anti-serpin E2 autoantibodies in sera and synovial fluids

Levels of autoantibodies reactive with rh serpin E2 in synovial fluids and sera. Dots represent values obtained for each individual and the mean of total samples in each group is indicated with a black bar. The red dots represent positive individuals. P values were calculated using the Student T Test and p ≤ 0.05 considered significant are indicated. The results are summarized in a table showing the mean levels of anti-serpin E2 autoantibodies detected by ELISA in synovial fluids or sera (upper panel). The percentage of synovial fluids or sera positive for anti-serpin E2 autoantibodies is given in the lower panel. (AU = arbitrary units).
(A) Levels of autoantibodies reactive with rh serpin E2 in synovial fluids of patients with OA (n=18), RA (n=44), PA (n=10) and AS (n=10) measured by ELISA. (B) Levels of autoantibodies reactive with rh serpin E2 in sera of healthy individuals (n=64) patients with OA (n=34), RA (n=183), PA (n=11) and AS (n=14) measured by ELISA.

**Figure 4: Anti-serpin E2 autoantibodies isolated from sera precipitate serpin E2**

(A) Levels of autoantibodies reactive with rh serpin E2 in serum samples from healthy individuals and RA patients used for the isolation of anti-serpin E2 autoantibodies. Dots represent values obtained for each individual and the mean of total samples in each group is indicated with a black bar. (AU = arbitrary units). (B) Autoantibodies isolated from human sera detected by Western blot with anti-human IgG antibodies. Anti-serpin E2 autoantibodies isolated from blood sera of healthy individuals (lanes 1-4) or patients with RA (lanes 5-8) and anti-PTTG1IP antibodies isolated from RA serum (lane 9). Heavy and light chain of IgG of expected sizes are indicated by arrows. Quantification of the levels of anti-serpin E2 autoantibodies isolated from sera of healthy individuals (n=4, white bar) or from sera of patients with RA (n=4, black bar) and anti-PTTG1IP autoantibodies isolated from sera of RA patients (n=2, white dotted bar) using densitometry. (C) Correlation between the amount of isolated anti-serpin E2 autoantibodies and % of precipitated serpin E2. (D) The ratio of % precipitated serpin E2 per densospot unit of isolated anti-serpin E2 autoantibodies (white bar) and isolated anti-PTTG1IP autoantibodies (black bar) ±SD.

**Figure 5: Correlation between the levels of anti-serpin E2 autoantibodies and the activity of uPA**

(A) uPA activity measured by uPA activity assay. Bars show downregulation of the uPA activity in the absence of rh serpin E2 (white bar), in the presence of rh serpin E2 (set as 100%, black bar), in the presence of rh serpin E2 preincubated with anti-serpin E2 antibodies isolated from sera of normal individuals (n=4, white striped bar) or from sera of RA patients (n=4, black striped bar) or rh serpin E2 preincubated with anti-PTTG1IP antibodies isolated from sera of RA patients (n=2, white dotted bar). Values are given as mean % inhibition of uPA ±SD. P≤0.05 calculated using Mann-Whitney U Test was considered significant and indicated with *. (B) Correlation between the levels of anti-serpin E2 autoantibodies isolated from sera of RA patients (black squares) and normal controls (white squares, n=4 each) and activity of uPA in presence of rh serpin E2.
(C) Correlation between the levels of anti-serpin E2 antibodies and the activity of uPA measured in sera (n=48). (D) Correlation between the levels of anti-serpin E2 antibodies measured in sera and activity of uPA in synovial fluids of the same patients (n=20).

Figure 6: Hypothetical pathways influenced by the interaction of serpin E2 with anti-serpin E2 autoantibodies

Occurrence of anti-serpin E2 autoantibodies which have the ability to interfere with the inhibitory activity of serpin E2 might cause a different outcome in RA.
Figure 1.
Figure 2.

Figure 3.
Figure 4.

- A: Anti-serpin E2 antibodies in sera (AU)
- B: Anti-serpin E2 antibodies (densosopt unit)
- C: $r^2=0.76$, p<0.01
- D: Ratio of precipitated serpin E2 (%) / densosopt units of isolated IgG
Figure 5.

Autoantibodies against serpin E2 impair the inhibitory function of serpin E2 towards serine proteases

Autoantibodies

Serpin E2 inhibiting

Role in RA:

- Plasmin → Matrix degradation (34)
- uPA → Invasion and proliferation of RA synoviocytes (36, 40)
- Thrombin → Angiogenesis, fibrin formation and inflammation (35, 38)
- Trypsin → Degradation of collagen type II (33)

Figure 6.