Increased Chromogranin A Levels Indicate Sympathetic Hyperactivity in Patients with Rheumatoid Arthritis and Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Sympathetic hyperactivity is an unfavorable disease consequence in rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) due to an increased risk of cardiovascular events. We aimed to identify a serum marker of the sympathetic nervous system, the adrenal chromogranin A (CHGA), in order to study sympathetic hyperactivity in RA and SLE.

Methods. Serum levels of CHGA were measured by radioimmunoassay in healthy subjects and patients with RA and SLE. CHGA immunofluorescence was performed in synovium of patients with RA and controls with osteoarthritis (OA). CHGA levels were measured in plasma, synovial fluid, and synovium superfusate in RA and OA controls.

Results. In healthy subjects, systemic CHGA levels correlated positively with age and plasma norepinephrine, indicating the sympathetic origin ($p < 0.01$). Serum CHGA levels were higher in RA and SLE than in healthy subjects ($p < 0.001$), which was particularly evident in female patients. Immunofluorescence revealed double-staining of CHGA and elastase-positive neutrophils in the synovium (but not with macrophages, T cells, fibroblasts, B cells, or tyrosine hydroxylase–positive cells). Density of CHGA+ cells was higher in RA synovium compared to OA controls. In OA controls and RA, CHGA levels were similar in plasma and synovial fluid, but levels in synovial tissue superfusate were markedly lower, which indicates that most of the CHGA is of systemic adrenal origin.

Conclusion. Increased level of CHGA is a good marker of systemic sympathetic hyperactivity. (First Release Dec 1 2007; J Rheumatol 2008;35:91–9)

Key Indexing Terms:
RHEUMATOID ARTHRITIS OSTEOARTHRITIS CHROMOGRANIN A
SYSTEMIC LUPUS ERYTHEMATOSUS SYMPATHETIC NERVOUS SYSTEM

Previous reports found an increased risk of atherosclerosis in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)[1-15]. The reasons for this phenomenon are currently being investigated, and it seems that the proinflammatory load is an important triggering factor[13,16]. A traditional risk factor is sympathetic hyperactivity, which in the form of hypertension belongs to the classical symptoms of the metabolic syndrome[17,18]. Such a sympathetic hyperactivity, particularly in relation to a relatively normal functioning hypothalamic-pituitary-adrenal (HPA) axis, has recently been claimed to be an unfavorable factor in patients with RA and SLE[19-24]. Sympathetic marker proteins such as neuropeptide Y, a neurotransmitter of the sympathetic nerve terminal, were used in order to detect an increased sympathetic activity by a simple radioimmunoassay[25]. However, no further adequate serum markers were tested to substantiate these findings and to facilitate diagnosis of sympathetic hyperactivity in patients with rheumatic diseases.

Chromogranin A (CHGA) is a marker for sympatho-adrenal activity, which has been described since the 1960s[26,27]. The CHGA serum level is an excellent indicator of sympathetic activity as demonstrated in healthy subjects[28-30]. CHGA is a 438 amino acid protein located in dense-core secretory granules of neuroendocrine cells necessary for catecholamine storage, and assembly and biogenesis of vesicles (as reviewed[31]). The CHGA precursor gene product is post-translationally processed yielding the biologically active peptides CHGA, vasostatin, pancreastatin, and parastatin[32]. CHGA has also been used as a diagnostic tool in neuroendocrine tumors[33]. However, the presence or relevance of CHGA has not been studied in patients with RA and SLE.

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The aim of our study was to determine serum CHGA in patients with RA and SLE and to compare these values with healthy subjects. Since we also found CHGA-positive (CHGA+) cells in synovial tissue of patients with RA and osteoarthritis (OA), we investigated whether local production of CHGA in the synovium might contribute to elevated serum levels in RA, and we used patients with OA as controls. In addition, we studied CHGA+ cells in the synovium to uncover the cell type producing CHGA in the local proinflammatory microenvironment.

MATERIALS AND METHODS

Healthy subjects and patients. In the first study, we investigated 92 healthy subjects [mean age ± SEM: 44.0 ± 1.7 yrs (range 18—75), women/men: 38/54], and health status was thoroughly verified by means of a 33-item questionnaire. The questionnaire addressed known diseases in the past and at present, current symptoms of diseases, current medication, prior vaccination, alcohol intake, smoking habits, family history, and surgical history. The questionnaire was adapted to the SENIEUR protocol[34]. This first study in healthy subjects was carried out to demonstrate the interrelation between serum levels of CHGA and a typical marker of the sympathetic activity (namely norepinephrine). We wanted to show that CHGA is a good marker of sympathetic activity when measured in the serum.

In the second study, we compared healthy controls to patients with RA and SLE. This study was carried out to demonstrate serum levels of CHGA in patients with inflammatory diseases in comparison to healthy subjects. Diagnosis of RA and SLE was based on the established criteria according to the American College of Rheumatology[35,36]. Clinical and laboratory data for subjects in the second study are recorded in Table 1, and C-reactive protein (CRP) was measured according to standard techniques in the Department of Clinical Chemistry, University Hospital Regensburg.

In the third study, we compared patients with RA and OA (as controls) in order to investigate CHGA+ cells in synovial tissue, synovial fluid levels of CHGA, and serum levels of CHGA. This study was carried out in order to demonstrate the origin of CHGA because inflamed tissue might be a strong producer of CHGA. If CHGA is derived from the inflamed tissue, it would not be a sign of sympathetic activity. The included patients underwent elective knee joint replacement surgery, and they were included without further selection. Clinical and laboratory data of the third study are recorded in Table 2, and erythrocyte sedimentation rate was measured by standard techniques.

All healthy subjects and patients were informed about the purpose of the study and gave written consent. The study was approved by the Ethical Committee of the University of Regensburg.

Collection of material. For the first and second study, healthy subjects and patients were investigated in the outpatient clinic and blood was drawn between 8:00 and 10:00 in the morning. In the third study, blood and synovial fluid were drawn during the procedure of arthroplastic surgery in the morning hours. Material was immediately centrifuged and stored at –80˚C. Synovial tissue samples were obtained immediately after opening the knee joint capsule. The preparation of the tissue for histology was as described[37]. Briefly, a piece of synovial tissue of up to 9 cm^2 was dissected. Fat tissue and tissue with a large number of blood vessels were removed. Four pieces of about 20 mm^2 of every patient were loaded into 4 superfusion chambers (superfusion technique see below), and 8 pieces of roughly 0.8 cm^2 were used for histology. Samples were fixed with 3.7% formaldehyde and then treated with sucrose 20% overnight at 4˚C. Samples were then placed in protective freezing medium (Tissue Tek, Sakura Finetek, Zoeterwoude, The Netherlands) and quick-frozen floating on liquid nitrogen. All tissue samples were stored at –80˚C.

Detection of CHGA+ cells, and double-staining with other cell types in RA and OA. Cryosections (8 μm) of at least 2 different formaldehyde-fixed synovial tissue samples from each patient were air-dried for 1 h and then rehydrated in phosphate buffered saline (PBS). Unspecific binding sites were blocked with PBS containing 10% fetal calf serum, 10% bovine serum albumin, and 10% normal goat serum for 1 h at room temperature. After 10 min washing with PBS, the sections were incubated with rabbit polyclonal antibodies against CHGA (Chemicon, Hampshire, UK) and with a mouse monoclonal antibody against macrophages (CD163; DakoCytomation, Carpinteria, CA, USA), T lymphocytes (CD3; DakoCytomation), fibroblasts (prolyl-4 hydroxylase; DakoCytomation), B lymphocytes (CD19; DakoCytomation), neutrophils (Neomarkers, Fremont, CA, USA), or tyrosine hydroxylase (the key enzyme for norepinephrine production in sympathetic nerve endings; Chemicon, Temecula, CA, USA). The samples were incubated 3 h at room temperature and then washed 3 × 5 min with PBS added with 0.3% Triton-

Table 1. Characteristics of healthy subjects and patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) in the second study. Data are given as means ± SEM (%).

Characteristic	First Study Healthy Subjects	RA	Second Study Healthy Subjects	SLE
Number	50	24	37	24
Age, yrs	53.6 ± 1.5	54.0 ± 2.0	38.4 ± 2.0	36.6 ± 2.8
Women/Men	26/24	14/10	26/11	20/4
Swollen joints	NA	8.1 ± 1.6	NA	NA
Tender joints	NA	8.4 ± 1.5	NA	NA
Pain (patient)	NA	4.0 ± 0.5	NA	NA
SLEDAI	NA	NA	NA	11.7 ± 2.0
C-reactive protein, mg/l	NM	25.0 ± 6.4	NM	7.1 ± 1.2
Medication				
Predisolone, n (%)	NA	14 (58)	NA	17 (71)
Daily prednisolone, mg	NA	6.1 ± 1.5	NA	17.1 ± 10.2
Methotrexate, n (%)	NA	13 (54)	NA	NA
Hydroxychloroquine, n (%)	NA	3 (13)	NA	1 (4)
Sulfasalazine, n (%)	NA	3 (13)	NA	NA
Azathioprine, n (%)	NA	1 (4)	NA	14 (58)
Cyclophosphamide, n (%)	NA	0 (0)	NA	0 (0)
NSAID, n (%)	NA	13 (55)	NA	3 (13)

NA: not applicable; NM: not measured; NSAID: nonsteroidal antiinflammatory drugs; SLEDAI: SLE Disease Activity Index.

Table 2. Characteristics of patients with rheumatoid arthritis (RA) and osteoarthritis (O) in the third study. Data are given as means ± SEM (%).

Characteristic	OA	RA
Number	8	10
Age, yrs	71.8 ± 1.5	66.1 ± 2.3
Women/Men	7/1 (87/13)	7/3 (70/30)
Erythrocyte sedimentation rate, 1st h	12.8 ± 3.6	31.4 ± 13.0
Medication		
Prednisolone, n (%)	0 (0)	6 (60)
Daily prednisolone, mg	0	4.0 ± 1.2
Methotrexate, n (%)	0 (0)	2 (20)
Hydroxychloroquine, n (%)	0 (0)	0 (0)
Sulfasalazine, n (%)	0 (0)	1 (10)
Azathioprine, n (%)	0 (0)	1 (10)
Leflunomide, n (%)	0 (0)	1 (10)
Opoid, n (%)	0 (0)	2 (20)
NSAID, n (%)	2 (25)	7 (70)

NSAID: non-steroidal antiinflammatory drugs.

X100. Staining was achieved with a secondary anti-rabbit Alexa Fluor 488 antibody (Molecular Probes, Invitrogen, Eugene, OR, USA) against CHGA and with a secondary anti-mouse Alexa Fluor 546 antibody against the second staining antibody (Molecular Probes). The sections were incubated 1 h 45 min in the dark at room temperature, and then washed 3 × 5 min with PBS with 0.3% Triton-X100.

Control staining was performed with unspecific rabbit IgG instead of the above-mentioned primary antibodies against CHGA. In a further analysis without double-staining, the density of CHGA+ cells was averaged from 17 randomly selected high-power fields of view (400×) and expressed per square millimeter.

Superfusion of synovial tissue. As described[37], we used a microsuperfusion chamber apparatus to superfuse slices of synovial tissue with culture medium (RPMI 1640, 25 mM HEPES, without FCS, 1% Pen/Strep, 30 μM mercaptoethanol, 0.57 mM ascorbic acid, 1.3 mM calcium, all additions from Sigma, Germany). These superfusion chambers had a volume of approximately 80 μl. Superfusion was performed for 120 min at a temperature of 37˚C and a flow rate of 66 μl/min (1 piece per chamber, 4 chambers in parallel). Synovial tissue pieces had a standard size of 5 mm in diameter using a precision biopsy punch (Stiefel, Offenbach, Germany). Using 4 chambers, we were able to investigate 4 slices in one experiment of one synovial tissue sample. At 120 min, superfusate was collected in order to measure CHGA in a fraction of approximately 1 ml (collected over 15 min).

Measurement of CHGA and norepinephrine. CHGA in serum, synovial fluid, or superfusate was measured by a commercial radioimmunoassay (Asbach Medical Products, Obrigheim, Germany). The detection limit for the assay was 1.5 ng/ml. Interassay and intraassay coefficients of variation were below 10%.

The amount of norepinephrine in serum was measured by radioimmunometric assay (IBL, Hamburg, Germany). The high-sensitive protocol used with this kit has a detection limit of 10 pg/ml. Test samples analyzed with HPLC showed that this radioimmunometric technique produced comparable results. Interassay and intraassay coefficients of variation were below 10%.

Presentation of the data and statistical analysis. All data are given as mean ± SEM. Correlations were calculated by correlation analysis (SPSS/PC, Advanced Statistics, v12.0.0, SPSS Inc., Chicago, IL, USA) and graphically demonstrated by a linear regression line. Group means were compared by the nonparametric Mann-Whitney test (SPSS). $p < 0.05$ was the significance level.

RESULTS

CHGA as a marker of the sympathetic nervous system in healthy subjects. In order to investigate whether serum CHGA is a marker of sympathetic activity, we correlated serum CGHA with age and plasma norepinephrine. Age was shown earlier to positively correlate with an increased sympathetic activity[38]. In our study, age correlated positively with serum CHGA (Figure 1A). In addition, plasma norepinephrine correlated positively with serum CHGA (Figure 1B), which indicates that CHGA measured with our radioimmunoassay is a marker of sympathetic activity.

Serum CHGA in patients with RA and SLE. Serum CHGA was increased in all patients with RA and SLE compared to healthy subjects (Figures 2A, 2B). The subdivision into female and male patients revealed that particularly women with RA and SLE demonstrated increased levels (Figure 2). However, men also tended to have increased serum CHGA as compared to healthy men, which was most probably not significant due to the lower number of male subjects investigated (type II error; Figure 2). Interestingly, serum CHGA was higher in women with RA as compared to men with RA, which delineates a sex-specific difference (Figure 2A). This was not observed in patients with SLE (Figure 2B).

CHGA+ cells in synovial tissue of patients with RA and OA. In a further analysis, we wanted to clarify whether CHGA is present in inflamed tissue. It might well be that the presence of CHGA in inflamed tissue might contribute substantially to systemic CHGA levels. Indeed, CHGA+ cells existed in synovium of patients with RA and OA (Figure 3). Double immunofluorescence revealed that CHGA+ cells did not double-stain with CD163+ macrophages, CD3+ T lymphocytes, prolyl-4-hydroxylase-positive fibroblasts, CD19+ B lymphocytes, and tyrosine hydroxylase-positive cells (Figure 3). However, CHGA+ cells double-stained with elastase-positive neutrophils (Figure 3).

A further analysis of patients with RA and OA demonstrated that patients with RA had a higher synovial density of CHGA+ cells as compared to patients with OA (Figure 4A). Further, the systemic inflammation marker CRP correlated positively with synovial density of CHGA+ cells in patients with RA (Figure 4B). This indicates that these cells might be involved in systemic inflammation. The correlation was also positive in OA but it did not reach the significance level (Figure 4B).

Comparison of CHGA levels in serum, synovial fluid, and synovium superfusate in RA and OA. In order to investigate whether the source of CHGA might be the inflamed tissue and not the sympathetic nervous system (particularly the adrenal glands), we investigated CHGA levels in serum, synovial fluid, and synovium superfusate of the same patient. Although density of CHGA+ cells was lower in OA than in RA (Figure 4A), systemic serum levels and synovial fluid levels of CHGA were similar in both patient groups (Figure 4C, indicated as nonsignificant). If CHGA is locally produced, one would have expected a higher synovial fluid level in RA because of the markedly higher density of CHGA+ cells (Figure 4A). In addition, superfusate levels of CHGA were lower by a factor

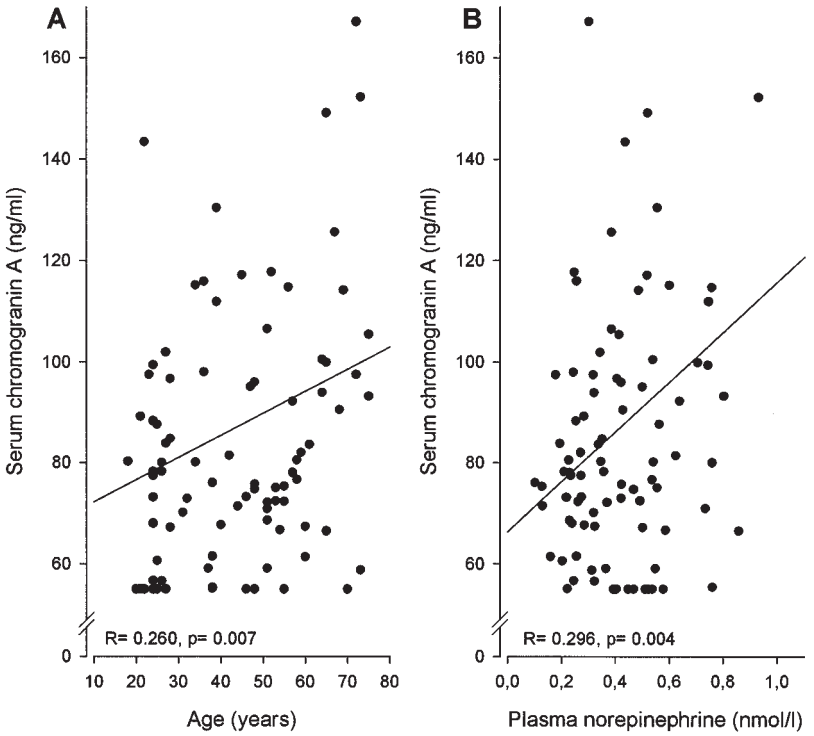

Figure 1. Correlation between age or plasma norepinephrine and serum chromogranin A. Graph depicts the linear regression line, correlation coefficient, and p value. Each symbol represents 1 healthy person.

of 100 as compared to serum and synovial fluid levels (Figure 4C). Despite the lower density of CHGA+ cells in OA than RA, no differences were observed in serum, synovial fluid, and superfusate levels between RA and OA (Figure 4C).

DISCUSSION

Our study demonstrates elevated systemic levels of CHGA in RA and SLE compared to healthy subjects. It also demonstrates CHGA+ cells in inflamed tissue of patients with RA, but these cells are most probably not the source for systemic CHGA in these patients.

By using a different sympathetic marker molecule (CHGA), our study attempted to corroborate other studies, which demonstrated sympathetic hyperactivity in patients with inflammatory diseases[19-24]. In our study, we used CHGA as a marker of adrenal origin from vesicles in sympatho-adrenal cells of the adrenal medulla (as reviewed[31]). In our recent studies, we demonstrated sympathetic hyperactivity using the sympathetic marker neuropeptide Y[25,39], which is mainly released from sympathetic nerve terminals and much less from adrenomedullary cells. Both markers from a different sympathetic source, adrenal medulla (CHGA) versus sympathetic nerve endings (neuropeptide Y), demonstrate sympathetic hyperactivity in patients with RA and SLE.

The question remained whether previously identified CHGA+ cells in inflamed tissue in RA might be a substantial source of elevated serum CHGA. If this would be the case, sympathetic hyperactivity would not be the reason for elevated CHGA serum levels in patients with RA. In our study, we tried to answer this question by comparing material from patients with RA and OA controls.

The density of CHGA+ cells was markedly higher in RA as compared to OA controls. Thus, we thought that elevated CHGA serum levels can be derived from exaggerated local production of CHGA from synovial cells in inflamed tissue in patients with RA. However, since OA controls compared to RA demonstrated a markedly lower density of CHGA+ cells and serum levels, synovial fluid levels, and superfusate levels were similar in RA versus OA, most of the CHGA should come from another source outside the joint. As mentioned above, this source is most probably the adrenal medulla because CHGA is released from this particular organ (as reviewed[31]). The detection of relatively high levels of CHGA in synovial fluid in RA and OA controls is thus only a sign of spillover into the joint cavity. Nevertheless, there is little production of local CHGA as substantiated in superfusion experiments. The role of the local production remains to be determined. Double immunofluorescence revealed that at least some neutrophils expressed CHGA, which probably is responsible for vesicle biogenesis in these cells similar to that in sympatho-adrenal cells of the adrenal medulla (as reviewed[31]). However, most CHGA+ cells do not double-stain with neutrophils, which indicates that other presently unidentified cells produce this sympathetic marker. It must be

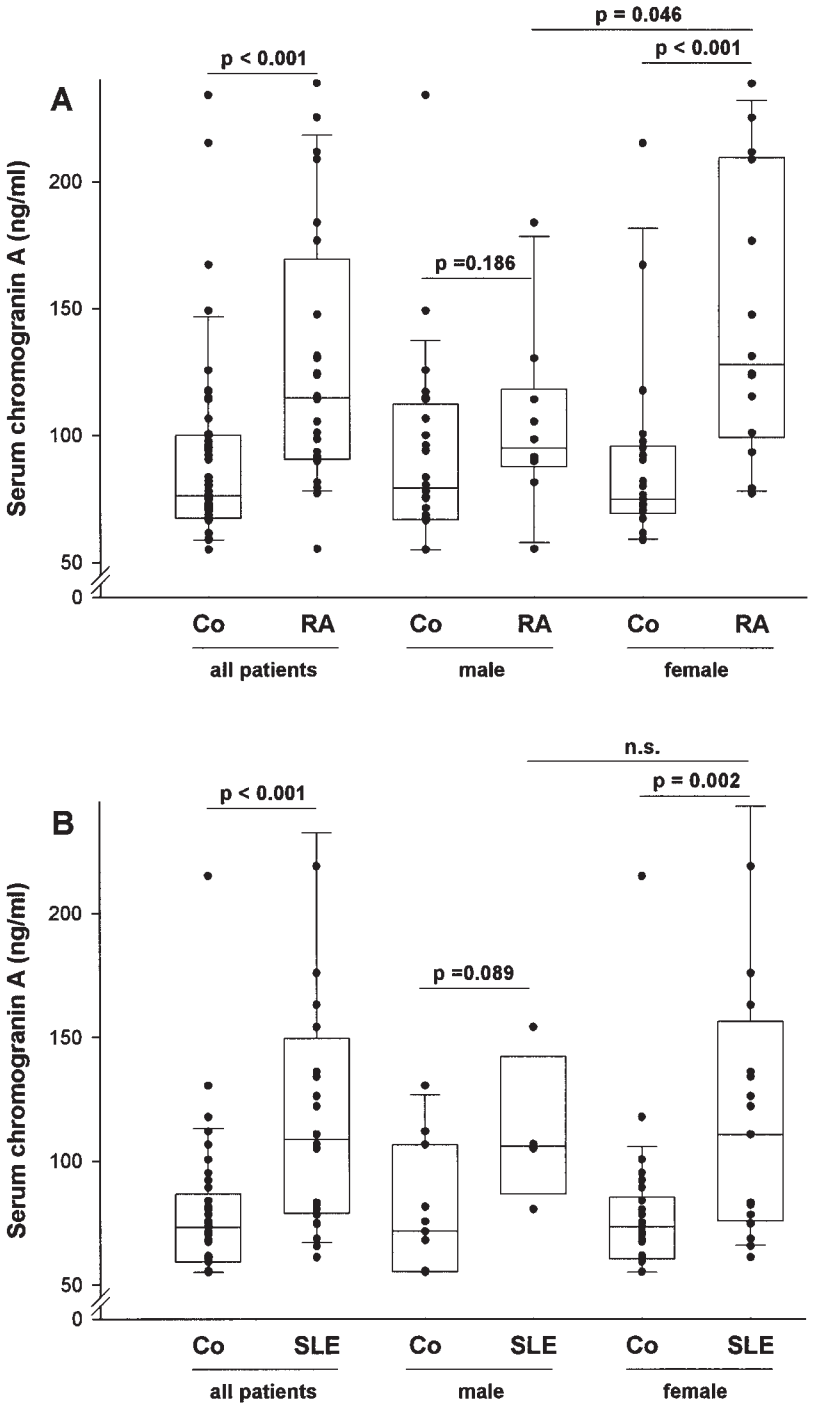

Figure 2. Serum levels of chromogranin A in patients with RA and SLE. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentiles. Each symbol represents 1 person. The p values are derived from Mann-Whitney test. n.s.: not significant, Co: control.

the subject of further studies to identify the cellular source of CHGA in RA synovial tissue.

Since we did not investigate inflamed tissue of patients with SLE, we cannot answer whether or not CHGA is derived from cells in inflamed tissue in these patients. However, since we know that patients with SLE have elevated neuropeptide Y serum levels, and thus an elevated sympathetic activity, we speculate that elevated serum CHGA levels in these patients are similarly derived from the adrenal medulla and not from inflamed tissue. We suggest that this needs to be substantiated by investigation of inflamed tissue of patients with SLE.

At this point the question arises why the sympathetic nerv-

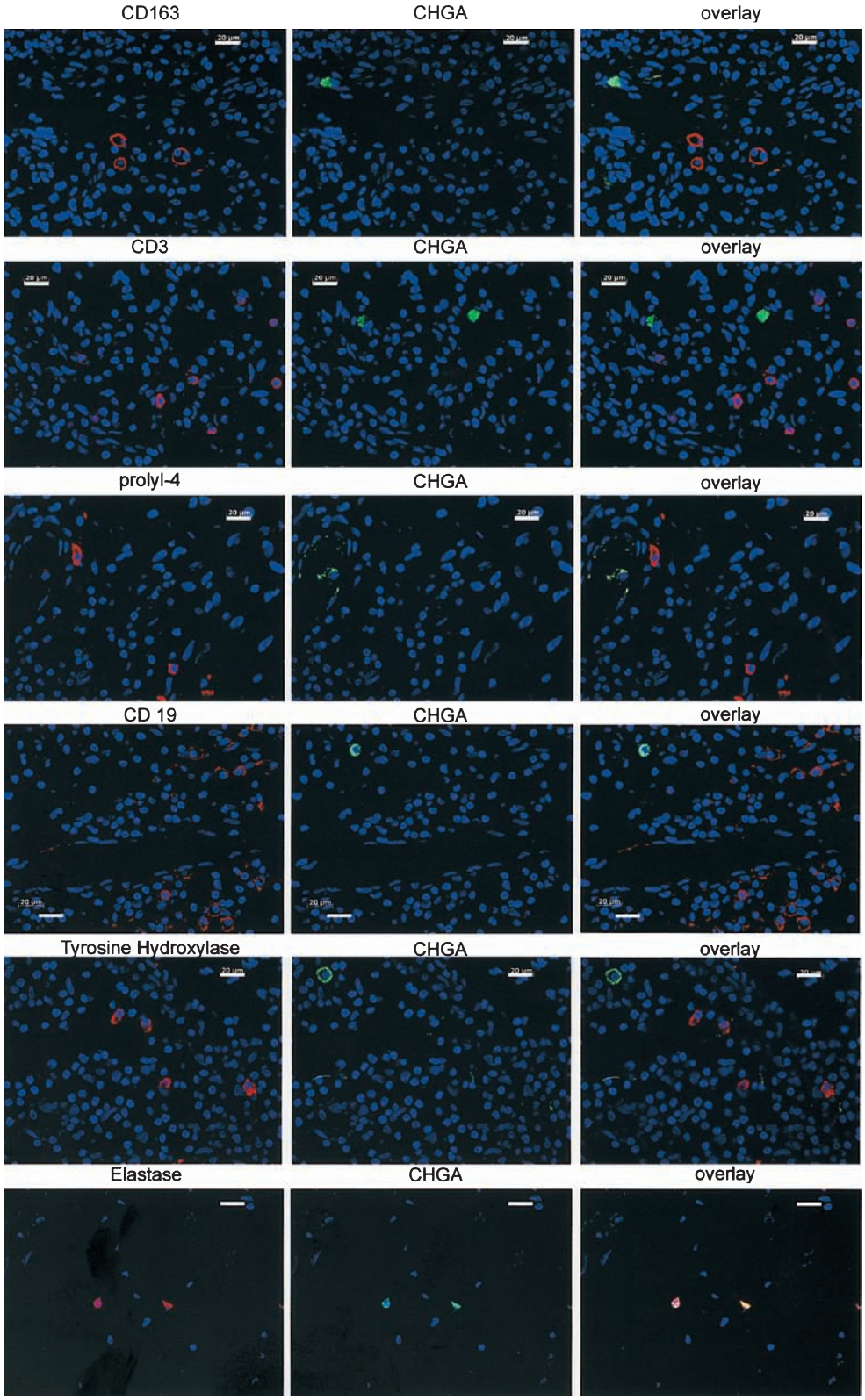

Figure 3. Double-immunohistochemistry of chromogranin A (CHGA)-positive cells and various synovial cells. In the left column, cells positive for CD163+ macrophages, CD3+ T lymphocytes, prolyl-4-positive fibroblasts, CD19+ B lymphocytes, tyrosine hydroxylase-positive cells, and neutrophil elastase are shown in the tissue of a patient with RA. In the middle column, CHGA+ cells of the same patient and the same synovial high-power field are shown. The right column shows the overlay of the 2 images to the left. Magnification 400×. Bar = 20 μm.

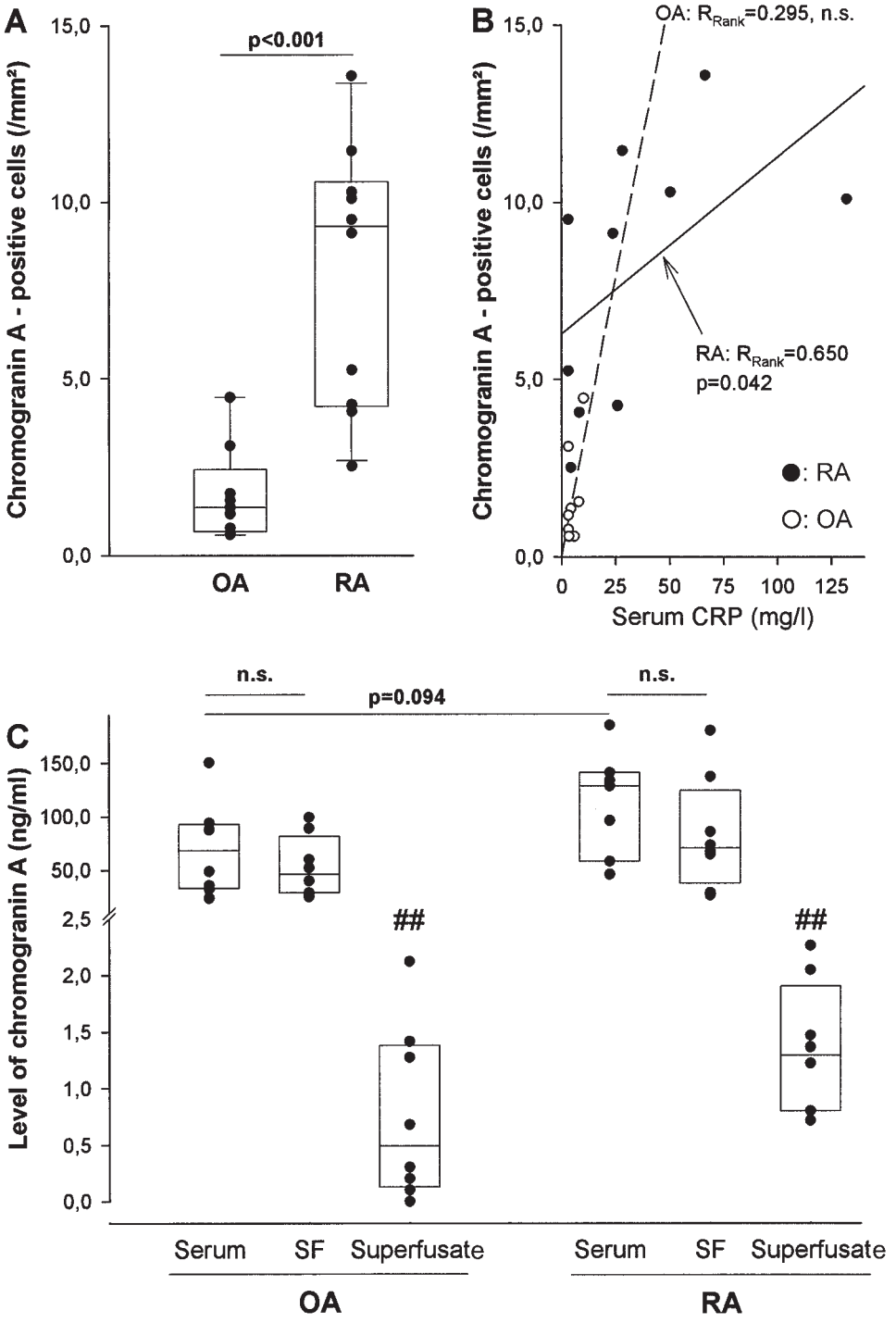

Figure 4. Density of chromogranin A (CHGA)-positive cells in synovium and CHGA levels in different body fluids. A. Density of CHGA-positive cells in OA versus RA. B. Correlation between serum level of CRP and density of CHGA-positive cells in RA and OA. The rank correlation coefficient and p value are given. C. Levels of CHGA in different body fluids of the same patients with OA and RA. Box plots show 10th, 25th, 50th (median), 75th, and 90th percentiles. Each symbol represents 1 person. The p values are derived from Mann-Whitney test or Spearman rank correlation analysis. $^{\#\#}p < 0.001$ vs serum and synovial fluid levels. n.s.: not significant; SF: synovial fluid.

ous system is activated in these patients. Sympathetic hyperactivity is found in the presence of a relatively low activity of the HPA axis, because serum cortisol levels are normal[25]. Coupling of the sympathetic nervous system and HPA axis is important to support the ß-adrenergic and glucocorticoid receptor pathways[40-47]. This leads to stronger cooperative

effects than using one system alone. Cooperative activity of both axes is observed in asthmatics when these patients use local glucocorticoids and local ß2-adrenergic agents[48,49]. Cooperation increases the bronchodilatory effect of each substance alone. A similar cooperativity can be observed in patients with septic shock[50]. In septic shock, combined treatment with norepinephrine and cortisol leads to improved effects on circulation, blood pressure, and glucose allocation. In patients with chronic inflammatory diseases, a relative loss of HPA axis hormones in relation to proinflammatory cytokines may lead to deficient vasopressive activity of sympathetic neurotransmitters and reduced glucose allocation, which may consequently lead to upregulation of the sympathetic tone. From this point of view, sympathetic hyperactivity is the consequence of an inadequate activity of the HPA axis. The activity of the sympathetic nervous system is upregulated in order to sustain important bodily functions such as glucose allocation and systemic circulation.

By using an alternative sympathetic marker molecule (CHGA), our study confirms sympathetic hyperactivity in patients with RA, and possibly also in SLE. We confirm sympathetic hyperactivity, which is an unwanted symptom because it is an important risk factor for premature atherosclerosis in patients with chronic inflammatory diseases.

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