Cystatin C Binds Serum Amyloid A, Downregulating Its Cytokine-Generating Properties

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ABSTRACT. ***Objective.*** To assess the interaction between cystatin C (CysC) and serum amyloid A protein (SAA).

Methods. Levels of CysC and SAA and antibodies against these proteins were assessed in the paired blood and synovial fluid (SF) samples of 90 patients with rheumatoid arthritis (RA). Age and sex matched individuals having normal iohexol clearance (n = 90) and SF following joint trauma (n = 40) were used as controls. *In vitro* experiments included assessment of interaction between CysC and SAA by ELISA and the influence of CysC on SAA functions.

Results. A pilot screening for cystatins C, E, and F in blood and SF of patients with RA found CysC to be by far the predominant extracellular cystatin. Circulating CysC levels were significantly lower in patients with RA compared to the matched controls (0.81 ± 0.03 vs 1.01 ± 0.03 mg/l; p = 0.05). These low CysC levels could not be explained by the presence of anti-CysC antibodies in patients with RA. In contrast, concentrations of CysC that accumulated in the inflamed SF were significantly greater in patients with erosive RA (1.66 ± 0.08 mg/l) compared to nonerosive RA (1.36 ± 0.06 mg/l; p = 0.003) and controls (1.18 ± 0.03 mg/l; p = 0.043). *In vitro* studies showed direct binding of CysC to SAA. CysC/SAA binding impaired proinflammatory effects of SAA, reducing its ability to trigger expression of proinflammatory cytokines.

Conclusion. Our study shows a relative deficiency of circulating CysC during systemic inflammation in RA. Physical interaction between CysC and the acute-phase protein SAA (1) provides an explanation for CysC deficiency; and (2) suggests that CysC is regulating inflammatory responses. We hypothesize that decreased systemic CysC levels predispose to accelerated atherosclerosis and development of amyloidosis in patients with RA. (J Rheumatol 2007;34:1293–301)

Key Indexing Terms:
CYSTATIN C SERUM AMYLOID A CYTOKINES RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by joint destruction and severe disability. Comorbid conditions associated with RA including the increased prevalence of atherosclerosis and amyloidosis are important causes of early mortality in this patient group[1].

Extracellular proteases are a group of enzymes consisting of matrix metalloproteinases (MMP), serine proteases, and cysteine proteases, essential for the reorganization of extracellular matrix. The role of MMP and serine proteases (plasmin and urokinase) in the pathogenesis of cartilage and bone destruction during RA is increasingly well understood[2-4]. Although several members of the cathepsin family of cysteine proteases are overexpressed in synovial tissues and at the site of bone erosions of patients with RA[5-7], information regarding cysteine proteases, i.e., cathepsins, and their endogenous inhibitors, cystatins, in the pathogenesis of chronic arthritides is scarce.

Cathepsins are one of the main families of cysteine proteases, playing key roles in such physiological processes as intracellular protein degradation (cathepsins B, H, and L), in bone remodeling (cathepsin K), and potentially in antigen presentation (cathepsin S, legumain) (reviewed by Varghese[8]). Cystatins control the activity of cathepsins subdivided into 3 functional classes: (1) intracellular, cytoplasmic cystatins/stefins; (2) cell secreted cystatins; and (3) intravascular cystatins of the kininogen family[9].

Cystatin C (CysC) is the most abundant extracellular inhibitor of cysteine proteases[10]. Structurally, CysC molecule expresses independent active sites for binding different types of cysteine proteases. Indeed, CysC is a potent inhibitor of papain- and legumain-types of cysteine proteases[11]. Addition-

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ally, a serpin-like domain with unclear functional role has been recognized[9]. Most human cells constitutively express and secrete CysC into biological fluids. CysC has been shown to accumulate in serum, pleural effusions, and ascitic and cerebrospinal fluids, but not in the tissues during inflammation and tumor progression[9,12]. The concentrations of CysC are sufficiently high to efficiently inhibit any lysosomal cysteine protease that is released into a body fluid. Due to its low molecular weight (13.5 kDa), CysC is efficiently eliminated from the serum, making it an excellent marker of the glomerular filtration rate[12].

Diverse biological functions have been discussed in relation to CysC. As an inhibitor of cysteine proteases, CysC controls the remodeling of extracellular matrix[10,11]. Hereditary deficiency of CysC is associated with formation of arterial aneurysms and intracranial hemorrhages. It has been suggested that imbalance in the local expression of cathepsins and CysC leads to atherosclerosis and formation of aneurysms[13,14]. Recently, CysC has been indicated as an inhibitor of excessive bone resorption[15,16] regulating osteoclastogenesis. CysC has been shown to promote DNA synthesis and proliferation of fibroblasts and smooth-muscle cells[17]. These properties of CysC are of potential importance for RA, for cancer metastasis, and also for osteoporosis. Immunomodulatory properties of CysC that are probably independent of its enzymatic activity have become more apparent. Transforming growth factor-ß (TGF-ß) is suggested as the main inducer of CysC expression[13,18], while CysC functions as an antagonist of TGF-ß receptor providing a negative feedback to its own expression[19]. A modulatory effect of CysC on antiinflammatory cytokines has been suggested[20-22]. Proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α (TNF-α) downregulate CysC production[22].

There is both clinical and experimental evidence supporting a protective effect of CysC against amyloidosis[23,24]. In contrast, a mutation of CysC gene [variant human CysC (L68Q)] followed by changes in its structural and chemical properties turns CysC into an amyloidogenic protein[25,26]. Indeed, CysC is found as a main constitutive component in the amyloid depositions of patients with hereditary amyloidosis of Icelandic type, cerebral amyloid angiopathy, and Alzheimer's disease[27,28].

Amyloidosis is one of the most severe and life-threatening complications of RA[29]. It is characterized by the vascular deposition of insoluble fibrillar amyloid A (AA). AA fibrils are derived from the acute-phase protein serum AA (SAA). It has been shown that SAA is not merely an outcome of inflammation but rather a direct participant able to trigger production of proinflammatory cytokines[30]. Clearly, not all patients with RA develop AA amyloidosis. Biopsy evidence of amyloid can be found in 4% to 26% of patients with longstanding RA[31]. A reversible character of AA depositions was revealed, with decrease of amyloid load following immunosuppressive treatment and its increase with relapse of RA[32].

We show that CysC is by far the predominant extracellular cystatin in patients with RA, with significant accumulation in the inflamed synovial fluid (SF). We also show an inverse relationship between low circulating CysC levels and their high intraarticular levels, suggesting systemic consumption of this molecule. Further, we prove a direct interaction between CysC and SAA molecules that results in neutralization of the proinflammatory properties of SAA.

MATERIALS AND METHODS

Patients. Plasma and SF samples were collected from 90 patients with RA who attended the rheumatology clinics at Sahlgrenska University Hospital in Gothenburg for acute joint effusion. RA was diagnosed according to the American College of Rheumatology criteria[33]. Clinical and demographic data of the RA patient population are presented in Table 1. At the time of SF and blood sampling all the patients received nonsteroidal antiinflammatory drugs. Disease modifying antirheumatic drugs (DMARD) were taken by 48 patients, of whom 35 took methotrexate (MTX), 5 salazopyrin, 2 leflunomide, 4 parenteral or oral gold salt compounds, 5 cyclosporin A (2 in combination with MTX, 1 in combination with leflunomide, 1 in combination with azathioprine), and the remaining 2 patients were treated with azathioprine. Nine patients were treated with a combination of DMARD (MTX 8 patients, azathioprine + cyclosporin A 1 patient) and TNF-α inhibitors (5 patients received infliximab, 4 patients had etanercept). One patient received MTX in combination with interleukin 1 (IL-1) receptor inhibitor. The remaining 42 patients had no DMARD treatment at the time of blood and SF sampling. Thirty-seven patients received oral corticosteroids (mean dose 8.25 mg prednisolone/day). Patients receiving monotherapy with glucocorticosteroids (n = 9, mean dose 12.1 mg/day) were considered as having no DMARD treatment. Recent radiographs of the hands and feet were obtained for all patients. Presence of bone erosions, defined as the loss of cortical definition of the joint, was recorded in proximal interphalangeal, metacarpophalangeal, carpus, interphalangeal, and metatarsophalangeal joints of forefeet. Presence of one erosion was sufficient to fulfill requirement of an erosive disease. Presence of rheumatoid factor (RF) of any immunoglobulin isotype was considered as positive.

The study was approved by the Ethical Committee of the University of Göteborg and the Ethics Committee of Sahlgrenska Hospital. Informed consent was obtained from all patients.

Control groups. Recent studies have indicated that circulating CysC levels are dependent on the glomerular filtration rate and the age of individuals[12]. To minimize the influence of these measures in our study, blood samples from individuals having normal plasma clearance of iohexol and matching the RA patients with respect to age and sex (male n = 29, female n = 61) were used in the control group. SF from 40 patients (age 23–88 yrs, male n = 21, female n = 19) with noninflammatory knee joint diseases was used as control. The group with noninflammatory joint diseases included 8 patients with knee osteoarthritis, 21 with anterior cruciate ligament rupture, 3 with chondrocalcinosis, 4 with rupture of meniscus, and 4 with knee joint contusion.

Collection and preparation of samples. SF was obtained from knee joints by arthrocentesis, aseptically aspirated into tubes containing sodium citrate (0.129 mol/l; pH 7.4). Blood samples were simultaneously obtained from the cubital vein and directly transferred into sodium citrate medium. Collected blood and SF samples were centrifuged at 800 g for 15 min, aliquoted, and stored frozen at –20°C until use.

Measurement of CysC/SAA binding. The 96-well flat-bottom polyester ELISA plate (Nunc, Denmark) was coated with a precursor of AA protein, apo-SAA (1 μg/ml, 50 μl/well; PeproTech, London, UK) in carbonate buffer (pH 9.6) overnight. The control wells were coated with equimolar amounts of bovine serum albumin (BSA). The plates were washed with phosphate buffered saline (PBS)/0.05% Tween-20 and blocked with 1% PBS-BSA (pH 7.4) at 37°C for 1 h. After washing, different amounts of recombinant CysC (0–1000 pg/ml, 50 μl/well) were added and incubated 1 h at 37°C. After washing,

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Table 1. Clinical characteristics of patients with RA.

	Erosive RA, n = 47	Nonerosive RA, n = 43	p*	Controls, n = 90
Sex, F/M	30/17	31/12	NS	61/29
Age, yrs	61.2 ± 2	54.2 ± 1.3	< 0.05	58.4 ± 1.4
Rheumatoid factor, +/–	39/8	8/35	< 0.001	NA
Disease duration, yrs	12.1 ± 1.3	7.7 ± 1.3	< 0.001	0
Treated with DMARD	34	14	< 0.03	0
Methotrexate	26 (55%)	9 (21%)		
Other**	8 (17%)	5 (12%)		
Oral corticosteroids	17 (36%)	20 (46%)		
Nontreated	13 (28%)	29 (67%)	< 0.05	
Creatinine, μmol	60.9 ± 2.6	61.0 ± 3.7	NS	64.2 ± 2.2
CRP, mg/l	42 ± 8	38 ± 7	< 0.03	NA
WBC count, × 10^9/l				NA
Blood	8.2 ± 0.4	7.34 ± 0.2	NS	
Synovial fluid	10.6 ± 2.5	13.0 ± 2.5	NS	

* Between groups of erosive and nonerosive RA. ** Other: sulfasalazine 5, gold salts 4, leflunomide 1, cyclosporine A 5 (in combination with methotrexate 2, azathioprine 1, leflunomide 1, sulfasalazine 1). CRP: C-reactive protein, WBC: white blood cells. NS: nonsignificant, NA: not assessed.

SAA-immobilized CysC was detected by addition of rabbit anti-human CysC antibodies (Dakopatts, Copenhagen, Denmark) for 1 h at room temperature, followed by goat anti-rabbit alkaline phosphatase conjugated antibodies (Sigma, St. Louis, MO, USA) and enzyme substrate.

Preparation of CysC/SAA complexes. Recombinant human CysC was produced as described[34]. To allow the reaction between SAA and CysC, 5 and 50 μg of recombinant apo-SAA was incubated with increasing amounts of CysC (0–100 μg) in Tris-HCl buffer, 50 mM, pH 7.4, for 30 min at 37°C. The ability of the SAA-CysC mixtures, and each of the components separately, to induce cytokine expression was assessed in cultures of human peripheral blood mononuclear cells (PBMC).

Cell culture preparation and stimulation conditions. PBMC were prepared from heparinized blood of healthy individuals by separation on a Lymphoprep density gradient, washed, and resuspended in complete medium (Iscove's medium containing 1% L-glutamine, 5 × 10^{-5} M ß-mercaptoethanol, 50 μg/ml gentamycin sulfate, and 10% heat inactivated fetal calf serum). Freshly isolated PBMC were resuspended to 2 × 10^6/ml and cultured with SAA-CysC complexes (see above), and each of the components separately in 24-well plates in a humidified atmosphere of 5% CO_2 at 37°C. For extracellular release of cytokines, supernatants were collected following 48 h of stimulation. Cell stimulation was performed in 2 independent experiments using PBMC cultures from 3 different blood donors. The results obtained in all the experiments were pooled.

CysC levels were determined by an automated particle-enhanced immunoturbidimetric assay using reagents obtained from Dako Cytomation (Copenhagen, Denmark) as described[12,35]. The procedure was implemented on the Cobas Mira Plus Instrument (Roche, Basel, Switzerland). The obtained values were recalculated using a standard curve and expressed in mg/l. The total analytical imprecision of the method, calculated using a control sample with a defined CysC value, was 3.2%. Cystatin E and cystatin F levels were detected by ELISA as described[36].

SAA levels were measured in blood of patients with RA and matched controls by an ELISA using the SAA kit from BioSource International (Camarillo, CA, USA) following manufacturer's instructions. Before analyses serum samples were diluted 1/2000 in diluent buffer provided in the kit. The absorbance of the human SAA in serial dilutions ranging from 9.4 to 300 ng/ml were plotted on a semi-log graph and the concentration of SAA in the samples was calculated.

Antibodies against CysC and SAA were measured in blood of RA patients and matched controls by ELISA. Ninety-eight-well plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with recombinant human CysC or apo-SAA (10 μg/ml, 50 μl/well) and incubated overnight at 4°C. After washing, the wells were blocked with 1% BSA-PBS (100 μl/well) for 1 h and the tested sera (diluted 1/50 in 1% BSA-PBS) were introduced to the plate. After 2 h of incubation, the plate was intensively washed with PBS-Tween 0.05%. The immunoglobulins bound to CysC or apo-SAA were visualized using horseradish peroxidase-labeled IgG F(ab)$_2$ mouse anti-human antibodies (Dako), added in concentration 0.5 μg/ml (100 μl/well) followed by an appropriate substrate. The reaction was measured by absorbance at 405 nm. The presence of antibodies was defined as the absorbance of the sample above the mean + 2 standard deviations (SD) of the controls.

Cysteine protease-inhibiting function of CysC was assessed by its ability to reduce papain (Sigma) cleavage of a synthetic substrate, Bz-DL-Arg-NHPhNO$_2$ (Bachem, Bubendorf, Switzerland) as described[37]. In brief, the cysteine protease activity of papain (0.1 M phosphate buffer, pH 6.5, containing 1 mM DTT, 1 mM ETDA) was initially tested against L-trans-epoxysuccinyl-Leu-3-methylbutylamido-4-guanidino-butane (E-64; Boeringer-Mannheim GmbH, Mannheim, Germany) and the active molar concentration of papain equal to 60 μM was chosen to use in further experiments; 60 μM papain was consequently incubated with increasing amounts of CysC (concentrations 0–0.1–1.0–10 μM) in the presence of 10 μM of Bz-DL-Arg-NHPhNO$_2$ substrate to achieve 80% inhibition of papain activity. The cleavage of substrate was monitored spectrophotometrically by the color development at 410 nm. The influence of CysC binding to apo-SAA on the cysteine protease-inhibiting function of CysC was assessed by comparing the papain-dependent substrate cleavage in the presence of CysC and SAA-CysC complex as described[37].

Determination of cytokine levels. The level of IL-6 in supernatants was determined by the proliferation of the IL-6-dependent cell line, B13.29. Amount of IL-6 was calculated by incorporation of [^{3}H]-thymidine after 72 h stimulation and compared to the standard dilutions of recombinant mouse IL-6 (Genzyme, Cambridge, MA, USA). Levels of IL-1ß were measured by an ELISA (R&D Systems, Abingdon, UK) following manufacturer's recommendations. Optical density of the tested samples was compared with the values obtained from serial dilutions of recombinant human IL-1ß provided in the kit.

Laboratory measures of disease activity. Serum levels of C-reactive protein (CRP) were measured with a standard nephelometric assay with established normal range 0ß5 mg/l. The erythrocyte sedimentation rate was measured by the Westergren method having normal range 0–20 mm/h. White blood cell counts (WBC) in blood and in SF were performed using an F300 microcell counter (Sysmex, Toa, Japan). Plasma creatinine levels were measured by a creatininase enzyme-based procedure on a Hitachi Modular P analysis system.

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Statistical analysis. The levels of CysC in the blood and SF samples were expressed as mean ± SEM. Comparisons between the matched blood and SF samples were analyzed by the paired t test. Comparison of CysC levels was performed between the patient blood samples and controls. For further comparison patient material was stratified according to radiological findings (erosive RA vs nonerosive RA). Differences in CysC levels in the blood and SF between the groups were calculated separately employing the Mann-Whitney U-test. For evaluation of possible influence of current treatment on CysC levels, patient material was stratified according to DMARD treatment (treated vs untreated). Comparison in the group pairs was performed using the Mann-Whitney U-test. For simultaneous comparison of CysC levels in more than 2 groups the equality of variance F test was employed. Interrelations between the CysC levels and SAA, duration of the joint disease, age, WBC, and CRP were calculated employing Spearman correlation coefficient. The presence of antibodies against CysC and against apo-SAA in the blood samples of patients with RA was defined as positive if the absorbance value of the patient sample was above the mean + 2 SD of the absorbance of the matched control group. For all statistical evaluations, p values < 0.05 were considered significant.

RESULTS

CysC levels in blood and SF of patients with RA. Levels of cystatin C, F, and E were measured in the paired blood and SF samples of patients with established erosive RA (Table 2). As shown in Table 2, CysC was the predominant extracellular cystatin detected. Levels of CysC in SF were higher then those measured in circulation of patients with RA. These observations led us to further studies on CysC.

CysC levels were significantly higher in SF than in circulation, both in patients with RA and in the control groups (Figure 1A, 1B). There were remarkably high CysC levels locally in the joints of patients with RA compared to the joints of noninflammatory controls (1.51 ± 0.05 mg/l vs 1.18 ± 0.03 mg/l; p = 0.04), but simultaneously low CysC levels in the circulation of patients with RA compared to the serum of matched blood donors (0.81 ± 0.03 mg/l vs 1.01 ± 0.03 mg/l; p = 0.05). To investigate this disproportion and to eliminate the role of demographic confounding factors, age and sex matched controls were found for each patient with RA, and CysC levels were compared within the identical age groups (Figure 1). We observed that serum CysC levels were lower in all age groups in patients with RA as compared to the controls. This difference was most pronounced in the patients below 50 years of age and leveled off in the group above 70 years. This finding indicates a decreased production or alternatively consumption of CysC in the circulation of patients with RA.

Compromised renal function is a known confounding factor predisposing to increased levels of CysC in circulation[35]. In all but 2 of the patients with RA studied, creatinine levels were within the normal range, with no difference between the erosive (60.9 ± 3.7 μmol) and nonerosive (61.0 ± 2.6 μmol) groups. Plasma creatinine levels did not differ significantly between the patients with RA and the age and sex matched controls (63.2 ± 5.1 μmol; Table 3). As expected, CysC levels in circulation were significantly related to the creatinine levels (r = 0.45, p < 0.0001), while in SF of patients with RA such a correlation was not found (r = 0.14, p = not significant). The levels of CysC in the circulation and in SF were strongly related (r = 0.72, p < 0.0001). In order to eliminate the influence of renal function on CysC analysis, we calculated a ratio between the CysC levels found in SF and in blood. In patients with RA, CysC levels were almost 2 times higher in SF compared to blood (1.51 ± 0.05 vs 0.81 ± 0.03 mg/l, ratio 1.86). The difference was less pronounced between the control groups (1.18 ± 0.03 vs 1.01 ± 0.03 mg/l, ratio 1.17). The CysC ratio obtained (SF/blood) was not correlated to creatinine levels (r = 0.06, NS). Thus, the difference in circulating CysC levels between patients with RA and the controls could not be explained by the difference in glomerular filtration rate.

CysC levels and joint destruction in patients with RA. Among the 90 pairs of SF and blood samples, 47 patients had destructive joint disease (erosive RA), and the remaining 43 patients had no changes of the joints as judged by the recent radiological examination of the hands and feet (nonerosive RA). Patients with erosive RA were, as expected, significantly more often positive for RF, had longer duration of joint disease, and were more often being treated with MTX compared to the group with nonerosive RA. Comparison of these patient groups showed that patients with erosive RA had significantly higher intraarticular levels of CysC compared to nonerosive patients (1.66 ± 0.08 mg/l vs 1.36 ± 0.06 mg/l; p = 0.003) and blood levels of CysC (0.88 ± 0.04 mg/l vs 0.74 ± 0.05 mg/l; p = 0.001; Figure 1). Further evaluation of the intraarticular

Table 2. Pilot study of cystatin C, E, and F levels (ng/ml) in blood and synovial fluid of RA patients.

Sex/Age	Radiographic Erosions	RF	DMARD	Cystatin C		Cystatin E		Cystatin F	
				Blood	SF	Blood	SF	Blood	SF
F 60	Yes	Pos	MTX	401	690	0.24	0.41	< 0.2	0.69
F 76	Yes	Pos	MTX	710	930	0.45	0.46	0.53	0.23
F 69	Yes	Pos	None	566	734	0.41	0.3	0.41	0.49
M 52	Yes	Pos	Lef	506	688	0.37	0.38	0.65	0.46
F 73	Yes	Neg	MTX	665	843	0.47	0.3	0.68	0.76
Mean				570	777	0.39	0.38	0.57	0.53
SD				124	106	0.09	0.06	0.12	0.21

RF: rheumatoid factor; DMARD: disease modifying antirheumatic drugs; MTX: methotrexate; Lef: leflunomide.

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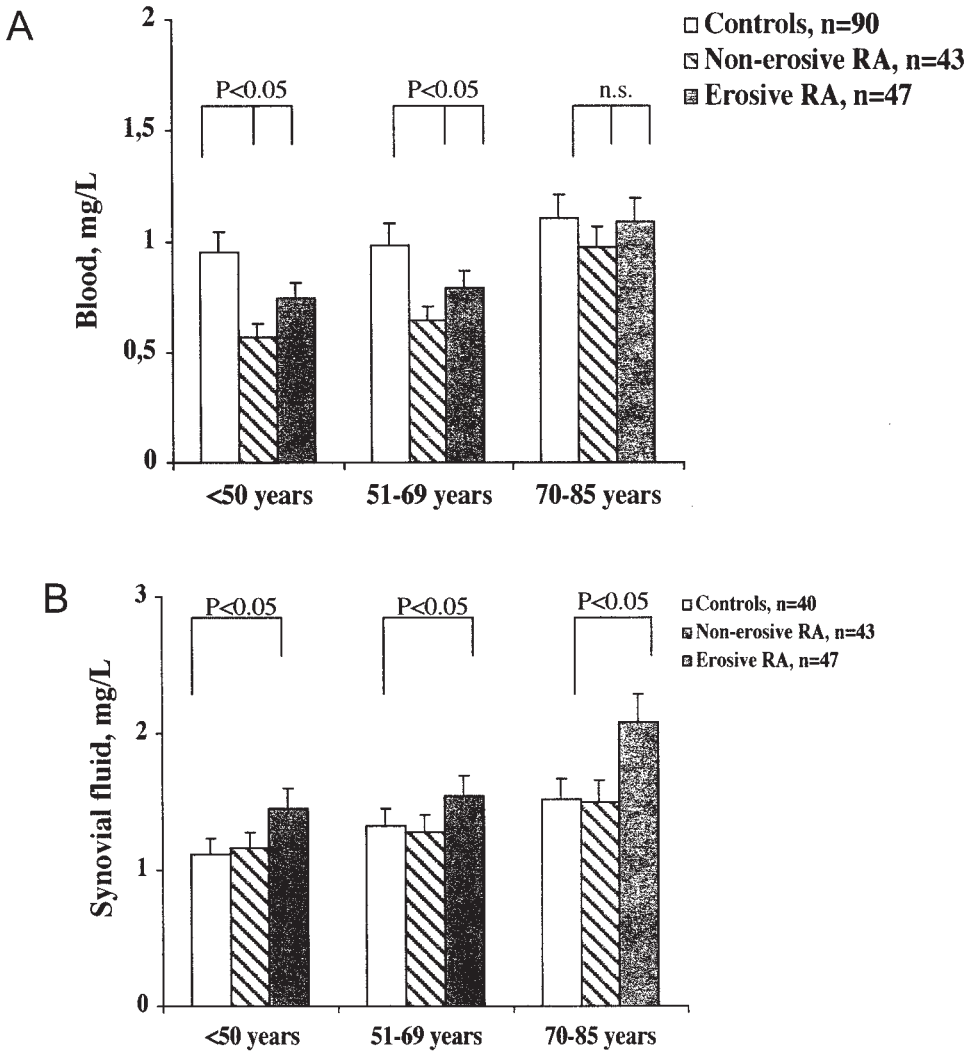

Figure 1. Levels of cystatin C (CysC) in patients with RA having erosive and nonerosive joint disease compared to age and sex matched controls. A. Circulating CysC levels are significantly decreased in patients with RA compared to controls. Decrease is more pronounced in younger patients and levels off in patients over 70 years old. B. Levels of CysC in synovial fluid are significantly higher in patients with RA than in the controls having noninflammatory joint diseases.

Table 3. Circulating levels of cystatin C, SAA, and creatinine in patients with RA and controls matched for age and sex.

	RA Patients	Controls	p
Cystatin C, mg/l	n = 90	n = 90	
20–50 yrs	0.62 ± 0.04	0.95 ± 0.04	= 0.003
51–69	0.75 ± 0.08	0.98 ± 0.06	< 0.05
70–85	1.06 ± 0.06	1.10 ± 0.08	NS
SAA, mg/l	n = 87	n = 58	
20–50 yrs	105 ± 6	26 ± 1	< 0.001
51–69	106 ± 11	29 ± 4	< 0.01
70–85	182 ± 8	NA	
Creatinine, μmol	n = 90	n = 90	
20–50 yrs	55.5 ± 2.6	67.2 ± 3.7	NS
51–69	58.7 ± 3.2	63.5 ± 2.2	NS
70–85	71.8 ± 2.8	68.7 ± 4.1	NS

NS: nonsignificant; NA: not assessed.

CysC levels with respect to age showed that high levels of CysC were detected in patients with erosive joint disease. In contrast, the CysC levels were similar between the patients having no joint destruction and controls (Figure 3). Neither CysC levels in blood nor levels in the SF correlated to the leukocyte counts in the respective compartment, indicating other cell sources than circulating leukocytes maintaining CysC levels. Treatment with MTX was associated with low levels of CysC in blood compared to patients not treated with DMARD (0.77 mg/l vs 0.84 mg/l; $p < 0.01$). However, this difference disappeared after the adjustment for age and erosivity. Oral corticosteroids were used by 37 of 87 patients. Patients treated with oral corticosteroids showed no difference in the levels of blood CysC compared to those who were not treated with corticosteroids (1.63 ± 0.07 mg/l vs 1.57 ± 0.08 mg/l; NS).

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Low levels of CysC in patients with RA are not related to circulating anti-CysC antibodies. The presence of anti-CysC and anti-SAA antibodies of IgG isotype was assessed in blood samples of 87 patients with RA and in 58 samples of the age and sex matched controls as described in Materials and Methods. The sample was considered positive if its absorbance value was above the mean + 2 SD of controls. In the tested collection of control samples the cutoff level for anti-CysC antibodies was 0.96 and for anti-SAA antibodies 0.51. Anti-CysC antibodies were present in 3 of 87 (3%) patients with RA and in 2 of 58 (4%) control samples (NS). The absorbance values of anti-CysC antibodies did not correlate to the levels of CysC in circulation. Thus, neutralization of CysC by CysC-specific antibodies cannot be considered a major reason for low CysC levels in circulation of patients with RA. Anti-SAA antibodies were found in 13 of 87 (15%) patients with RA and in 2 of 58 (4%) controls ($p < 0.05$). No correlation was found between the absorbance values of anti-SAA antibodies and the levels of SAA in circulation of patients with RA.

SAA levels and their relation to CysC. Levels of SAA were measured in blood of 87 patients with RA and in 58 controls matched to the patient group by age and sex. No sera from the control group above the age of 70 years were available for SAA determination. As expected, SAA levels were significantly higher in patients with RA compared to the controls (Table 3). SAA levels were related to the destruction of joints, being higher in RA patients with nonerosive disease compared to those with erosive RA (133 ± 8 vs 123 ± 10 mg/l; $p < 0.05$). SAA levels were strongly correlated to the level of CysC ($r = 0.50$, $p < 0.0001$) and CRP ($r = 0.53$, $p < 0.0001$). Weaker correlation was also found between the SAA levels and the age of patients ($r = 0.30$, $p = 0.026$). Patients with RA aged ≥ 70 years had significantly higher levels of CysC ($p = 0.005$), SAA ($p = 0.002$), and CRP ($p = 0.05$) compared to the younger patients with RA (Table 3).

Effect of interaction between SAA and CysC on the function of these proteins. First, we assessed if there is a direct binding between CysC and SAA. For this purpose, ELISA wells were coated with a defined amount of recombinant apo-SAA (50 ng/well) and incubated with increasing concentrations of recombinant CysC that varied from 0.05 to 50 pg/well. We observed that the absorbance increased in direct proportion to the amount of CysC added to the well, indicating that binding of CysC to SAA occurred in dose-dependent fashion (Figure 2). Binding of recombinant CysC was not observed in the control wells coated with equimolar solution of bovine albumin. This finding indicates a direct binding between CysC and SAA.

Next, the effect of SAA-CysC complex formation on the function of SAA was assessed. Human PBMC were treated with preformed complexes consisting of apo-SAA (50 μg) and 2 different concentrations of CysC (100 μg and 10 μg). Additionally, control PBMC cultures were treated with apo-SAA or CysC separately. We found that apo-SAA induced the expression of IL-1ß and IL-6, while CysC did not trigger production of these cytokines. In contrast, CysC was an efficient inhibitor of basal cytokine production in nonstimulated PBMC cultures. Preincubation of apo-SAA with 2 molar excess of CysC reduced the expression of proinflammatory cytokine IL-1ß by 55% and IL-6 by 75% (Figure 3A, 3B). These observations show that CysC binds and neutralizes proinflammatory properties of apo-SAA. We also observed that CysC exhibits antiinflammatory properties downregulating the production of IL-6 and IL-1ß cytokines independent of apo-SAA stimulation.

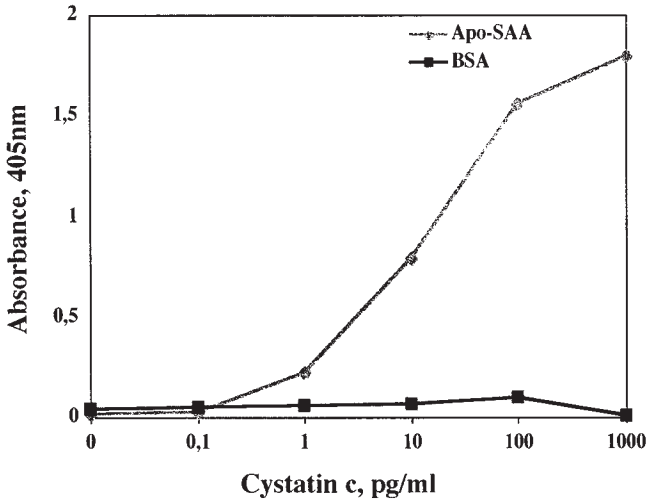

Figure 2. CysC binds precursor of serum amyloid A protein (apo-SAA). A 96-well plate was coated overnight with recombinant apo-SAA, blocked with 1% BSA, and incubated with increasing amount of CysC. Binding of cystatin C to apo-SAA occurred in direct proportion to the amount of CysC introduced to the system. No CysC binding was found in wells coated with BSA.

Further, we assessed the influence of CysC-SAA binding on its activity as a cysteine protease inhibitor. The activity of CysC preparation was titrated against the chosen amount of papain to achieve about 80% reduction of papain activity, as described in Materials and Methods. To assess the influence of apo-SAA on CysC activity, a 10× excess of apo-SAA as compared to CysC concentration was added to the papain-CysC mixture. An alternative experiment was performed when the CysC titration procedure was carried out in the presence of excess apo-SAA in the papain solution. Titration curves of papain activity were similar in the presence and absence of apo-SAA. These experiments showed that apo-SAA could neither inhibit the cysteine protease capacity of papain nor reduce the CysC inhibitory activity toward papain.

DISCUSSION

CysC is the most abundant human extracellular inhibitor of cysteine proteinases[10,34]. In agreement with this, our pilot study showed that CysC levels in joint fluid and in circulation were many times higher compared to those of cystatin F and cystatin E. This finding together with the low selectivity of

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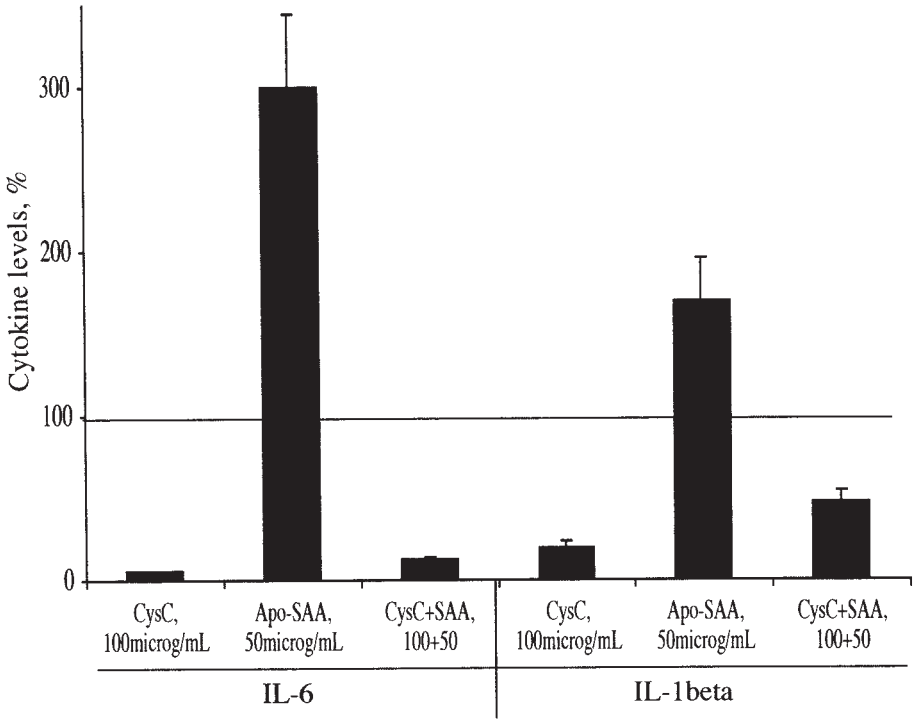

Figure 3. Complex formation between CysC and apo-SAA leads to reduction of SAA's ability to induce cytokine expression. Human PBMC purified from heparinized blood of healthy individuals (n = 3) and adjusted to 2 × 10^6/ml concentration were incubated with cystatin C/apo-SAA complexes (100 + 50) and corresponding amounts of each of the components separately. Complexes between CysC and apo-SAA were prepared using 2-molar excess of CysC. After 48 h incubation supernatants were collected and the amount of IL-6 and IL-1ß produced was measured. Results show mean ± SD of 3 independent experiments. Levels of cytokines in nonstimulated PBMC cultures are set to 100%.

cystatins in their interaction with substrate(s) makes CysC the main regulator of cysteine proteinase activity in circulation and in other body fluids. Further assessment of CysC in our larger population of RA patients and sex and age matched controls emphasized (1) a significant decrease of CysC levels in circulation of patients with RA compared to the controls, and (2) local accumulation of CysC in the inflamed joints of patients with RA.

The level of CysC in circulation of patients with RA was significantly decreased compared to the matched controls. This difference in circulating CysC levels was more pronounced in the younger patients with RA and leveled off after the age of 70. The low CysC could be a result of insufficient production or of high turnover and consumption of CysC. We assessed several possible mechanisms of CysC consumption. One was a redistribution of CysC from circulation to the inflammation site. This explanation may be attractive regarding the intraarticular accumulation of CysC observed simultaneously in the same patients with RA. Indeed, the level of CysC in SF was about 2-fold higher than in the circulation. This proportion between the circulating and intraarticular CysC levels was conserved in all patient groups irrespective of age and the presence of erosivity. In agreement with previous reports focusing on the levels of circulating CysC in direct relation to renal function[12,35] we observed an increase of CysC levels with age; levels were highest in the group of RA patients above the age of 70. However, levels of CysC in SF showed a similar increase as a function of age, supporting the assumption of redistribution and local expression of CysC by synovia of patients with RA reported by others[7].

The other possible reason for low circulating CysC addressed in our study is increased elimination due to immune complex formation. Production of antibodies targeting molecules participating in joint inflammation is a key process in the pathogenesis of RA. The determination of antibody production against CysC showed no increase in the level of antibodies interacting with CysC in patients with RA compared to the age and sex matched controls. Thus, low levels of circulating CysC in the group of patients with RA may not be explained by an excessive immune complex formation, making CysC a less probable target for B cell responses in RA. On the other hand, we observed that CysC readily binds the precursor molecule to the AA protein apo-SAA. This interaction results in the loss of one of SAA's functions, namely its ability to induce cytokine production. In contrast, CysC-SAA complex retained CysC enzymatic activity as an inhibitor of

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cysteine proteases. Thus, interaction with SAA gives CysC clearly defined antiinflammatory properties in addition to and independently of its ability to regulate extracellular proteolysis, e.g., inside the inflamed joints. In the clinical setting, complex formation between CysC and SAA could potentially lead to its elimination from circulation with subsequent deposition in the surrounding tissues. Interestingly, high frequency of antibodies targeting SAA has previously been described in RA not complicated by amyloidosis[38].

Extracellular deposition of polymerized SAA is the essence of secondary amyloidosis, a severe complication of RA. The understanding of CysC binding properties on the polymerization and deposition of SAA fibrils could help identify patients with RA susceptible to the development of secondary amyloidosis. In our patients with RA we found only a weak correlation between the circulating levels of CysC and SAA. However, a significant production of anti-SAA antibodies found in a significant subpopulation of patients with RA favors the existence of several parallel mechanisms regulating SAA levels during RA. Colocalization of CysC with another amyloid-forming molecule, beta-amyloid peptide, has been found in the brains of patients with such neurological disorders as cerebral amyloid angiopathy, hereditary cerebral hemorrhage with amyloidosis[24], Down syndrome, and Alzheimer's disease[27]. There is clinical and experimental evidence supporting a protective effect of CysC against cerebral amyloidosis[23,24].

In our study, a CysC deficiency was observed in the majority of RA cases overrepresented among the younger patient group. The prognostic value of this acquired CysC deficiency for survival and morbidity during RA requires further evaluation. Recent studies clearly indicate an association between CysC deficiency and such vascular pathologies as atherosclerosis and development of arterial aneurysms. Imbalance characterized by profound decrease of CysC and augmented expression of cysteine proteases within atherosclerotic plaques has been implicated for the progression of vascular damage[13,14]. Low levels of circulating CysC in association to its gene polymorphism have recently been depicted as a possible predictor of atherosclerotic progression[39]. In animal models, lack of CysC has been shown to promote formation of atherosclerotic plaques in predisposed recipients[40,41]. Acquired CysC deficiency may be an important factor responsible for increased risk of myocardial infarction and cerebrovascular events in patients with RA[42,43].

Our study provides new insight into the role of CysC during RA. Our finding of low circulatory CysC levels simultaneously with high intraarticular levels in an RA population strongly suggests redistribution of this molecule. Indeed, our data point to a direct interaction between CysC and SAA, leading to functional consequences for proinflammatory cytokine production. Further studies related to *in vivo* consequences of systemic CysC elimination in models of RA/amyloidosis are under way.

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