Inflammatory cell infiltrate and RANKL/OPG expression in rheumatoid synovium: Comparison with other inflammatory arthropathies and correlation with outcome

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Abstract

Objectives

To evaluate if the immunofluorescence analysis of synovial tissue (ST) using antibodies against RANKL/OPG, conjugated with the immunophenotyping of lymphocytes and macrophages, could be of diagnostic and prognostic value in rheumatoid arthritis (RA) patients.

Methods

3-year prospective study of 103 consecutive patients submitted to closed needle biopsy for diagnostic purposes. ST was analyzed with routine histologic techniques and immunofluorescence, using monoclonal antibodies against RANKL, OPG, CD163, CD68, CD4, CD8, interferon-γ and CD19. Patients were prospectively evaluated with a clinical, laboratorial and radiological protocol. At the end of the follow-up patients were divided according to the final diagnosis. Results of the initial histologic evaluation were compared between the main diagnostic groups and in RA patients histologic data was correlated with clinical and radiologic outcome measures.

Results

The RANKL/OPG ratio and the inflammatory infiltrate were significatively higher in RA (n=25) as compared to the same ratio observed in other inflammatory joint diseases (OIJD, n=48) and in osteoarthritis (n=17). The difference between RA and OIJD was specifically confirmed when the comparison involved spondyloarthropathy (n=26). Final HAQ score and radiologic outcome were correlated with the density of intimal CD68+ macrophages. Radiologic progression was correlated with subintimal CD4+ lymphocytes and CD68+ macrophages and intimal CD68 and CD163+ macrophages.

Conclusion

The quantification of the RANKL/OPG ratio and of the number of lymphocytes in the ST might be useful to differentiate RA from other inflammatory joint diseases. The ST number of CD4+ lymphocytes and macrophages are probable predictors of radiologic progression in RA patients.

Key words

Rheumatoid arthritis, synovial tissue, RANKL, OPG, prognosis, diagnosis.

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This work was supported by a grant from Comissão de Fomento da Investigação em Cuidados da Saúde, Ministério da Saúde P.I. nº 163/01 and by Programa Operacional Ciência Tecnologia Inovação (POCTI), financed by the EU and by national funds of the MCES.

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Received on March 15, 2004; accepted in revised form on November 30, 2005.

Introduction

An extensive effort has been made to understand the basic pathologic aspects beneath the clinical manifestations of Rheumatoid Arthritis (RA) that might be important for the early diagnosis and prognosis of this condition (1). Gallagher *et al.* have suggested that some features of synovitis were more severe in RA as compared to other inflammatory disorders and that there could be a correlation with clinical severity (2).

Recently, using a composed histologic score to compare RA with other joint diseases we have concluded that only the number of cells in the lining layer, the percentage of vessels with inflammatory infiltrate, the dimension of the lymphocyte clusters and the percentage of lymphocytes in the synovium could have some discriminatory interest (3). Veale *et al*. reported a reduced synovial membrane macrophage number and lining layer hyperplasia in psoriatic arthritis as compared to RA (4). In addition, Smeets *el al*. showed a significant increase in the mean score for lymphocytes in rheumatoid synovium as compared with reactive arthritis synovial samples (5). A recent study of early arthritis patients suggested that the number of plasma cells, B lymphocytes and macrophages were significantly increased in RA in comparison to other forms of arthritis (6).

On the other hand, a later publication depicted that active RA patients had a significantly higher lymphocytic infiltrate as compared to active spondyloarthropathy (SpA) patients (7). The same group also reported that CD163 positive macrophages were observed in a higher number in the synovial tissue of SpA patients as compared to RA patients (8). We had independently shown that in rheumatoid synovium CD163 is a very specific macrophage marker, probably down regulated by interferon-gamma (IFN-γ) (9).

In addition, immunohistological analysis of synovial tissue detected higher levels of receptor activator of NF-κB ligand (RANKL) positive cells in patients with active RA and SpA as compared with patients with inactive RA, osteoarthritis (OA) and normal subjects (10, 11). In contrast, the same group has found lower levels of osteoprotegerin (OPG) positive cells in RA patients, in comparison with SpA and OA patients (12).

Recent immunohistochemical studies have also searched for predictors of the clinical course of RA. In fact, it was proposed that a possible relationship between the intensity of T and B lymphocyte infiltrate (13, 14), the perivascular inflammatory infiltrate (3), the number of macrophages (15, 16) and the outcome of RA could exist.

Although many data have been published, the question remains whether synovial biopsy has any value in the differential diagnosis and prognosis of inflammatory joint diseases (17). In an attempt to elucidate this relevant issue we performed a 3-year prospective study of 103 consecutive patients submitted to closed needle biopsy for diagnostic purposes. Results of the initial histologic evaluation (which included the use of monoclonal antibodies against RANKL, OPG, CD163, CD68, CD4, CD8, IFN-γ and CD19) were compared between the main diagnostic groups and in RA patients histologic data were correlated with clinical and radiologic outcome measures evaluated at the end of the follow-up.

Material and methods

Patients

One hundred and three consecutive patients with active arthritis for a mean period of 9.9 ± 16.7 months were submitted to a closed needle biopsy (18) for diagnostic workout, between January 2000 and December 2001, in the Rheumatology Department of Santa Maria Hospital, Lisbon, Portugal. Seven patients were excluded from further analysis due to the following reasons: lost for follow-up (5 cases); concomitant diagnosis of septic arthritis and rheumatoid arthritis (1 case); insufficient amount of synovial tissue (less than 8 mm^2, 1 case). The remaining 96 patients were subsequently submitted to a clinical, laboratorial and radiological follow-up with a mean duration of 19.3 ± 7.6 months. All patients gave informed consent and the Santa Maria Hospital Ethics Committee approved the study.

Clinical, laboratory and radiological follow-up

All patients were evaluated accordingly to a previously published protocol (PMAR) (19) that includes the following variables: identification; personal medical history; familial medical history; epidemiological data; Graffar score (20); educational score (the number of years of education); onset of disease; systemic manifestations (subcutaneous nodules; pulmonary fibrosis confirmed by chest roentgenograms and lung function tests; echocardiographic evidence of pericardial effusion or pleural effusion shown by chest roentgenograms; Felty's syndrome (less than 2×10^9/l granulocytes and splenomegaly); cutaneous vasculitis (leukocytoclastic vasculitis histologically proved); non-compressive neuropathy confirmed by electromyography; the diagnosis of Sjögren's syndrome was based on the clinical symptoms of dry eyes and dry mouth, confirmed by a positive Schirmer's test and/or keratoconjunctivitis sicca, with involvement of salivary glands documented by positive lip biopsy and/or salivary scintigraphy; actual medication; prior medication; disease activity [morning stiffness; grip strength; patient and physician visual analogue scale for global assessment of disease activity; visual analogue scale of pain; joint count including painful, swollen and structurally damaged joints; calculation of the Disease Activity Score –DAS28 (21,22)]; American College of Rheumatology- ACR functional class (23); Health Assessment Questionnaire (HAQ) (24); laboratory tests [hemoglobin, platelet count, rheumatoid factor, Westergren erythrocyte sedimentation rate (ESR), C reactive protein (CRP)]; radiological evaluation (hands and feet).

The protocol was applied at the time of the biopsy and in each follow-up visit, which were scheduled at 3-month intervals. Radiological evaluation was performed at inclusion and after 1 year of follow-up, and a blinded observer (JEF) using the Sharp/van der Heijde method analyzed radiographs (25). Final modified Sharp score was considered to be the radiological outcome and the difference between initial and final modified Sharp scores was designated as the radiologic progression.

Synovial tissue

At least 4 synovial samples, from different locations, were obtained from each patient. Tissue samples were embedded in OCT compound (Tissue Tek, Elkhart, IN), snap frozen in liquid nitrogen and stored at -70ºC. Each sample received a code and was blinded stored. Tissues were sectioned serially, to a thickness of 5 μm, at -25ºC, taken up onto slides, air dried for 1 hour and stored at -70ºC until immunofluorescence processing. At least two slides of each case were also stained with haematoxylin and eosin.

Antibodies for immunofluorescence

Serial sections of each tissue were incubated with the following mouse-anti-human monoclonal antibodies: IgG1 anti-CD68 diluted at 1:200 (PGM1 clone; Dako, High Wycombe, UK), IgG1 anti- CD163 diluted at 1:400 (Ber Mac clone; Dako, High Wycombe, UK), IgG1 anti-CD4 diluted at 1:20 (Dako, High Wycombe, UK), IgG1 anti-CD8 diluted at 1:40 (Dako, High Wycombe, UK), IgG1 anti- CD19 diluted at 1:30 (Dako, High Wycombe, UK), IgG1 anti-human IFN-γ diluted at 1:200 (Mabtech, Sweden), IgG2b anti-human RANKL diluted at 1:80 (R&D Systems, Minneapolis, USA) and IgG1 anti-human OPG diluted at 1:20 (R&D Systems, Minneapolis, USA). Negative control experiments were performed by using isotype-matched irrelevant antibodies and by omitting the primary antibodies. Preblocking with recombinant IFN-γ, RANKL and OPG was performed as an additional specificity control.

Immunofluorescence methods

Serial cryostat sections were thawed at room temperature, fixed in acetone (BDH Laboratory Supplies) at 4ºC, during 10 minutes, air dried for 2 minutes and washed in PBS for 30 minutes at room temperature. Sections were then rinsed in PBS and incubated with primary antibodies for 16-24 hours at 4°C, washed in PBS during 30 minutes (3 washes of 10 minutes) and then incubated with appropriate secondary antibodies conjugated to fluorescein isothyocianate (dilution 1:100, Sigma Chemicals, St. Louis, Mo, USA), for 1 hour at room temperature. Slides were further washed with PBS for 30 minutes (3 washes of 10 minutes). Sections were finally incubated with DAPI (KPL, USA) during 5 minutes. A last wash with PBS during 30 minutes was also performed. (9)

Sample observation

Samples were examined and photographed with a Leica DMR microscope equipped for epifluorescence. The synovial sections were coded and randomly analyzed by two blinded observers (NCD and AF). The inter-reader and intra-rater reproducibility was confirmed by the random and blinded re-observation of one in each 10 slides by a third observer (JEF). All sections were examined under 400x magnification. The surface area was estimated using an eyepiece with a squared graticule. For each patient a mean area of 40 ± 15 mm^2 was studied. The number of positively stained cells in every high power field was recorded and the mean count per mm^2 was calculated. In the subintima the ratio CD68/CD163 and the ratio RANKL/OPG were calculated for each individual patient.

In addition, a semiquantitative histological score, adapted from Rooney *et al*. (26), was used to analyze the haematoxylin and eosin slides. The score was simplified in accordance with our previous results (3) and includes 4 variables: score 1- the mean number of synoviocytes in the lining layer, with a possible number of layers ranging from 1 (score 0) to more than 10 (score 10); score 2- the mean percentage of vessels with perivascular infiltrates of lymphocytes, which were characterized as aggregates of lymphocytes that were contiguous with the vessel wall and were no more than 10 cells in diameter- less than 5% (score 0), 5-10% (1), 10-20% (2), 20-30% (3), 30-40% (4), 40-50% (5), 50-60% (6), 60-70% (7), 70-80% (8), 80-90% (9), 100% (10); score 3- the mean number of lymphocytes in the diameter of the focal aggregates of lymphocytes, including perivascular

infiltrates with more than 10 cells in diameter- less than 11 (score 0), 11-15 (1), 15-20 (2), 20-25 (3), 25-30 (4), 30-35 (5), 35-40 (6), 40-45 (7), 45-50 (8), 50-55 (9), more than 55 (10); score 4- the mean percentage of lymphocytes per high power field (under 400x magnification)- 0 (score 0), 1-10% (1), 10-20% (2), 20-30% (3), 30-40% (4), 40-50% (5), 50-60% (6), 60-70% (7), 70-80% (8), 80-90% (9), 90-100% (10). Total score was considered to be the mean of the 4 scores.

Data analysis

All the data were introduced in a Microsoft Access 2000 database and all the calculations were made with SPSS Manager 10. Data were presented as mean ± standard deviation (sd). Means from unpaired samples were compared, using the independent-sample Student t-test or the Mann-Whitney test according to the type of distribution. Means from the same group were compared with the paired sample Student t-test or the Wilcoxon test according to the type of distribution. The Pearson's or the Spearman's correlation coefficients were used to determine the correlation between two variables, as appropriate for the type of distribution. P values less than 0.05 were considered significant.

Results

Clinical, laboratorial and radiological follow-up

After a mean follow-up of 19.3 ± 7.6 months a diagnosis was possible to be established for the 96 patients that were actually included in the analysis. Twenty-five patients fulfilled the ACR diagnostic criteria for RA (27), 48 had other inflammatory joint diseases (OIJD), 17 patients were classified as knee OA (ACR diagnostic criteria) (28) and 6 patients had an infectious agent isolated from the joint (septic arthritis- SA). In the OIJD group 26 patients were diagnosed as SpA, according to the European Spondyloarthropathy Study Group criteria (29), of which 18 met criteria for reactive arthritis (ReA) (30), 4 were cases of ankylosing spondylitis [AS, modified New York Criteria (31)], 3 were undifferentiated SpA (US) (29) and 1 was a psoriatic arthritis (PA, Wright and Moll criteria) (31).

Of the remaining 23 patients with OIJD, 11 corresponded to cases of crystal associated arthritis (6 cases of calcium pyrophosphate dehydrate crystals identified in the synovial fluid and 5 cases of monosodium urate monohydrate crystals identified in the synovial fluid), 2 were sarcoidosis, 1 was a Sjögren's syndrome, 1 was a paraneoplastic polyarthritis (lung adenocarcinoma) and 7 were undifferentiated arthritis, based on the exclusion of other rheumatic diseases (32). Social status (measured with the Graffar score), educational background (number of years of education), duration of the disease before biopsy and duration of follow-up were comparable between all diagnostic groups, with the exception of septic arthritis cases which were submitted to a biopsy significantly earlier than the other patients (Table I).

At the end of the follow-up period there was an improvement in the ESR, HAQ score and patient global evaluation in all patients (Table II). Initial and final ESR, HAQ and patient global evaluation were higher in RA than in other diagnostic groups, with the exception of the initial ESR in septic arthritis, which was higher than the initial ESR in RA patients (Table II). Two patients died

Table I. General characteristics of the studied population.

Diagnosis	Number	Age (years ± sd)	Disease duration (months ± sd)	Follow up duration (months ± sd)	Educational score (years ± sd)	Graffar score (score ± sd)
RA	25	53.9 ± 15	11.6 ± 11.1	19.4 ± 8.2	6.1 ± 3.8	3.4 ± 0.8
OIJD	48	41.5 ± 20.9*	9.3 ± 20.3	19.7 ± 7.3	8 ± 5.1	3.2 ± 1.2
OA	17	61.9 ± 13.9	8.1 ± 3.7	18.4 ± 7.9	6.7 ± 5.9	3.4 ± 1.3
SA	6	70.2 ± 6.5*	1.4 ± 2.4*	17.5 ± 7.9	5.3 ± 5.8	3.7 ± 1.2
Total	96	50.1 ± 19.9	9.9 ± 16.7	19.3 ± 7.6	7.1 ± 5	3.3 ± 1.1

RA: rheumatoid arthritis; OIJD: other inflammatory joint diseases; OA: osteoarthritis; SA: septic arthritis. *p < 0.05 vs. RA.

Table II. Initial and final disease-related variables for the studied population.

Diagnosis	ESR (mm/h ± sd)		HAQ (score ± sd)		Patient Global VAS (mm ± sd))	
	Initial	Final	Initial	Final	Initial	Final
RA (n = 25)	43.1 ± 32.9	28.2 ± 24.7#	1.5 ± 0.9	0.67 ± 0.66#	64.4 ± 30.9	44.9 ± 29.7#
OIJD (n = 48)	41.4 ± 33.5	12.2 ± 7.8*#	0.73 ± 0.8*	0.16 ± 0.57*#	57.6 ± 29.8	29.7 ± 24.5*#
OA (n = 17)	18.7 ± 13.7*	15.2 ± 11.9*	0.53 ± 0.72*	0.29 ± 0.44*	56.2 ± 28.9	33.4 ± 30.7#
SA (n = 6)	80.2 ± 37.2	19 ± 16.2#	1.3 ± 1.1	0.23 ± 0.44#	44.3 ± 36.1	13 ± 17.6*
Total (n = 96)	40.8 ± 33.2	18.6 ± 18.3#	0.94 ± 0.92	0.34 ± 0.65#	58.6 ± 30	29.2 ± 28.3#

RA: rheumatoid arthritis; OIJD: other inflammatory joint diseases; OA: osteoarthritis; SA: septic arthritis. ESR: erythrocyte sedimentation rate; HAQ: Health Assessment Questionnaire; VAS: Visual Analogue Scale. *p < 0.05 vs. RA; #p < 0.05 initial vs final.

Table III. Initial and final disease-related variables for RA patients.

	ESR (mm/h ± sd)	NTJ (n ± sd)	NSJ (n ± sd)	VASg (mm ± sd)	VASp (mm ± sd)	VASph (mm ± sd)	DAS (score ± sd)	HAQ (score ± sd)	Grip (mmHg ± sd)	Mstiff (mim ± sd)	Sharp (score ± sd)
Initial	43.1±32.9	6.6±6.9	6.1±6.9	64.4±30.9	68.4±31.3	60.4±30.1	5.1±1.6	1.5±0.9	57.8±71.6	68.4±54.6	43.3±47.5
Final	28.2±24.7*	2.9±5.3*	2.4±3.5*	44.9±29.7*	48.5±29.3*	30.5±23.2*	3.6±1.5*	0.67±0.66*	77.3±73.7	36.4±48,5*	49.2±47,8*

ESR: erythrocyte sedimentation rate; NTJ: number of tender joints; NSJ: number of swollen joints; VASg: patient Global Visual Analogue Scale; VASp: patient Pain Visual Analogue Scale; VASph: physician Global Visual Analogue Scale; DAS28: Disease Activity Score; HAQ: Health Assessment Questionnaire; Grip: grip strength; Mstiff: morning stiffness; Sharp: modified Sharp score. *$p < 0.05$ initial vs final.

Table IV. Histological characteristics of the studied population.

	RA (n = 25)	OIJD (n = 48)	OA (n = 17)	SA
ICD68 (cells/mm^2 ± sd)	546.7 ± 907.5	341.3 ± 518.4	227.2 ± 336.3	199.9 ± 243.0
ICD163 (cells/mm^2 ± sd)	302.1 ± 440.4	250.0 ± 324.4	225.6 ± 320.1	273.6 ± 193.5
IOPG (cells/mm^2 ± sd)	130.1 ± 227.7	102.8 ± 196.5	180.3 ± 250.8	18.9 ± 36.7
SICD68 (cells/mm^2 ± sd)	85.5 ± 81.7	65.7 ± 83.2	52.8 ± 82.6	73.2 ± 24.9
SICD163(cells/mm^2 ± sd)	67.1 ± 56.9	66.8 ± 84.9	36.3 ± 34.1	86.5 ± 47.8
SICD68/163 (ratio ± sd)	1.5 ± 1.1	1.3 ± 1.7	1.1 ± 1.1	1.0 ± 0.5
SIRANKL(cells/mm^2 ± sd)	63 ± 82.6	31.1 ± 67.7*	12.9 ± 21.9*	45.1 ± 66.0
SIOPG (cells/mm^2 ± sd)	39.6 ± 64.9	39.8 ± 49.6	50.6 ± 55.3	45.3 ± 71.2
SIRANKL/OPG(ratio ± sd)	1.9 ± 2.5	0.6 ± 1.2*	0.3 ± 0.5*	1.2 ± 0.5
CD4 (cells/mm^2 ± sd)	78.4 ± 77.3	28.2 ± 48.8*	32.3 ± 59.9*	32.7 ± 40.9
CD8 (cells/mm^2 ± sd)	40.8 ± 34.3	15.2 ± 26.9*	9.2 ± 17.1*	25.3 ± 33.9
CD19 (cells/mm^2 ± sd)	51.4 ± 66.8	7.4 ± 21.6*	6.3 ± 17.7*	21.2 ± 32.3
IFN-g (cells/mm^2 ± sd)	80.4 ± 78.6	43.2 ± 88.1	12.9 ± 21.9	86.2 ± 69.2
S1 (score ± sd)	1.5 ± 2.1	0.5 ± 0.8*	0.4 ± 1.4	1.5 ± 2.1
S2 (score ± sd)	2.6 ± 3.4	0.9 ± 1.9*	0.5 ± 1.2*	1.3 ± 2.2
S3 (score ± sd)	0.4 ± 0.8	0.09 ± 0.3*	0*	0.2 ± 0.1
S4 (score ± sd)	2.1 ± 2.4	0.8 ± 1.1*	0.5 ± 0.8*	2.1 ± 3.1
TS (score ± sd)	1.7 ± 1.8	0.8 ± 1.3*	0.4 ± 0.8*	1.2 ± 1.3

RA: rheumatoid arthritis; OIJD: other inflammatory joint diseases; OA: osteoarthritis; SA: septic arthritis. I: intima; SI: subintima; S1-4 and ST: simplified histological scores as described in Material and methods.
*$p < 0.05$ vs. RA.

during the second year of follow-up: one due to lung adenocarcinoma (the patient with the paraneoplastic polyarthritis) and another one with a respiratory infection (one of the patients with septic arthritis, a 78 year-old men with diabetes and living in a nursing home).

At the time of the biopsy, RA patients (Table III) had a mean age of 53.9 ± 15 years, rheumatoid factor was detected in the serum of 17 (68%) patients, the mean DAS score was 5.1 ± 1.6, the mean HAQ score was 1.5 ± 0.9, the mean morning stiffness was 68.4 ± 54.6 minutes, the mean grip strength was 57.8 ± 71.6 mmHg and the mean modified Sharp score was 14.4 ± 18.2. From a functional point of view (ACR classification) RA patients could be classified as follows: 11 (44%) in class I, 5 (20%) in class II, 6 (24%) in class III and 3 (12%) in class IV. The characteristics of the RA population studied were similar to the disease pattern of RA previously described in Portugal (33).

At the moment of enrollment all RA patients had received non-steroidal anti-inflammatory drugs and 7 had already started a low dose of prednisone [less than 7.5 mg; 2 of them had also started methotrexate (MTX)]. At the end of the follow-up period 21 patients were on a low dose of prednisone, 14 patients were on MTX (3 of them with associated infliximab, 1 with anakinra and 1 with etanercept), 2 were on sulfasalazine, 1 was on leflunomide, 1 on intramuscular gold salts and 1 on azathioprine. All the biologic drugs were started after the second radiologic assessment.

In the last evaluation all the clinical measurements had improved (Table III): the mean DAS score was 3.6 ± 1.5 ($p = 0.001$), the mean HAQ score was 0.7 ± 0.6 ($p = 0.0001$), the mean morning stiffness was 36.4 ± 48.5 minutes ($p = 0.017$), the mean grip strength was 77.3 ± 73.7 mmHg [not significant (NS)]. Although the final mean modified Sharp score (radiological outcome) increased to 20.3 ± 18.4 ($p = 0.0001$), corresponding to a mean increase in the modified Sharp score (radiological progression) of 5.4 ± 3.5. At final review, from a functional point of view (ACR classification) RA patients could be classified as follows: 11 (44%) in class I, 6 in class II (24%), 8

Table V. Histological characteristics of RA and OIJD patients.

	RA (n = 25)	All SpA (n = 26)	ReA (n = 18)	AS, US, PA (n = 8)	CIA (n = 11)	Others (n =11)
ICD68 (cells/mm^2 ± sd)	546.7 ± 907.5	415.0 ± 648.3	548.8 ± 754.8	147.4 ± 186.0	289.9 ± 311.7	198.9 ± 197.2
ICD163 (cells/mm^2 ± sd)	302.1 ± 440.4	268.4 ± 396.1	349.4 ± 458.8	106.3 ± 135.1	283.5 ± 234.0	163.6 ± 156.9
IOPG (cells/mm^2 ± sd)	130.1 ± 227.7	136.3 ± 238.9	141.5 ± 248.3	126.0 ± 233.3	36.9 ± 87.7	84.8 ± 138.2
SICD68 (cells/mm^2 ± sd)	85.5 ± 81.7	76.5 ± 105.8	83.9 ± 116.7	61.7 ± 83.9	65.0 ± 39.9	37.3 ± 28.6
SICD163(cells/mm^2 ± sd)	67.1 ± 56.9	70.2 ± 103.5	58.2 ± 63.8	94.3 ± 158.7	70.2 ± 52.5	53.6 ± 58.8
SICD68/163 (ratio ± sd)	1.5 ± 1.1	1.1 ± 1.3	1.5 ± 1.4	0.5 ± 0.6*	1.8 ± 2.4	1.2 ± 1.6
SIRANKL(cells/mm^2 ± sd)	63 ± 82.6	36.5 ± 83.7*	16.7 ± 31.6*	74.1 ± 132.1	21.8 ± 39.1*	21.2 ± 45.3*
SIOPG (cells/mm^2 ± sd)	39.6 ± 64.9	46.9 ± 59.3	45.1 ± 66.2	50.4 ± 45.8	30.3 ± 39.1	30.9 ± 24.9
SIRANKL/OPG(ratio ± sd)	1.9 ± 2.5	0.6 ± 1.2*	0.5 ± 0.8*	0.9 ± 1.8	0.74 ± 1.3	0.6 ± 0.9*
CD4 (cells/mm^2 ± sd)	78.4 ± 77.3	37.3 ± 59.4*	31.8 ± 46.4*	48.3 ± 81.8	12.8 ± 23.3*	20.4 ± 32.2*
CD8 (cells/mm^2 ± sd)	40.8 ± 34.3	15.7 ± 20.7*	15.7 ± 21.9*	15.7 ± 19.1*	20.5 ± 40.8	8.2 ± 24.4*
CD19 (cells/mm^2 ± sd)	51.4 ± 66.8	7.9 ± 25.7*	6.7 ± 26.7*	10.6 ± 24.8*	9.4 ± 18.6*	3.8 ± 11.6*
IFN-g (cells/mm^2 ± sd)	80.4 ± 78.6	30.2 ± 58.0*	30.8 ± 65.9*	29.2 ± 41.0*	71.4 ± 142.2	47.2 ± 82.9
S1 (score ± sd)	1.5 ± 2.1	0.6 ± 0.9	0.5 ± 0.8	0.8 ± 1.2	0.4 ± 0.7*	0.2 ± 0.5*
S2 (score ± sd)	2.6 ± 3.4	1.1 ± 1.9*	1.1 ± 2.2	0.9 ± 1.5*	1.3 ± 2.3	0.05 ± 0.2*
S3 (score ± sd)	0.4 ± 0.8	0.05 ± 0.3*	0*	0.2 ± 0.5	0.3 ± 0.5	0*
S4 (score ± sd)	2.1 ± 2.4	1.0 ± 1.4*	1.0 ± 1.3	1.1 ± 1.5	0.7 ± 0.8*	0.4 ± 05*
TS (score ± sd)	1.7 ± 1.8	0.9 ± 1.4	0.48± 1.1	1.2 ± 1.9	1.0 ± 1.7	0.2 ± 0.2*

RA: rheumatoid arthritis; SpA: spondyloarthropathy; ReA: reactive arthritis; AS: ankylosing spondylitis; US: undifferentiated spondyloarthropathy; PA: psoriatic arthritis; CIA: crystal-induced arthritis. I: intima; SI: subintima; S1-4 and ST: simplified histological scores as described in Material and methods. *$p < 0.05$ vs. RA.

(32%) in class III and none in class IV. Interestingly, a positive correlation could be detected between the radiological progression and both the initial DAS score (r = 0.53, p = 0.006) and the initial HAQ score (r = 0.46, p = 0.021). In addition, the final HAQ score was positively correlated with the initial HAQ score (r = 0.59, p = 0.002) and inversely correlated with the initial grip strength (r =-0.47, p = 0.017).

Histologic analysis and final clinical diagnosis

As can be more detailed in Table IV, significant differences were observed between RA synovium and the synovial tissue from patients with other inflammatory joint diseases (OIJD) and OA. In fact, the lymphocytic infiltration, as suggested by the semiquantitative histological score, and confirmed by monoclonal antibodies against CD4, CD8 and CD19, was higher in RA patients as compared to OIJD patients. Of particular interest was the difference in the ratio RANKL/OPG, which was significatively higher in RA patients as compared to OIJD patients. When RA and SpA synovial tissue were directly compared (Table V) the same pattern emerged, although in this case a difference in IFN-γ positive cells (higher number in RA patients) could be also depicted. If SpA were analyzed without the ReA cases an additional feature was identified: the CD68/CD163 ratio was higher in RA patients as compared with SpA patients (Table V). The comparison of rheumatoid synovium with synovial tissue from patients with crystal associated arthritis (Table V) only showed a significative higher number of CD4, CD19 and RANKL positive cells in RA cases. No major differences, using our histologic and immunohistologic parameters, could be found between the characteristics of rheumatoid synovium and those of septic arthritis (Table IV), although, this comparison was based in very few septic arthritis cases.

Histologic analysis and clinical and radiological outcome of RA patients

The density of intimal CD68 positive cells was the only histologic variable that correlated with both the final HAQ score (r = 0.44, p = 0.027) and the final modified Sharp score (radiological outcome; r=0.42, p = 0.039). The radiological progression (the difference between the final and the initial modified Sharp scores) correlated, not only with the density of intimal CD68 positive cells (r = 0.45, p=0.024), but also with the density of CD68 positive cells in the subintima (r = 0.41, p = 0.049), the density of intimal CD163 positive cells (r = 0.41, p = 0.05) and the density of CD4 positive cells in the subintima (r = 0.46, p= 0.021). RA patients with erosions present in their final radiologic evaluations (n = 10) had a significatively higher score for perivascular inflammatory infiltrate (3.9 ± 3.6 vs. 1.4 ± 2.3; p = 0.05) and a higher density of intimal CD68 (1179.8 ± 1296.1 vs. 185.9 ± 212.7 cells/mm^2; p=0.01) and CD163 positive cells (623.1 ± 604.4 vs. 119.6 ± 138.3; p = 0.006) as compared to RA patients without erosions (n = 15). No correlation was detected between the ratio RANKL/OPG (or the number of positive cells for RANKL and OPG) and RA outcome variables.

Discussion

Our data suggest that the evaluation of RANKL/RANK/OPG system expres-

sion in the synovial tissue can be useful for the differential diagnosis of RA. In fact, our results have documented that the RANKL/OPG ratio of immunoreactive cells is significatively higher in rheumatoid synovium as compared to the same ratio observed in the synovial tissue found in other inflammatory joint diseases (OIJD) and in OA. The difference between RA and OIJD was specifically confirmed when the comparison involved SpA as a whole and ReA in particular. The inflammatory infiltrate, as depicted by the histological scores and by the use of monoclonal antibodies against T and B lymphocytes, was also significatively higher in RA as compared to OIJD and OA. This fact confirms previous immunohistochemistry data (5-7) but raises also the possibility that simplified scores based on routine histology procedures (haematoxylin and eosin staining) might still be of interest for the diagnosis of RA. In addition, not only IFN-γ positive cells were significantly higher in RA than in ReA, as previously suggested (4), but also this difference could be observed when comparing RA and other SpA. Interestingly, we have confirmed a previous observation from Baeten *et al*. (8) of a lower subintimal CD163 expression in RA as compared to SpA (considering only AS, US and PA), which might partially be explained by the higher IFN-γ production in rheumatoid synovium and by its down regulating effect on CD163 (9).

Our results support a hypothetical prognostic role for the immunofluorescence detection of CD4 positive lymphocytes and macrophages in rheumatoid synovium. As a matter of a fact, radiologic outcome was correlated with the density of intimal CD68 positive macrophages and radiologic progression with subintimal CD4 positive lymphocytes and CD68 positive macrophages and intimal CD68 and CD163 positive macrophages. Moreover, patients that developed erosions at the final radiologic evaluation had a higher density of intimal CD68 and CD163 positive macrophages and also a higher perivascular lymphocyte infiltration. Intimal and subintimal macrophages have been previously correlated with radiologic outcome in RA patients (16). The relevance of the number of macrophages in rheumatoid synovium as a prognostic indicator in RA patients is also reinforced by the correlation between the density of CD68 positive cells and the final HAQ score that we have found in the present study. Our results, regarding the relation of perivascular lymphocytes and the density of CD4 positive lymphocytes with radiologic progression in RA, are in line with our previous retrospective analysis of 26 RA patients, in which we had depicted an association between high number of vessels with perivascular lymphocytes and the development of bone erosions (3).

Although RA seems to evolve with high levels of RANKL expression and low levels of OPG expression (34), in our study, the number of RANKL immunofluorescence positive cells in rheumatoid synovium and the RANKL/OPG ratio did not correlate with outcome measures of RA. There can be several explanations for this result. First of all, issues linked to sample selection bias and to the fact that needle biopsies do not collect samples from the peripheral parts of the joints, where the expression of RANKL is most relevant, might significantly limit the relevance of the immunohistology quantification of the RANKL/OPG ratio for predicting the progression and outcome of RA. However, this observation might be also explained by the fact that TNF-α, present in RA joints in high amounts, is a major osteoclastogenic factor, which is probably able to act both through the enhancement of RANKL expression and also by a mechanism independent of RANKL-RANK interaction (35). Furthermore, TNF-α is able to stimulate osteoclast differentiation in the presence of minimum amounts of RANKL, probably through synergy at the level of NF-κB and stress-activated protein kinase/cjun NH_2-terminal kinase signaling pathways (36). In support of a role for TNF-α in bone erosion independent from RANKL is also its induction of IL-1 release from rheumatoid fibroblasts and macrophages, which act as a major survival and activation signal for nascent osteoclasts (37). In addition, TNF-α is able to induce metallo-proteinases activity and contribute to the destruction of articular cartilage matrix in a process parallel to osteoclast driven joint aggression (38). Finally, several sources documented that TNF-α and IL-1 are chiefly released from macrophages activated by CD4 positive lymphocytes (39), the 2 cell types shown to be clearly correlated with radiological progression.

In conclusion, based on our immunofluorescence analysis of the synovial tissue we have shown that the quantification of the RANKL/OPG ratio and of the number of B and T lymphocytes in the synovium might be useful to differentiate RA from other inflammatory joint diseases. In addition, the synovial number of CD4 lymphocytes and macrophages are probable predictors of radiologic progression in RA patients.

Acknowledgements

The authors are grateful to Professor Luís Graça for his critical reading of the manuscript, and to Mrs. I. Campos and Mrs. M.C. Azevedo e Silva for their technical assistance.

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