Monocyte scintigraphy in rheumatoid arthritis: the dynamics of monocyte migration in immune-mediated inflammatory disease

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Monocyte Scintigraphy in Rheumatoid Arthritis: The Dynamics of Monocyte Migration in Immune-Mediated Inflammatory Disease

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Abstract

Background: Macrophages are principal drivers of synovial inflammation in rheumatoid arthritis (RA), a prototype immune-mediated inflammatory disease. Conceivably, synovial macrophages are continuously replaced by circulating monocytes in RA. Animal studies from the 1960s suggested that macrophage replacement by monocytes is a slow process in chronic inflammatory lesions. Translation of these data into the human condition has been hampered by the lack of available techniques to analyze monocyte migration in man.

Methods/Principal Findings: We developed a technique that enabled us to analyze the migration of labelled autologous monocytes in RA patients using single photon emission computer tomography (SPECT). We isolated CD14+ monocytes by ClinIMACS in 8 patients and labeled these with technetium-99m (99mTc-HMPAO). Monocytes were re-infused into the same patient. Using SPECT we calculated that a very small but specific fraction of 3.4 × 10^-3 (0.95 – 5.1 × 10^-3)% of re-infused monocytes migrated to the inflamed joints, being detectable within one hour after re-infusion.

Conclusions/Significance: The results indicate monocytes migrate continuously into the inflamed synovial tissue of RA patients, but at a slow macrophage-replacement rate. This suggests that the rapid decrease in synovial macrophages that occurs after antirheumatic treatment might rather be explained by an alteration in macrophage retention than in monocyte influx and that RA might be particularly sensitive to treatments targeting inflammatory cell retention.

Introduction

Macrophages in the inflamed synovial tissue of rheumatoid arthritis (RA) patients play a central role in the sustenance of synovial inflammation and promotion of tissue destruction [1–3]. Conceivably they are continuously replaced by circulating monocytes [4]. The dynamics of this replacement is a matter of controversy. Data on the effects of anti-rheumatic treatments on monocytes in RA patients, but at a slow macrophage-replacement rate. This suggests that the rapid decrease in synovial macrophages that occurs after antirheumatic treatment might rather be explained by an alteration in macrophage retention than in monocyte influx and that RA might be particularly sensitive to treatments targeting inflammatory cell retention.

Newly developed imaging techniques, such as Single Photon Emission Computed Tomography (SPECT), Positron Emission Tomography (PET) and more recently bioluminescence and fluorescence reflectance imaging, offer the possibility to portray the in vivo dynamics of cell migration in patients [16]. The application of these imaging modalities to analyze the behavior of monocytes is hampered by the relative scarcity of these cells in the peripheral blood and the technical difficulties of specific cell isolation at the GMP level and efficient labeling to result in an adequate detection signal. These problems might be addressed by the combination of scintigraphic imaging with sophisticated cell isolation procedures, such as immunomagnetic cell selection [17].

We recently developed a procedure using a combination of immunomagnetic cell selection with CD14 coated beads and an improved labeling procedure with technetium-99m (99mTc)-hexamethylpropylene-amine-oxide (HMPAO) and SPECT to visualize the migratory behavior of autologous monocytes [18,19].
We applied this method in patients with active RA to test the hypothesis that synovial inflammation is maintained by a continuous influx of monocytes into the synovial compartment and to analyze the dynamics of such influx.

Results

Eight RA patients (4 male and 4 female) were included into the study. The median age of the patients was 52 years (range 39 to 59 years) and the mean disease duration was 19 (range 10–38) years. Erosions were present in all patients. Two patients had nodular disease. Four patients were seropositive for IgM rheumatoid factor. The mean (±SD) disease activity score evaluated in 28 joints (DAS28) at screening was 5.8 ± 0.8. All patients were treated with stable dosages of methotrexate.

Applying immunomagnetic cell selection with CD14 labeled beads, on average 19.9 ± 10^6 (10.4–36.9 ± 10^6) monocytes were isolated, with a mean recovery of 40.8% (24–69%) CD14 positive cells. This resulted in a cell suspension with a purity of 90.4% (79–96%) CD14 positive cells as determined by FACS analysis.

Labeling with 99mTc-HMPAO resulted in a mean radioactivity of 211 (43–393) MBq. Having shown that CD62L expression on monocytes did not change after the bead isolation procedure and that 99mTc-HMPAO labeling did not affect the monocyte migratory capacity in vitro (unpublished observations), we decided to re-infuse labeled monocytes in RA patients. Re-infusion was well tolerated in all patients. No signs of increased complement activation could be demonstrated one hour after re-infusion of radioactively labeled monocytes: C3b/c (mean ±SD): 26.4 ± 13.5 and C4b/c 8.3 ± 1.5 before treatment versus 26.0 ± 12.3 and 16.2 ± 10.0 1 hour after re-infusion, respectively.

Migration of labeled monocytes was visualized using scintigraphy. The majority of monocytes was initially trapped in the lungs, followed by redistribution in liver, spleen and bone marrow (Figure 1), following the pattern of labeled leukocytes [19]. As expected, renal activity with visualization of the urinary bladder was seen in all patients. Furthermore, physiological bowel uptake could be detected from one hour post infusion. Significant uptake of radioactivity in stomach and/or thyroid was not observed. In 2 patients whole-body imaging was feasible up to 20 hours post infusion.

Small but distinct uptake was found in the joints of all patients, with a mean of 9 (range 1–25) positive joints (Figures 1 and 2).

There was an increased signal in di-articular joints in all patients at all time points, with a maximal signal at one hour post re-infusion (Figure 3). We calculated that a median of 4027 (interquartile range [IQR] 2094–8370) labeled monocytes migrated into the biopsied joints that were analyzed in more detail, representing 3.4 ± 10^-2 (0.95–5.1 ± 10^-2) % of re-infused monocytes. The results were confirmed after 2 weeks, when the scans were repeated: there was no change in clinical parameters, the number of joints with increased signal on the scintigraphic images, and joint signal intensity. Using these numbers, an estimate was made about the extent of monocyte influx into the biopsied joints. The median monocyte concentration in peripheral blood was 1.9 ± 10^9 (1.6–2.3 ± 10^9) at the time of blood withdrawal. Assuming an average blood volume of 5 liters, a median of 323,000 (272,000–1,150,000) monocytes entered the biopsied joints (3.4 ± 10^-3 % of total circulating monocytes).

Figure 1. Scintigraphic images of labeled autologous monocytes in a patient with rheumatoid arthritis. Anterior and posterior (A,B) whole body images of a patient 2 hours after infusion of monocytes labeled with 283 MBq 99mTc-HMPAO. Transient pulmonary accumulation occurs, with an equivalently increased uptake in liver, spleen and bone marrow (A,B). A reference source is placed just below the knees. Panel C and D show the same images but with masking of the pulmonary, bladder and source signal. Increased articular uptake is observed in di-articular joints as the shoulders, elbows, knees and small hand joints.

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Since macrophages are the dominant synovial inflammatory cell population and the extent of their tissue infiltration correlates with disease activity [1–3,20], we compared scintigraphic signal intensity with synovial macrophage infiltration and disease activity parameters. Synovial macrophage infiltration was assessed by immunohistochemical staining of arthroscopic biopsies from selected joints. These biopsy samples had been obtained one day after the second scintigraphy. Six out of 8 synovial biopsies passed synovial tissue quality control. The number of CD163 positive macrophages in the synovium correlated significantly with the number of scintigraphy positive joints one, two and three hours after re-infusion (r = 0.89, P = 0.019; r = 0.89, P = 0.019; r = 0.94, P < 0.01, respectively). Furthermore, there was a significant correlation between the number of CD163 positive macrophages and the percentage of monocytes shown by scintigraphy two hours after re-infusion (r = 0.89, P = 0.019). The other immunohistologic markers were not significantly correlated with scintigraphic data (data not shown).

Subsequently, the relationship between scintigraphic signal and disease activity parameters was analyzed. Of interest, the swollen joint count correlated significantly with the number of positive joints shown by scintigraphic scans at day 14 (r = 0.78 and r = 0.90; P < 0.01). There was also a positive correlation between the number of swollen joints and the number of positive joints shown by scintigraphic scans at day 1 (r = 0.97 and r = 0.73, respectively; P < 0.01, day 14: r = 0.78 and r = 0.90; P < 0.01). There was also a positive correlation between the number of swollen joints and the number of positive joints shown by scintigraphic scans at day 14 (r = 0.81, r = 0.76 and r = 0.81; all P < 0.01, at respectively 1, 2 and 3 hours after infusion).

Discussion

In the present study we used a recently developed procedure, that visualizes the migratory behavior of monocytes [18,19], to test the hypothesis that synovial inflammation in RA is maintained by influx of monocytes into the synovial compartment. The results suggest that while there is indeed a continuous influx of circulating monocytes into the synovial compartment, their numbers are small, indicating that only a relatively small fraction of synovial macrophages is replaced per day.

We found that a median of $3.4 \times 10^{-3}$ (0.95–5.1 $\times 10^{-3}$) % of labelled monocytes entered the synovial compartment after re-
such numbers considerably, these data indicate that in RA patients
the number of synovial macrophages in large inflamed joints exceeds
the synovial compartiment, being a median of 323,000 cells. Since the
similar percentages of unlabeled circulating monocytes enter the
joint sublining, where they play a pivotal role in inflammatory cell
retention and survival [23].

It was recently shown that a range of anti-rheumatic treatments
induces a significant decrease in synovial macrophages associated
with clinical improvement within hours to weeks after initiation of
therapy [5,6]. It was hypothesized that these treatments ultimately
affect migration of monocytes and/or retention of macrophages
[21]. In studies on infliximab, an effective biological treatment for
RA which blocks TNFα [22], a marked decrease in synovial
macrophage numbers occurred already 24 hours after initiation of
treatment, which could not be explained by induction of apoptosis
[8–11]. The results of the current study suggest that this effect may
mostly be explained by an effect of TNFα blockade on macrophage
retention. Of interest, a decrease in VCAM-1 and ICAM was found
shortly after infliximab treatment [8,10]. While these are important molecules involved in adhesion of monocytes to the vascular endothelium, they are in RA patients also abundantly expressed by cells in the intimal lining layer and the synovial
sublining, where they play a pivotal role in inflammatory cell
retention and survival [23].

Thus, the data indicate that in line with animal models on
chronic inflammatory lesions, macrophage renewal by monocytes
is slow in chronic synovitis [12–15]. Together with the previous
studies showing a rapid decrease in cellularity after successful
antirheumatic therapy (even in the absence of apoptosis), these
data support the notion that therapeutic strategies aimed at
interfering with retention of inflammatory cells at the site of
inflammation might be capable of inducing clinical improvement
in immune-mediated inflammatory disease. Accordingly, recent
studies have shown benefit of interfering with adhesion molecules:
anti-function associated antigen (LFA)-1 antibody (efalizumab)
treatment in psoriasis and anti-CD4β1-integrin (VLA-4) antibody
(natalizumab) treatment in multiple sclerosis [24,25].

In conclusion, we developed monocyte scintigraphy, which allowed
us to demonstrate the dynamic influx of monocytes into the synovial
compartment of RA patients. This approach provides insight into the
pathogenesis of this immune-mediated inflammatory disease and
supports the notion that blocking only the influx of inflammatory cells
may be insufficient to induce clinical improvement.

Materials and Methods

Patients

Patients fulfilling the American College of Rheumatology (ACR)
1987 revised classification criteria for RA [26] were included into
the study. All patients had active RA, as defined by a disease
activity score evaluated in 28 joints (DAS28) >3.2 [27]. Patients
were on stable disease-modifying antirheumatic drug (DMARD)
treatment at inclusion.

Ethics Statement

Approval was granted by the Medical Ethics Committee of the
Academic Medical Center/University of Amsterdam (AMC). Each patient gave written informed consent prior to participation.

Isolation of Monocytes

Hundred milliliters of peripheral blood was taken from each
patient. CD14+ monocytes were isolated using a positive selection
procedure with magnetic-activated cell sorting according to the
manufacturer’s protocol (MACS®Miltenyi Biotec, Bergisch Gladbach,
Germany). After selection, the percentage of CD14+, CD3, and CD66 positive cells was determined by fluorescence-activated
cell sorting (FACS) analysis. The CD14+ enriched cells were
resuspended in 10 ml buffer containing 0.9% (w/v) NaCl, 20% (w/v)
human serum albumin (Sanquin Blood Supply Foundation
division of Plasma Products, Amsterdam, the Netherlands) and
3.8% (w/v) TNC (NVI, Bilthoven, the Netherlands) for labeling.

Radiopharmaceuticals

Exemetazime (Ceretec™, RVG16226) was supplied as a ready-
for-labeling kit (GE Healthcare B.V., Amersham, Cygne Centre,
Eindhoven, the Netherlands). 99mTc-pertechnetate was obtained
from a 99Mo-carrying Ultratechnekow® FM generator (DRN
4329, Tyco Healthcare, Mallinckrodt Medical, Petten, the
Netherlands) and was eluted in accordance with the instructions
of the manufacturer. Radiochemical purity control (RPC) essays
were done by means of chromatography on ITLC-SG strips, using
a mobile phase of 0.9% sodium chloride (NaCl) [28]. Radiolabel-
ing of cells was performed as described earlier [18]. Briefly, the
cells were centrifuged and freshly prepared 99mTc-HMPAO of
very high specific activity in a low volume was added to the
monocyte cell pellet. After incubation the excess of 99mTc-
HMPAO was diluted and subsequently removed from the cell
pellet after centrifugation. The labeled monocytes were re-
suspended in 0.9% NaCl and re-infused into the same patient.

Scintigraphy

An average of 20×10⁶ monocytes labeled with 200 MBq 99mTc-
HMPAO was injected intravenously within 15 minutes after
radiolabeling. Whole body imaging was performed at 15 minutes
and 1, 2, 3, and 20 hours post infusion using a dual head
gammacamera (140 keV, window 15%, 256×1024 matrix, 10 cm/min)
fitting with low energy all purpose collimators (Siemens Ecam).
Detail images of the hands (palmar) and feet (plantar) were acquired in a 256x256 matrix for 5 minutes. This procedure was repeated twice weeks after the baseline scintigraphy.
The incubation of antibody and fixation with FACSTMlysis solution (BD Biosciences, CA) was performed. Purified monocytes were resuspended in PBS (1% bovine serum albumin (Sigma-Aldrich, MO). Red cells were lysed by washing cells twice with BD FACSTMlysis solution (BD Biosciences, Minneapolis, MN). Cell migration rate was quantified by flow cytometry for non-labeled monocytes and scintillation gamma counter for labeled monocytes. Comparison of migrating cell percentage for non-labeled and Tc- HMPAO labeled monocytes did not demonstrate an impairment of monocyte migratory capacity in the in vitro chemotaxis assay (data not shown).

Monocyte activation was tested by assessing CD62L expression by flow cytometry on monocytes in unmanipulated whole blood and after the bead isolation procedure using CD62L-PE conjugated antibody (BD Biosciences). Antibody concentrations were used according to the manufacturer’s protocol. Antibody staining was performed using 50 µl of whole blood. Blood cells were incubated with antibodies for 15 min and washed twice with PBS containing 1% bovine serum albumin (Sigma-Aldrich, MO). Red cells were lysed by washing cells twice with BD FACSTMlysis solution (BD Biosciences, CA). Purified monocytes were resuspended in PBS containing 1% bovine serum albumin (Sigma-Aldrich, MO) and incubated with antibody and fixed with FACSTMlysis solution (BD Biosciences, CA). Samples were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, NJ). Frequency of CD62L expression on monocytes did not change after the bead isolation procedure (data not shown).

In Vivo Assessment of Complement Activation after Re-Infusion

To exclude the possibility that re-infusion of labeled monocytes induced complement activation, complement activation products were measured in the serum before and one hour after re-infusion. Activation of C3 (C3b/c) and C4 (C4b/c) was assessed with an ELISA as described before in detail [29]. In brief, monoclonal antibodies recognizing neo-epitopes on activated C3 and C4 were used as capturing antibodies. Biotinylated polyclonal rabbit anti-human C3 and polyclonal sheep anti-human C4 antibodies were used as detecting antibodies.

Arthroscopy and Synovial Biopsy

The day after the second set of scans, all patients underwent a mini-arthroscopy under local anesthesia from an actively inflamed knee, ankle or wrist, to obtain synovial tissue samples [30]. Biopsies were taken with a 2.3-mm grasping forceps (Storz, Tuttinglen, Germany) from 6 or more sites within the joint to minimize sampling error. The tissue samples were snap frozen en bloc in Tissue Tek OCT (Miles, Elkhart, IN) after collection. Frozen blocks were stored in liquid nitrogen until sectioning. Sections of 5 µm were cut using a cryostat and mounted on Star Frost adhesive glass slides (Knittelgla¨ser, Braunschweig, Germany). Sealed slides were stored at −80°C until immunohistochemical staining was performed.

Immunohistochemical Analysis

Synovial tissue sections were stained using the following monoclonal antibodies to analyze the cell infiltrate: anti-C3b55 (67:Serotec, Oxford, UK) to detect fibroblast-like synoviocytes (FLS), anti-C668 (EBM11: DAKO, Glostrup, Denmark) to detect macrophages and anti-CD3 (SK7, Becton Dickinson, San Jose, CA) for T-cells as described previously [31]. The scavenger receptor CD163 (Ber-MAC3; DAKO) was stained to detect alternatively activated tissue macrophages. Staining of cellular markers was performed using a three-step immunoperoxidase method [32]. For control sections the primary antibody was omitted or irrelevant immunoglobulins were applied. Tissue quality was assessed by analyzing the presence of an intimal lining layer.

Digital Image Analysis

All sections were analyzed at random by trained technicians who were blinded for clinical and scintigraphic data. The analysis was done by computer-assisted image analysis as previously described in detail [32]. In short, images were acquired and analyzed using a Syndia algorithm on a Qwin-based analysis system (Leica, Cambridge, UK). For all markers 18 high-power fields were analyzed. Positive staining of cellular markers was expressed as positive cells per mm² (counts/mm²). CD66b+ macrophages were analyzed separately for the intimal lining layer and the synovial sublining.

Statistics

Associations between joint signal parameters and swollen joint count, tender joint count, DAS28, ESR, CRP and immunohistochemical markers were expressed by Spearman’s correlation coefficients. The changes in joint signal intensity and clinical parameters after two weeks were tested with the Wilcoxon signed rank test for paired non-parametric data.

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Author Contributions

Conceived and designed the experiments: RMT CAW RJB SED CV DW ESI PPT. Performed the experiments: RMT CAW RJB SED DW ESI PPT. Contributed reagents/materials/analysis tools: RJB SED CV DW ESI BLFvES PPT. Wrote the paper: RMT CAW RJB SED CV DW ESI DMG BLFvES PPT.

References


