CHAPTER 4

CHARACTERISATION OF SYNOVIAL MAST CELLS IN KNEE OSTEOARTHRITIS: ASSOCIATION WITH CLINICAL PARAMETERS

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ABSTRACT

Objective: To investigate the presence of mast cells in the osteoarthritic (OA) synovium and their association with clinical parameters in comparison with rheumatoid arthritis (RA) samples.

Methods: Synovial tissues of 56 symptomatic OA and 49 RA patients were obtained. Two to three paraffin slides were used to quantify inflammation using haematoxylin and eosin staining (synovitis score 0-9), and numbers of mast cells (per 10 high-power fields) using double immunofluorescence for CD117 and tryptase. Average scores per patient were used for analysis. Knee radiographs of OA patients were scored according to the Kellgren and Lawrence (KL) system and pain was determined in OA patients at baseline by visual analogue scale.

Results: Median (range) of mast cells was significantly higher in OA samples 45 (1-168) compared to RA samples 4 (1-47) (p-value < 0.001), despite a lower median (range) synovitis score in OA (2.5 (0-6.0)) compared to 4.6 (0-8.0) in RA samples. The synovitis score was significantly correlated with the number of mast cells (in OA Spearman's rho (p-value) 0.3 (0.023) and RA 0.5 (p-value < 0.001)). Clinically, the number of mast cells was associated with an increased KL-grade (p-value 0.05) in OA patients, but not with self-reported pain.

Conclusion: Prevalence of mast cells in OA synovial tissue is relatively high and associates with structural damage in OA patients, suggesting a role of mast cells in this disease.

INTRODUCTION

Osteoarthritis (OA) is a common rheumatic disorder that has been considered to be a non-inflammatory condition[1]. Synovitis, however, could play a role in the pathophysiology of OA[1-6] as it is a predictor of cartilage destruction[7,8] and a determinant of pain[9,10].
Although the biological processes underlying these associations are poorly understood, it has been suggested that immune cells infiltrating the synovial tissue could be important determinants of synovial inflammation[6,11]. Most frequent types of immune cells found in OA are macrophages, T cells and mast cells[11]. In contrast to macrophages and T cells, mast cells have been less frequently investigated in OA[12-22]. Interestingly however, it has been reported in the 1980s and 90s that mast cells were the only type of immune cells found in equal numbers[14,15,17,23] or higher[12,18] in OA compared to rheumatoid arthritis (RA) and healthy controls[15-18,20,23]. These observations suggest a role for these cells in the pathophysiology of OA, although little additional studies have been performed since then. Moreover, no studies investigated the relationship between mast cells and clinical symptoms and signs of OA.
Several mechanisms have been put forward through which mast cells could contribute to the disease process in RA and these could also apply in OA. For example, mast cells are thought to attract other immune cells through cytokine release, chemokine release or direct cell to cell contact, which leads to activation of the synovium and ultimately to inflammation and bone destruction. Other possible mechanisms include the activation of fibroblasts and other synovial cells, stimulation of angiogenesis, upregulation of adhesion molecules on endothelium and others[24]. Furthermore, mast cells could contribute to pain in OA, as they have been implicated in pain perception in several disorders[25]. How mast cells exert these effects is also poorly understood, but release of soluble mediators and enzymes have been suggested as possible mechanisms.
The contribution of mast cells to radiographic changes and pain is still unknown. To get insight into the potential role of mast cells in OA, we investigated the difference in mast cell number or degranulation status in OA compared to RA and at different stages of disease. Furthermore, we investigated the association of the number of mast cells with the grade of synovitis, structural damage and pain in OA.

METHODS

Osteoarthritis (OA) Patients

The OA patients participated in the geMstoan study (GEneration of Models, Mechanism & Markers for Stratification of OsteoArthritis patieNts), an observational study in established and end-stage knee OA patients[26]. This study has been approved by the ethics committee

of the Leiden University Medical Center (LUMC) and the Diaconessenhuis Leiden. All patients provided written informed consent. Inclusion and exclusion criteria are described elsewhere[26]. Two groups of patients with symptomatic radiographic primary knee OA, following the ACR criteria,[27] have been included in the geMstoan study: one group with end-stage disease that were planned to receive an arthroplasty ("late OA") and another group with mild to established OA that had no indication for an arthroplasty ("early OA").

Rheumatoid Arthritis (RA) patients

Tissue samples were obtained as leftover material from patients who underwent arthroplasty ("late RA"). Arthroscopy samples ("early RA") were collected within a study described in [28], including patients with RA; excluded were those on oral prednisolon > 10 mg/day, recent (less than six weeks) change of disease modifying anti-rheumatic drugs (DMARDs) or recent joint injection. The study was approved by the Medical Ethical Committee of the LUMC, Leiden.

Synovial tissue collection

Synovial samples were either collected during arthroplasty or arthroscopy. Synovial samples were collected from patients admitted for arthroplasty for OA or RA to the orthopaedic department of the LUMC or Diaconessenhuis Leiden. Arthroscopy was performed at the department of Rheumatology for research purposes only, using a small-bore 2.7 mm arthroscope (Storz, Turrlingen, Germany) with sterile technique, as described previously[26,28,29]. Synovial tissues obtained during arthroscopy and arthroplasty were fixed in formalin for approximately 24 hours and then transferred to 70% ethanol where they were stored until embedded in paraffin.

Histological staining of biopsies

Four micrometer thick sections were stained with haematoxylin and eosin (H&E) after deparaffinization. For each patient, 2-6 H&E stained synovial tissue samples were scored for 3 features, according to Krenn et al.[30]: lining cell layer, synovial stroma and inflammatory infiltrate. The grading system of Krenn et al. was modified as described earlier[26]. All synovial tissue samples were scored by 2-3 independent observers (BDL, SNA and ALD for OA, BDL and ALD for RA) who were blinded to imaging clinical data; an average score per feature was calculated (0-3) for each patient. Furthermore, a total synovitis score was calculated (sum of averaged scores of all 3 features (0-9)). Only for the OA samples, in case the scoring of one observer was evidently different from the other two, one rescoring was performed by that observer.

Mast cell staining and scoring

Four micrometer sections of synovial tissue from patients with OA were deparaffinized, rehydrated and treated with DAKOpen (DAKO, Glostrup, Denmark). After antigen retrieval by heating 30 min in prewarmed EDTA pH9 in 96ºC, the sections were blocked with blocking solution (10% Normal Donkey Serum (DS) in 1%BSA/PBS) (Sigma Aldrich, Saint Louis, Missouri (MO), USA) for 30 min. at RT. Sections were incubated first with 1:100 polyclonal rabbit anti-human CD117 (DAKO, Glostrup, Denmark) and 0.125 µg/ml monoclonal mouse anti-human tryptase antibody (Merck Millipore, Darmstadt, Germany) in 1%DS/1%BSA/PBS for 1 hour at RT, followed by wash with PBS and incubation with 1:1000 polyclonal donkey anti-rabbit IgG Alexa Fluor 488 for mast cells and 1:1000 polyclonal donkey anti-mouse IgG Alexa Fluor 568 for tryptase (both Invitrogen, Carlsbad, California, USA) in 1%DS/1%BSA/PBS., for 1 hour at RT.

Control rabbit Ig (Antibodies online, Aachen, Germany) and control mouse IgG1 (DAKO, Glostrup, Denmark) were included as isotype controls.

Slides were covered with Vectashield Hard Set mounting medium with DAPI (Vector laboratories, Burlingame, California (CA), USA) and analyzed on a fluorescence imaging microscope (Axio Scope A1, Zeiss, Oberkochen, Germany) coupled to a MRc5 camera using AxioVision 4.9.1. software.

Degranulated mast cells were defined as $CD117^{+}$ or $CD117^{-}tryptase^{+}$ cells for which at least 2 tryptase granules next to the cells were readily detectable. All other $tryptase^{+}$ cells were defined as non-degranulated mast cells. Degranulated, non-degranulated and total number of mast cells (degranulated + non-degranulated), were determined in 10 adjacent high-power fields in the middle of the tissue section, starting on the left side of the section at the lining layer. Examples of mast cell stainings are presented in Figure 1. Direction of sub sequential high power fields was from left to right and from top to bottom. Mast cells in OA tissues were counted manually by three independent blinded observers (BDL, SNA and ALD), while two blinded observers counted the mast cells in RA tissues (BDL, ALD). Average (between all observers) total number of mast cells per 10 high power fields and percentage of degranulated cells were used for analyses.

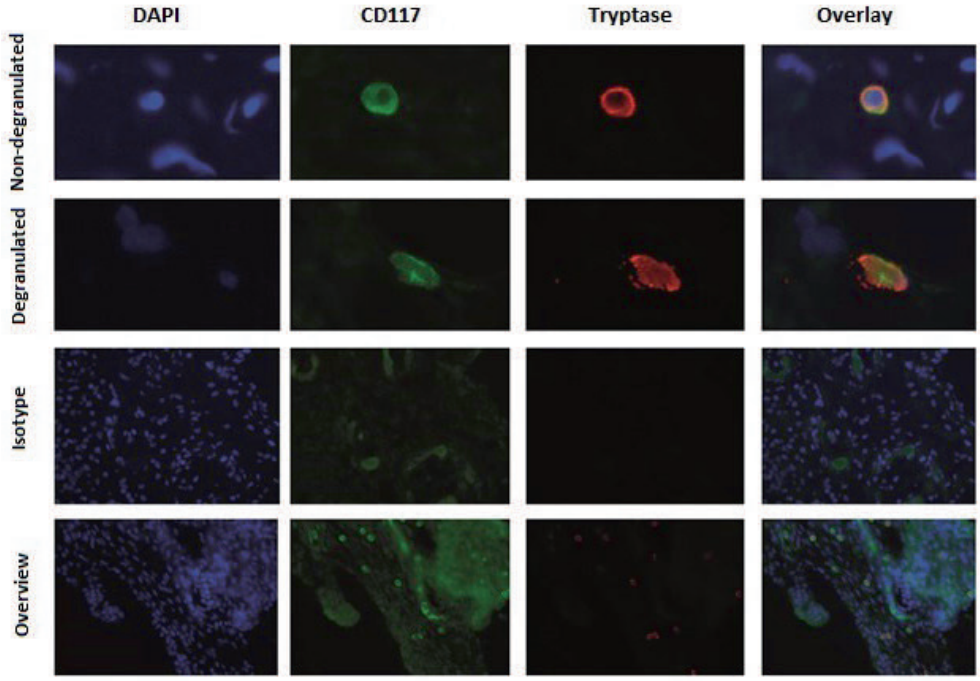

Figure 1. Paraffin sections were stained for DAPI, CD117 and tryptase as described in Materials and Methods. Individual stainings and the overlay (right column) are presented, as well as examples of degranulated and non-degranulated mast cells, isotype stainings and overview image on a lower magnification.

Scoring knee radiographs

Radiographs (posterior anterior (PA) fixed flexion) were obtained of all patients in the geMstoan study. Radiographs were scored, blinded for patient characteristics, by an experienced musculoskeletal radiologist (HK) according the Kellgren- Lawrence (KL) scale[31]. Reproducibility was as earlier described[26].

Clinical data

In the geMstoan patients, demographics and disease characteristic were collected via standard questionnaires. Pain was measured by the visual analogue scale (VAS, 0-100), in whichparticipants were asked to place an X at a 100 mm line that represented the general pain intensity for that knee. A score of 100 represents worst possible pain intensity.

Statistics

For comparison between groups, unpaired t-test, Mann-Whitney-U test or Kuskal-Wallis test were used as indicated. Correlations were investigated using Spearman's rank correlation test. Linear regression was used to investigate association between mast cells (variable) and

inflammation (outcome). Statistical analyses were calculated performed using SPSS 22.0 and GraphPad Prism version 6.02.

RESULTS

Patient characteristics:

Fifty-six patients with symptomatic knee OA and 49 RA patients were included. Their characteristics are summarized in Table 1. Twenty-two patients with "early" OA and 23 patients with "early" RA underwent knee arthroscopy and synovial biopsies were obtained. Thirty-four patients with "late" knee OA and 26 "late" RA patients were included and synovial biopsies of the knee were obtained.

Table 1. Characteristics of 56 patients with knee osteoarthritis (OA) and 49 patients with rheumatoid arthritis (RA).

	All	early	late
OA, nr. of patients	56	22	34
Age (years), mean (sd)	61.9 (8.1)	60.4 (7.3)	62.8 (8.6)
Female, n (%)	36 (64.3)	16 (72.7)	20 (58.8)
BMI (Kg/m²)	29.2 (20.5-49.9)	29.2 (24.1-43.9)	29.2 (20.5-49.9)
VAS pain (0-100)	64.5 (4-95)	42.5 (4-84)	68.0 (32-95)
KL grade (0-4)	3.00 (1-4)	2.0 (1-4)	3.0 (1-4)
KL 1 (n= 4 (7.3%)	4 (7.3)	3 (13.6)	1 (3.0)
KL 2 (n=13,23.6%)	13 (23.6)	11 (50.0)	2 (6.1)
KL 3 (n=21, 38.2%)	21 (38.2)	6 (27.3)	15 (45.5)
KL 4 (n= 17,30.9%)	17 (30.9)	2 (9.1)	15 (45.5)
Total synovitis score (0-9)	2.5 (0-6.0)	1.8 (0-4.8)	2.9 (0-5.6)
Lining layer	0.8 (0-2.8)	0.5 (0-2.8)	1 (0-2.5)
Stromal activation	0.9 (0-2.7)	0.7 (0-2.7)	1 (0-2.3)
Infiltrate	0.5 (0-3.0)	0.2 (0-1.8)	0.9 (0-3)
RA, nr. of patients	49	23	26
Age (years), mean (sd)	62.3 (11.9)	64.0 (14.2)	63.0 (9.7)
Female, n (%)	32 (68.1)	15 (68.2)	17 (68.0)
Total synovitis score (0-9)	4.6 (0-8.0)	4.5 (0-7.0)	5.1 (0-8.0)
Lining layer	1.5 (0-3.0)	1.0 (0-3.0)	1.5 (0-2.3)
Stromal activation	2.0 (0-3.0)	2.0 (0-3.0)	1.5 (0-3.0)
Infiltrate	1.6 (0-3.0)	1.1 (0-3.0)	2.0 (0-3.0)

Numbers represent median (range) unless otherwise specified. Abbreviations: BMI= body mass index; KL= Kellgren-Lawrence

Mast cells in OA vs RA samples

First, we have quantified mast cells in a set of synovial tissues obtained from both "early" and "late" OA and RA patients. Mast cells were mainly located in the sub-lining layer in both OA and RA samples (data not shown). Median (range) of mast cells per patient per 10 HPF was 45 (1-168) in the OA samples and this was significantly higher than 14 (1-47) in the RA samples (p-value < 0.0001, Fig. 2a). Similarly, mast cells were more abundant in both the early and late OA samples than in their RA counterparts (Fig. 2b). A relatively small proportion of the mast cells were degranulated in both OA and RA samples (Fig. 2c) and no significant differences could be detected between any of the groups (Fig. 2d), indicating that the abundance rather than the degranulation state of the mast cells is associated with having OA.

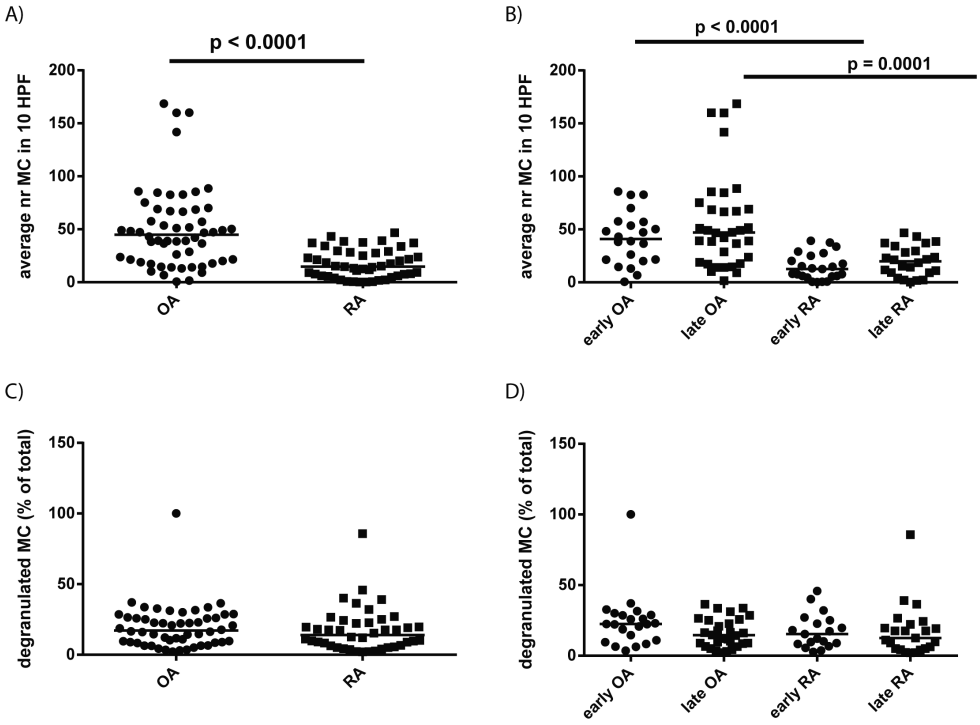

Figure 2. Total mast cell numbers in 10 high power fields in A) total osteoarthritis (OA) and Rheumatoid Arthritis (RA) population, B) early and late OA and RA populations are depicted. The percent degranulated mast cells in the total OA and RA population (C) or early and late OA and RA populations (D) is depicted. Each dot represents one patient. The median per group is indicated. P-values for differences between groups (as indicated) tested by Mann-Whitney (A-C) or Kruskal-Wallis (D) are depicted.

Association with histological synovial inflammation

Patients with OA had lower histological synovitis scores than patients with RA; this was observed for the three separate features (lining layer, stromal activation and infiltrate), as is depicted in Table 1. Moreover, a higher total synovitis score could be found in the RA compared to the OA samples (Fig. 3a and Table 1), despite a lower number of mast cells, and these differences were still present when arthroscopy or arthroplasty samples from OA and RA were compared (Fig. 3b). Next, we studied whether the higher abundance of mast cells could be related to the presence of higher synovitis in general[26].

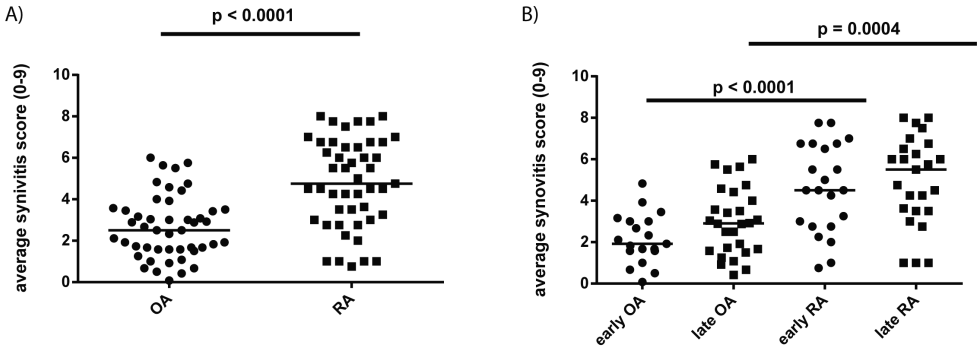

Figure 3. Synovitis scores determined by H&E staining (as described in Materials and Methods) in A) total osteoarthritis (OA) and Rheumatoid Arthritis (RA) population or B) early and late OA and RA populations are depicted. Each dot represents one patient. The median per group is indicated. P-values for differences between groups (as indicated) tested by student's t-test are depicted.

Mast cell numbers associated with the synovitis score both in the OA and the RA samples (Fig. 4), although the effect size of this association was lower in OA than in RA (beta (95% CI) = 0.48 (1.35 – 4.52) for RA, (beta(95%CI) = 0.35 (1.82 – 14.68 for OA), as shown by univariate linear regression analyses.

Interestingly, as shown in fig.4, there is an increased number of mast cells in OA synovium compared to RA synovium, even in the absence of significant inflammation (synovitis score ≤ 1). Moreover, mast cells explained only 12% of the variance in total synovitis score in OA, while this was 23% in RA, in univariate linear regression analyses.

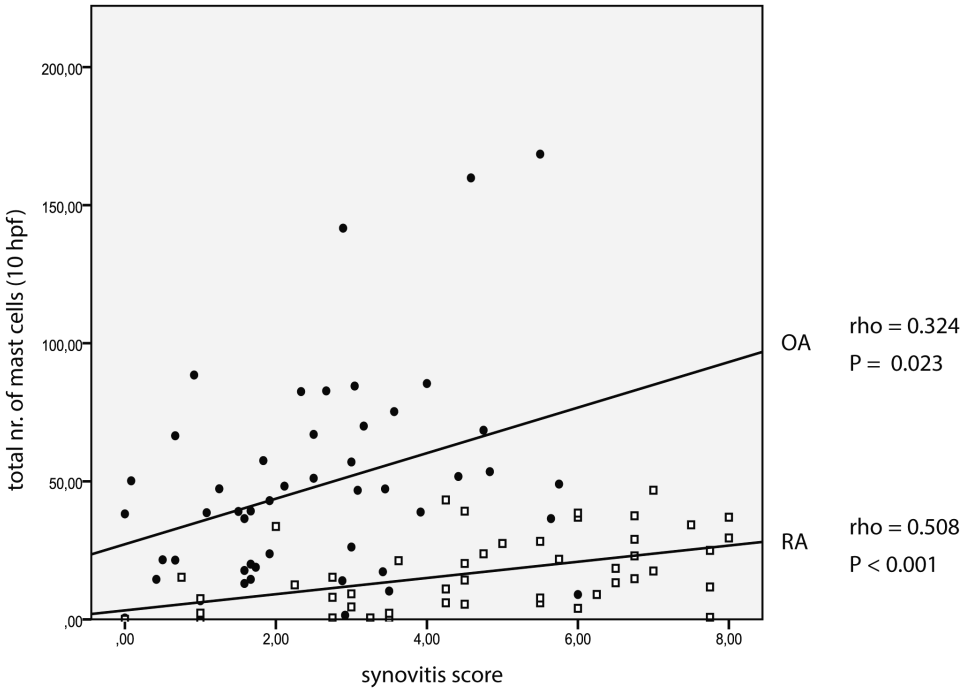

Figure 4. The correlation between total number of mast cells and synovitis scores (determined by H&E staining) is presented in osteoarthritis (OA, closed circles) and Rheumatoid Arthritis (RA, open squares) patients. Each dot represents one individual. Spearman's correlation coefficient and p-values are presented for each group.

Association of mast cells with clinical features in OA

To understand the possible contribution of mast cells to the disease process in OA, we further investigated the association between mast cell numbers and clinical disease parameters.

To ensure that enough variation is present in VAS pain values and radiographic severity scores, we performed these analyses in the whole OA group. As depicted in Fig.5a, there is no significant association between the number of mast cells and VAS pain. Because a trend for higher number of mast cells in females than in males was observed (median (range) 50 (1-169) for females, 39 (2-67) for males, p = 0.060 (Mann-Whitney-U test)), we have also stratified our analyses for gender and found that there is no correlation between mast cells abundance and VAS pain in any of the groups (data not shown).

To evaluate the association with radiographic damage, we studied the amount of mast cells present in patients with a KL score of 1/2 vs a KL score of 3/4. As shown in Fig. 5b, there is an increased number of mast cells in the KL3/4 (median (range), 49 (2-169) compared to KL1/2 group (median (range) 26 (1-82) (p = 0.05 (Mann-Whitney-U test)). This association remains in stratified analyses in females (Fig. 5c), and in males, although it does not reach statistical significance in males (Fig. 5d).

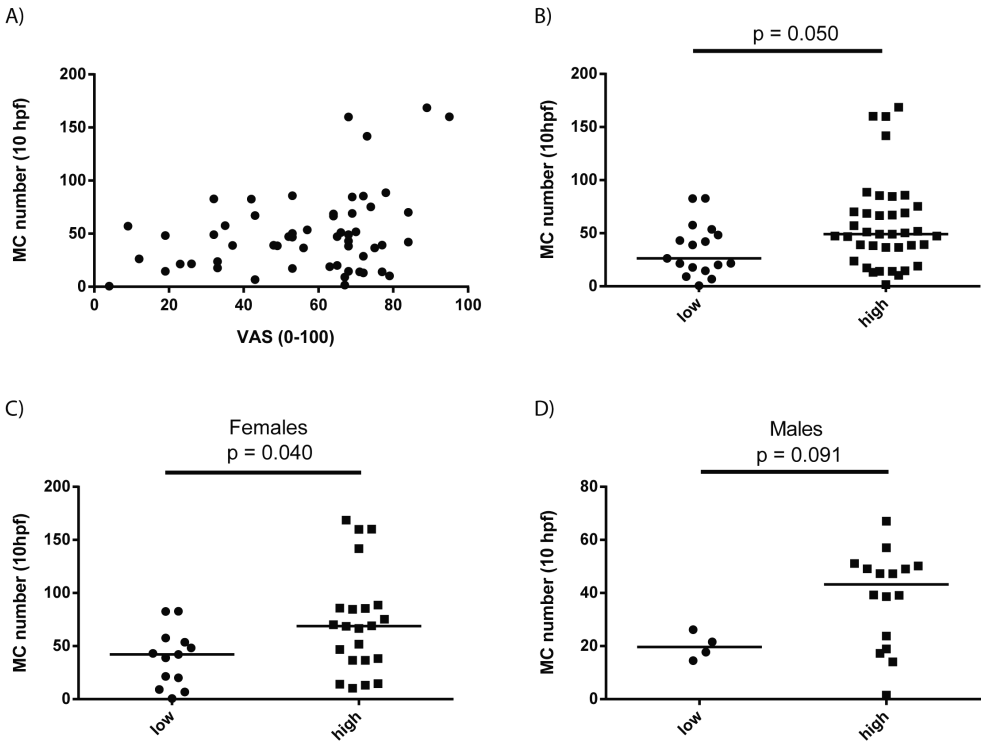

Figure 5: *A) The correlation between number of mast cells and visual analog scale (VAS) pain is depicted. Differences in mast cell numbers between patients with low Kellgren-Lawrence (KL) scores (KL1 and KL2) and high KL scores (KL3 and KL4) were evaluated in the total osteoarthritis (OA) population (B), as well as in females (C) and (D) males. P-values were determined by Mann-Whitney.*

DISCUSSION

In this study, we investigated the presence and degranulation state of mast cells in OA and RA samples at different disease stages. Our data indicate that there is an increase in mast cell number in OA compared to RA synovium, while there is no difference in mast cell number or degranulation state between end-stage disease (late) compared to mild disease (early) in either disease, confirming and expanding previous findings. Furthermore, we found that higher numbers of mast cells are associated with more synovitis and more structural damage in OA patients. To our knowledge, this is the first published report to find an association between mast cells synovial infiltration and clinical parameters in OA, suggesting a role of these cells in disease pathogenesis.

Mast cells were readily detected in OA synovial tissue in present study. By staining for tryptase and CD117, we detected a higher number of mast cells in the OA compared to RA synovium. Previous studies comparing OA and RA synovial mast cells reported controversial results. Although the underlying reason for this is unclear, most previous studies have used toluidine blue to detect mast cells and this staining is possibly less sensitive than the tryptase staining employed by us and others[32]. Moreover, in contrast to previous studies that also used tryptase staining, we have counterstained the tissue sections with CD117, allowing also detection of mast cells that have partially or totally degranulated. Using this technique, we could assess the number of degranulated mast cells in situ, while most previous studies have investigated degranulation in synovial fluid[33] or rather the capacity of synovial mast cells to degranulate upon ex vivo stimulation[13,14].

Our data indicate no differences in mast cell number or degranulation between “early” and “late” disease in either group of patients. Moreover, percentage of degranulated mast cells was around 20% in both diseases and independently of disease stage, which is in line with an earlier study[15]. This indicates that, independently of disease severity, a certain percentage of mast cells is degranulated in the synovium. Moreover, neither the number nor the percentage of degranulated mast cells was associated with inflammation, pain or radiographic damage (data not shown), while the number of mast cells was. Mast cells can be activated by several stimuli and degranulation is only one of the possible outcomes of mast cell activation. Our data suggest that other mechanisms than degranulation probably contribute to synovitis or radiographic damage in OA.

Both in OA and RA, mast cell numbers were correlated with the degree of synovitis. This indicates that mast cells could be recruited or stimulated to proliferate in the presence of synovitis or that a higher number of resident mast cells leads to recruitment of inflammatory cells into synovium. Indeed, previous studies have suggested that stem cell factor (SCF) secreted by synovial cells could lead to recruitment and hyperplasia of mast cells into synovium and this could be associated with histologic inflammation[23,34]. Whether this mechanism plays a role in our patient cohort, remains to be elucidated. Interestingly, OA patients have consistently higher numbers of mast cells than RA patients, independently of synovitis score and even in the absence of significant inflammation. Moreover, mast cells seem to contribute less to synovitis in OA than in RA. Taken together, these data suggest that the higher number of mast cells in OA than in RA is to a certain extent mediated by mechanisms unrelated to synovitis. This is not surprising, considering that the mechanisms involved in mast cells activation in OA and RA could be very different. For example, it is conceivable that in RA, autoantibodies could contribute to mast cells activation, while in OA other factors, such as matrix degradation products could be of importance. Indeed, low molecular weight hyaluronic acid, fibronectin or fibronectin fragments are potentially able to act as danger signals for cells expressing TLR2,-4 or CD44 and some of these receptors have been previously shown to be expressed on mast cells[35].

Our data suggest that mast cells are associated with the degree of radiographic damage in OA patients. Although the mechanisms involved in this association are unclear, it is likely that synovitis is not the main mechanisms mediating this association, as synovitis is not associated with radiographic damage in our population (data not shown) and is only weakly associated with mast cell numbers.

Mast cells could contribute to cartilage damage and/or osteophyte formation through various mechanisms. For instance, tryptase released by mast cells could contribute to cartilage damage through activation of protease-activated receptor 2 (PAR2), which has been identified in human OA and RA synovium[20] and cartilage[36] and has been shown to be critical for the induction of OA in a mouse model[37]. Similarly, mast cells have been previously associated with osteopenia, as a result of activation of osteoclasts, indicating that they can influence bone turnover (reviewed in[24]) Moreover, previous reports have suggested that increased numbers of mast cells could lead not only to increased osteoclast activation, but also to increased osteoblast numbers[38]. Possible mechanisms include release of tryptase and activation of PAR-2 on osteoblasts[39] and release of oncostatin M[40] and possibly other mechanisms with direct or indirect effects on osteoblasts.

The present study has some limitations. Firstly, only a relatively low number of patients were included in the present study, most of whom were females. This could explain the fact that most significant associations found were only detected in females. Furthermore, the present study is cross-sectional, precluding establishment of cause and consequence. Similar studies in a longitudinal manner could be very informative in assessing whether high mast cells numbers are a cause of inflammation/radiographic damage or a consequence of active disease in arthritis patients. Moreover, both RA and OA patients were taking medication at the time of the study. Although it is nowadays known that some of the anti-rheumatic drugs, such as corticosteroids and cyclosporine could affect mast cell function[41-44], the effect of most drugs taken by the patients in our cohort on mast cells is still unknown. Finally, the present study lacks comparison with normal controls, associated with difficulties of obtaining synovial tissues from healthy subjects age-matched with our geMstoan population.

Taken together, our data show for the first time an association between mast cell numbers and radiographic damage in OA patients, suggesting a possible role of mast cells in this disease.

Acknowledgments

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