Variability of RANKL and Osteoprotegerin Staining in Synovial Tissue from Patients with Active Rheumatoid Arthritis: Quantification Using Color Video Image Analysis

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ABSTRACT. ***Objective.*** To assess the interpatient, interbiopsy, and intrabiopsy variability of receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) immunostaining within synovial tissue from rheumatoid knee joints with active synovitis, using digital image analysis.

Methods. Synovial biopsy specimens were obtained from patients with rheumatoid arthritis (RA) and active synovitis. Immunohistologic analysis was performed on frozen synovial tissue biopsy specimens from 6 patients using a monoclonal antibody (Mab) to detect RANKL (626) or OPG (805 or 8051). Patients with a minimum of 4 synovial biopsies were included in the study. Sections were evaluated by computer assisted image analysis to assess between-patient, between-biopsy, and intra-biopsy variability of OPG and RANKL protein expression. The study was designed to deliberately maximize the variability.

Results. Computerized image analysis of staining with Mab to RANKL and OPG revealed variance for each antibody across the 3 components of the total variability.

Conclusion. Our study shows that variability in synovial immunostaining of RANKL and OPG protein is a significant and complex problem. We discuss methods to reduce this variability and suggest that the auspices of OMERACT may be employed to advance the study of synovium in collaborative international studies. (J Rheumatol 2003;30:2319–24)

Key Indexing Terms:
BONE RHEUMATOID ARTHRITIS OSTEOPROTEGERIN
RECEPTOR ACTIVATOR OF NUCLEAR FACTOR κB LIGAND
QUANTITATIVE ANALYSIS IMMUNOHISTOCHEMISTRY

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects synovial tissue, leading to cartilage damage and eventually localized bone erosion. The inflammation and tissue destruction in RA is thought to involve cell–cell interactions between lymphocytes, monocytes/macrophages, and type A and B synoviocytes. Understanding the pathobiology of the disease, being able to predict its severity and progression[1,2] and to monitor the effects of particular treatment regimes[3], is paramount for outcome.

The inflammatory infiltrate in the synovium of patients with RA contains monocytes/macrophages with the potential to become osteoclasts[4,5] and T cells that are involved in the soluble and membrane-bound production of receptor activator of nuclear factor κB ligand (RANKL)/TRANCE[6-9]. RANKL and its receptor, RANK, have been identified as key factors stimulating osteoclast formation and bone lysis[8-11]. RANKL binds directly to RANK on preosteoclasts and osteoclasts, enabling signal transduction for the differentiation of osteoclast progenitors as well as activation of mature osteoclasts[12,13]. Osteoprotegerin (OPG) is a soluble tumor necrosis factor (TNF) receptor-like molecule that is a naturally occurring inhibitor of RANKL[14]. OPG acts as a decoy molecule by binding to RANKL with high affinity and essentially blocking RANKL from interacting with RANK[12,14,15].

The concentrations of RANKL and OPG within the joint could affect the progression of bone damage within the

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rheumatoid joint[5]. Thus it is important to determine the distribution, the sites of origin, the level of production, and the targets of these mediators within the joint. Studies to date have not investigated the variation of RANKL and OPG (protein or mRNA expression) within a single joint, so conclusions drawn from *in situ* studies involving single biopsies may not be truly representative. Apart from the interpatient variability, there is histological variability between biopsies taken from the same joint (interbiopsy variability) and also variability within each single biopsy (intrabiopsy variability or experimental error). The purpose of measuring this variability is governed by the need to assess changes longitudinally in response to treatments that could affect expression of these markers. For significant changes to be measurable with treatment the interbiopsy and intrabiopsy variability of staining in a joint need to be low, so that a smaller number of biopsies become feasible and representative of the whole joint.

We investigated the interpatient and interbiopsy and intrabiopsy variability in concentrations of RANKL and OPG protein within these joints. We used digital image analysis (DIA) to assess concentrations of RANKL and OPG. Video image analysis is a sensitive method that allows determination of both the relative mean concentration of antigen [measured by the mean optical density (MOD)] and the total amount of antigen in a field [measured by integrated optical density (IOD)]. We used 2 different monoclonal antibodies (Mab) to detect OPG as we found they detected different forms of OPG[16]. Mab 805 detects dimeric OPG present on endothelial cells of the vasculature, while Mab 8051 detects monomeric and dimeric OPG on the outer synovial lining and vessels (to a lesser degree).

MATERIALS AND METHODS

Patients. Six RA patients with active synovitis in a knee, fulfilling the American College of Rheumatology criteria for RA[17], were included in the study. Details of patients and medication at time of surgery are summarized in Table 1. The study protocol was approved by the institutional Medical Ethics Committee and informed consent was obtained from each patient.

Synovial tissue. A small-bore arthroscopy (2.7 mm arthroscope; Dyonics, Andover, MA, USA) was performed under local anesthesia as described[18]. Biopsies of synovial tissue were obtained from all accessible regions of the knee joint, but mainly from the suprapatellar pouch. Synovium was identified macroscopically by the presence of blood vessels. Biopsies from macroscopically inflamed (identifiable villous formation and hyperemia) and noninflamed areas were chosen to maximize possible variation. Patients with a minimum of 4 distinctly separate biopsies were included in the study. Variability in cytokine and cell adhesion molecule expression in this patient group has been described[19]. The samples were separately snap-frozen in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, USA) and stored at –80°C until used. Five-micrometer sections were cut on a cryostat and mounted on APTS (Sigma, St. Louis, MO, USA) coated glass slides. The first section of each tissue was stained with hematoxylin and eosin for routine histopathological assessment. Sections with good morphology and synovial lining were selected for study. The glass slides were boxed and stored at –20°C until immunohistologic analysis.

Immunohistochemistry. Serial sections were stained with the following mouse Mab: anti-human OPG antibodies (Mab 805 and Mab 8051) and anti-human RANKL (Mab 626) (all from R&D Systems, Minneapolis, MN, USA), as described[16,20]. To eliminate variability in immunohistochemial staining, all staining was performed at one time with each antibody. Exclusion of the relevant primary antibody and use of an irrelevant primary antibody (X63) was performed to create negative controls for nonspecific binding. Anti-CD3 (Becton Dickinson, Mountain View, CA, USA) was used as a positive control.

Antibodies were detected according to a 3-step immunoperoxidase method[16,20-22]. Briefly, endogenous peroxidase activity was inhibited using 0.1% sodium azide and 1% hydrogen peroxide in Tris/phosphate buffered saline buffer followed by incubation with the primary Mab to RANKL (Mab 626) or OPG (Mab 805 or 8051) or anti-CD3. Synovial tissue sections were incubated with affinity purified, horseradish peroxidase (HRP) conjugated goat anti-mouse antibody (Dako, Glostrup, Denmark). A tertiary antibody, affinity purified HRP conjugated swine anti-goat Ig (Tago, Burlingame, CA, USA), was added to enhance detection. HRP activity was then detected using hydrogen peroxide as the substrate and amino ethylcarbazole (AEC; Sigma) as the dye. Slides were counterstained briefly with hematoxylin solution and mounted in Gurr Aquamount (BDH, Poole, UK).

Microscopic analysis. After immunohistochemical staining, sections stained with anti-human RANKL (Mab 626) and OPG (Mab 805 and Mab 8051) antibodies were analyzed in random order by a computer assisted color video image analysis system (Video Pro 32, Leading Edge P/L, Adelaide, South Australia) analyzing 4–7 high power fields (HPF) for each section as described[3,20-24]. Images were recorded using a video camera and digitized using a PV100 multimedia 16 bit color video digitizer card in an Intel 80486 DX computer. The resultant image was displayed on a SVGA monitor in a 640 × 480 pixel window with 24 bit resolution. The image windows used by the Video Pro 32 system are composed of up to 640 × 442 pixels that separate into 8 bit brightness and color values for each pixel, so that the system can define the color to be measured with great precision. Thresholds were set for each section to detect and measure identified reaction product. All sections stained with a single antibody were analyzed in the same session by one observer (MDS). The measurements included the mean optical density (MOD) field, which is a measure of the average cellular concentration of RANKL or OPG, and the IOD, which is calculated as the MOD × area of AEC staining and is proportional to the total amount of RANKL or OPG staining.

Statistical analysis. In this study, multiple (4–9) biopsies were taken from a knee joint on each patient and within each biopsy 4–7 HPF were analyzed. The questions to be answered were: (1) Is the variability between patients greater than the variability between biopsies in the same patient? (2) What is the estimate of variation within a patient? (3) What is the estimate of variation between patients? The 3 response measurements considered separately were 805, 8051, and RANKL.

The data were analyzed using a random-effects variance components analysis. A 2-level nested model was considered, with multiple biopsies performed on each patient in the study and multiple high power fields taken from each biopsy. Both patient and biopsy were considered as random factors as the instances of each could be considered as random samples from the population of all patients and biopsies that we wished to investigate. They therefore represent a source of variation in the response measurements rather than specific levels of interest, as when constructing a fixed factor model.

The focus of the inference is the partitioning of the total observed variation into 3 distinct sources: variability across different patients; variability across biopsies on a single patient; and variability across HPF within a biopsy. This last source of variation constitutes the experimental error.

The model for the response measurement Y_{ijk} is represented as[25] $Y_{ijk} = \mu + A_i + B_{j(i)} + E_{ijk}$, where A_i, $B_{j(i)}$, and E_{ijk} are random variables with zero means and variances σ_A^2, σ_B^2, and σ_E^2, respectively. A_i is the patient variation, $B_{j(i)}$ is the biopsy within-patient variation, and E_{ijk} is the residual or experimental error (HPF variation within biopsy).

The maximum likelihood technique[26] was chosen for the variance components analysis as this method is more suited to unbalanced data sets, that is, differing numbers of biopsies within patients and differing numbers of HPF within biopsies. The maximum likelihood technique gives an asymptotic variance-covariance matrix of the variance estimates, which was then used to establish 95% confidence intervals and for testing hypotheses ($\alpha = 0.05$) about the variance components.

RESULTS

Clinical and demographic features. Clinical and demographic data of the patients with active RA included in the study are presented in Table 1. The median age of the patients was 66 years; one of the 6 patients was a woman.

Representative examples of the histological fields are illustrated in Figure 1. Figure 1A shows that RANKL protein was detected on mononuclear cells within inflammatory infiltrates in the synovial membrane[20]. Two distinct patterns of staining of OPG in synovial tissue were seen[16]. As shown in Figure 1B, Mab 805 was exclusively associated with endothelial cells lining synovial blood vessels, while Mab 8051 detected OPG mainly in the lining layer of the synovial membrane (Figure 1C).

The factor level data is given in Table 2, which shows the number of HPF per biopsy for each patient. The coefficients of variation for IOD on one HPF for RANKL, Mab 8051, and Mab 805 were 2.59%, 4.9%, and 3.08%, respectively; for MOD, they were 2.15%, 2.99%, and 3.85%, respectively.

Table 3 gives estimates for the amount of variation attributed to variation across patients, variation across biopsies within a single patient, and the estimate of experimental error across multiple HPF within a single biopsy. The percentages in brackets indicate the percentage of variance for each component of the total variance for each antibody. The data in Table 3 indicate considerable variance for each component. The asymptotic covariance matrix was produced giving the standard errors (SE) of each estimate; 95% confidence intervals (CI) were then computed (Table 4). To test for significant variance differences, the 95% CI were compared for overlapping limits. Table 4 shows that with staining for OPG with Mab 8051 there were no significant variance differences for MOD and IOD. Similarly, there were no significant variance differences for RANKL protein. However, for OPG staining detected with Mab 805, while there were no differences in variance for IOD, there were significant differences for MOD, where experimental error was significantly smaller than the between-biopsy variation.

The estimate of patient variability for Mab 805 staining using MOD is shown in Table 3 as zero. Four different estimation techniques were used on this data and each one converged to an estimate of zero for this variable. Examining the data and iteration history of the procedures leads us to believe that this is because the true value of this patient variability is very small. No SE or confidence limits are calculated for this estimate (Table 4).

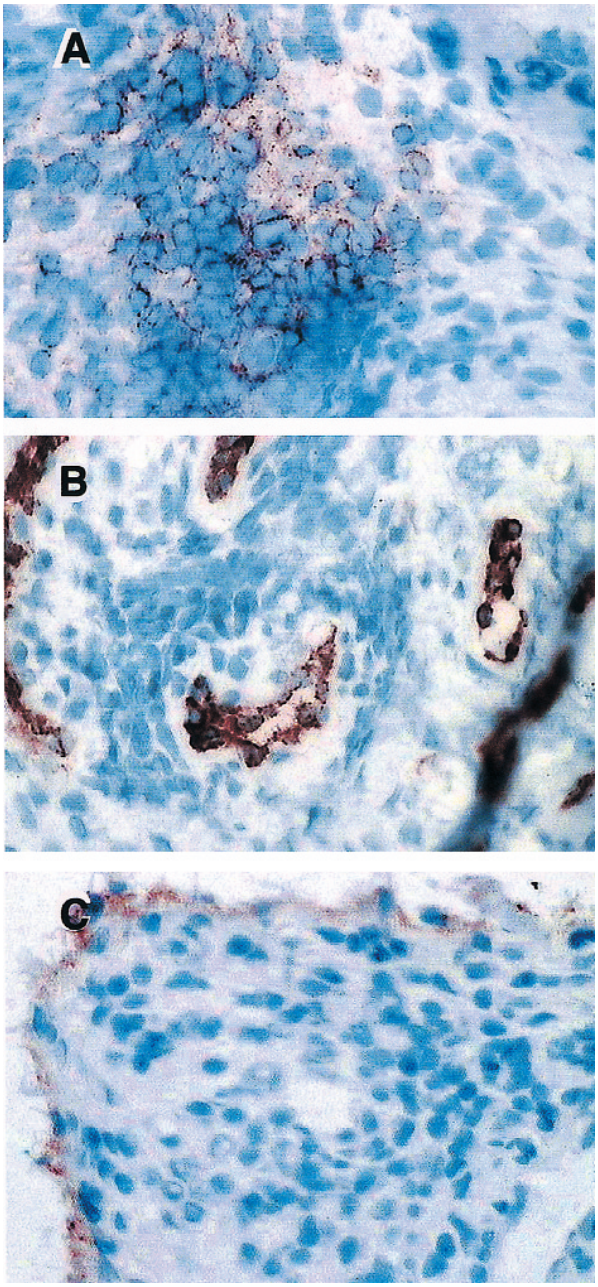

Figure 1. A. RANKL staining in the lymphoid aggregates in the synovial tissue from a patient with active RA using Mab 626. B. OPG staining on the endothelial cells of the vessels in synovial tissue from a patient with active RA using Mab 805. C. OPG staining on the lining in synovial tissue from a patient with active RA using Mab 8051. All antibodies were detected with immunoperoxidase staining with AEC (red) and counterstained with hematoxylin (blue). All magnifications are ×400.

DISCUSSION

Our aim was to compare interpatient variability with interbiopsy variability and with variability within each biopsy (intrabiopsy variability) for receptors and ligands that are potentially involved in the progression of bone damage within the rheumatoid joint. We selected patients with a

Table 1. Demographic and clinical details of patients with multiple site biopsies.

Patient	Age, yrs	Sex	No. of Biopsies	Disease Duration, mo	Medication at Time of Biopsy	CRP, mg/l	RF	Erosions
1	58	M	4	24	Azathioprine	79	+	–
2	59	M	5	120	IM gold/SSZ	110	+	+
3	67	F	5	72	IM gold/MTX	21	+	+
4	72	M	9	120	SSZ	47	+	+
5	64	M	4	324	IM gold/MTX	28	+	+
6	70	M	4	36	IM gold/MTX	10	+	+

SSZ: sulfasalazine, MTX: methotrexate, IM gold: intramuscular sodium aurothiomalate, CRP: C-reactive protein, RF: rheumatoid factor.

Table 2. Factor level information: number of high power fields/biopsy/patient.

Patient	No. of Biopsies	Number of High Power Fields/Biopsy		
		Mab 805	Mab 8051	Mab 626 (RANKL)
1	4	5,7,5,4	6,6,6,6	7,6,5,6
2	5	6,6,6,7,5	6,6,6,6,7	7,6,6,5
3	5	6,6,5,6,5	5,5,6,6	5,5,6,5,5
4	9	6,6,5,4,6,6,7,6,5	6,6,6,5,7,7,5,5,6	7,9,6,4,7,6,5,5,5
5	4	7,4,5,4	7,6,5,6	7,6,8,6
6	4	6,5,4,5	5,6,6,6	6,7,6,5

Table 3. Variance estimates.

	Mab 805		Mab 8051		RANKL	
	IOD	MOD	IOD	MOD	IOD	MOD
Var (pt)	14,343,624 (34.7)	0	2,347,321 (59.2)	9.825×10^{-3}	56,583,293 (54.0)	21.55×10^{-3}
Var (Biop (pt))	9,994,784 (24.2)	7.889×10^{-3}	531,451 (13.4)	7.686×10^{-3}	27,563,452 (26.3)	4.39×10^{-3}
Var (error)	17,008,170 (41.1)	2.827×10^{-3}	1,089,005 (27.4)	14.23×10^{-3}	20,637,669 (19.7)	6.898×10^{-3}

Var (pt) is the estimate of the amount of variation that can be attributed to the variation across patients. Var (Biop (pt)) is the variation across biopsies within a single patient. Var (error) is the estimate of the experimental error across the multiple HPF within a single biopsy. Data in brackets represent the percentage of the total variance by each component for each antibody. IOD: integrated optical density, MOD: mean optical density.

Table 4. The standard error (SE) of the variance estimate and 95% confidence intervals (CI).

Variance Component	IOD			MOD		
	Var (pt)	Var (Biop(pt))	Var (error)	Var (pt)	Var (Biop(pt))	Var (error)
Mab 805						
SE	9,893,745	3,737,780	2,039,893	NA	2.139×10^{-3}	3.391×10^{-4}
Lower CI	0	2,668,734	13,009,980	NA	3.696×10^{-3}	2.163×10^{-3}
Upper CI	33,735,364	17,320,834	21,006,360	NA	12.082×10^{-3}	3.192×10^{-3}
Mab 8051						
SE	1,446,018	208,185	127,017	6.956×10^{-3}	2.933×10^{-3}	1.660×10^{-3}
Lower CI	0	123,407	840,050	3.809×10^{-3}	1.937×10^{-3}	10.979×10^{-3}
Upper CI	5,181,518	939,495	1,337,960	23.460×10^{-3}	13.434×10^{-3}	17.486×10^{-3}
RANKL						
SE	36,602,751	8,991,338	2,390,951	131.432×10^{-4}	16.147×10^{-4}	9.991×10^{-4}
Lower CI	0	9,940,430	15,951,402	0	12.252×10^{-4}	53.316×10^{-4}
Upper CI	128,324,696	45,186,474	25,323,934	473.085×10^{-4}	75.547×10^{-4}	84.640×10^{-4}

NA: not applicable (see text). For abbreviations see Table 3.

range of disease duration and disease activity, all with a knee effusion. We also biopsied both macroscopically inflamed and noninflamed areas (an advantage of arthroscopic techniques over "blind" biopsy techniques) with the aim of maximizing interbiopsy variability.

Our study shows that the staining detected by computerized image analysis, using these Mab, reveals considerable variation across the 3 components of the total variability. In an ideal system, the intrabiopsy variability or experimental error will be minimal, less than the between-biopsy variability, which would be less than between-patient variability. In a clinical trial with synovial histological endpoints, the numbers of patients and biopsies in the trial are limited by patient acceptability and the power of the study. Clearly, in a longitudinal study, where changes in synovial histology are studied over time and comparisons made between different time points, the amount of variability is critical. The greater the error or variability, the more "noise" there is in the system and therefore the more difficult it is to detect the true difference between time points or groups being compared. Hence, it is important to be aware of these limitations and develop strategies to minimize this variability.

How could the study be improved to minimize variation? First, inflamed synovium can be identified by vascularity and villus formation, and by selecting inflamed synovium only, the interbiopsy variance could be reduced. The biopsy site contributes to histological variability within a joint; e.g., synovium from a region adjacent to cartilage is possibly more inflamed than synovium further from cartilage, such as the suprapatellar pouch[24,27]. Studies of homogenous patients (e.g., early RA) who have been biopsied under direct vision using arthroscopy have suggested greater uniformity in synovium harvested from different sites in the same joint[28,29].

As the sample size increases, the sampling distributions of the mean MOD and IOD approach a normal distribution, with the mean equal to the population mean and standard deviation (SD) of the population (SD/√n). The sample mean is an unbiased estimate of the population mean for any sample size, but the standard deviation of that estimate (i.e., the SE) decreases as the sample size increases. The consequence is that if the MOD or IOD are expressed as means, then the greater the number of HPF that contribute to that mean, the more "accurate" (i.e., the lower the standard error) is the sample estimate of the mean. Thus, the intrabiopsy error can be reduced by measuring all the relevant HPF within the defined area of the biopsy. Kraan, *et al*[29], measuring cytokine and adhesion molecule expression in the synovial membrane, attempted to address the problem of how many HPF to measure. They found a limited analysis of one region with 6 HPF allowed adequate discrimination (between synovial tissue from active joints versus remission) compared with an extended analysis of 3 regions with a total of 60 HPF. However, this discrimination was obtained between the 2 extremes of active disease and complete clinical remission. Complete clinical remission is a rare endpoint in clinical trials of treatments in RA, so that discrimination was obtained with optimized conditions. Similarly Bresnihan, *et al*[28], using a quantitative scoring system, found that a limited number of HPF (up to 17 for T cell infiltration) were sufficient to reliably measure the amount of lymphocytic infiltration in RA synovial tissue and that similar results were seen in early synovitis patients (arthroscopic biopsies) compared to those seen in late disease (arthroplasty biopsies). Their study did not address the intrabiopsy variability, and they concluded that their observations may not apply to the measurement of more sparsely distributed staining (such as that seen with RANKL in our study).

It is not valid to compare variance between antibodies because of different targets for the Mab and different affinities for the primary and secondary antibodies. Previous synovial biopsy studies have shown problems with interbiopsy variation due to variability in lymphocyte infiltration[19,24]. Youssef, *et al*[19] observed greater homogeneity in cell adhesion molecule staining than cytokine staining, and suggested that this occurred because cell adhesion molecules are mainly found on vascular endothelium. As Mab 805 detects OPG on vascular endothelium compared to RANKL, which is found mainly on T lymphocytes, this finding could explain why our results with Mab 805 MOD showed less intrabiopsy variation than interbiopsy variation.

Nevertheless, we have demonstrated variance for each of the antibodies where that variance consists of 3 contributing components. Our experimental design maximized that variance and we have discussed methods that will reduce it. The statistical analysis of variance is complex, as there are 3 levels/dimensions contributing to this variance. Previous studies[19,28,30] have not considered this complexity. Unfortunately, because of the complexity and the design of the study, where variance was deliberately maximized, power calculations to determine how many patients/biopsies are required for studies were not possible. However, our results suggest that most of the benefit obtained by carrying out additional studies could also be obtained by increasing the number of HPF per biopsy. More statistical power at this level of the analysis may allow for statistical separation of the inter- and the intrabiopsy variability, permitting further comparisons across staining techniques. This approach also has the advantage of being under the control of the researcher and is not dependent on the number of subjects available for a further study.

Synovial tissue is now being evaluated in several research centers as an endpoint/outcome measure in longitudinal therapeutic trials, particularly with biological agents[3,22,31]. While synovial tissue obtained by arthroscopy remains a research tool, it offers the potential of identifying important targets and providing insight into the mechanisms

of action of antirheumatic treatments. Standardization of techniques, quality control with exchange of biopsy material/staining methods, reference materials, and other techniques need to be developed to allow international collaboration. The expertise of OMERACT (Outcome Measures in Rheumatoid Arthritis Clinical Trials) should be made available to facilitate these endeavors so that variability in synovial membrane immunohistochemstry within and between research centers is minimized.

Our findings show Mab 805 can be used in longitudinal studies for RA synovium, but using our technology and methods, further refinements in the methods are required before the detection of OPG on the lining (with Mab 8051) and detection of RANKL in lymphoid aggregates (with Mab 626) can be performed.

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