Noninvasive In Vivo Imaging of Monocyte Trafficking to Atherosclerotic Lesions

Moritz F. Kircher, MD; Jan Grimm, MD, PhD; Filip K. Swirski, PhD; Peter Libby, MD; Robert E. Gerszten, MD; Jennifer R. Allport, PhD; Ralph Weissleder, MD, PhD

Background—Monocytes play a key role in atherogenesis, but their participation has been discerned largely via ex vivo analyses of atherosclerotic lesions. We sought to establish a noninvasive technique to determine monocyte trafficking to atherosclerotic lesions in live animals.

Methods and Results—Using a micro–single-photon emission computed tomography small-animal imaging system and a Food and Drug Administration–approved radiotracer ([indium 111] oxyquinoline, 111In-oxine), we demonstrate here that monocyte recruitment to atherosclerotic lesions can be visualized in a noninvasive, dynamic, and 3-dimensional fashion in live animals. We show in vivo that monocytes are recruited avidly to plaques within days of adoptive transfer. Using micro–single-photon emission computed tomography imaging as a screening tool, we were able to investigate modulatory effects on monocyte recruitment in live animals. We found that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors rapidly and substantially reduce monocyte recruitment to existing atherosclerotic lesions, as imaged here in vivo.

Conclusions—This novel approach to track monocytes to atherosclerotic plaques in vivo should have broad applications and create new insights into the pathogenesis of atherosclerosis and other inflammatory diseases. (Circulation. 2008; 117:388-395.)

Key Words: imaging ■ atherosclerosis ■ plaque ■ cells

In the past 2 decades, the concept of atherogenesis has changed dramatically from the notion of a lipid storage disease to the recognition that inflammation drives this process.1–5 Substantial evidence identifies monocytes as key cellular participants in the initiation and development of atherosclerotic plaques.5 Adhesion of monocytes to the arterial endothelium and their migration into the intima occur early in atherogenesis.6 Monocyte accumulation characterizes fatty streaks, the earliest grossly detectable lesion of human and experimental atherosclerosis.2 Monocytes differentiate into macrophages7 and secrete inflammatory cytokines that stimulate smooth muscle cell proliferation and migration8 and produce proteolytic enzymes that can degrade collagen and render the growing plaque’s cap thin and susceptible to rupture.1,9

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This key role of monocytes in all phases of atherogenesis highlights the need to understand better the dynamics of monocyte recruitment to developing plaques and to establish clinically viable imaging tools to visualize this process in high-risk patient groups or in the development and testing of novel therapies. To date, the data describing monocyte recruitment to atherosclerotic plaques have derived mainly from excised tissue specimens (eg, in experimental animals or endarterectomy or autopsy samples) that have undergone analysis either histologically,10 by polymerase chain reaction methods,11,12 or by flow cytometry.7 Thus, the analysis of monocyte trafficking to lesions has often been restricted to a static view that does not reflect the dynamics of this process. The ability to assess recruitment of monocytes in vivo would...
be of benefit in advancing our understanding of atherogenesis, evaluating antiinflammatory drugs designed to inhibit monocyte recruitment, and stratifying patients at risk for developing complications of atherosclerosis.

Here, we present an approach that enables noninvasive, dynamic, quantitative and high-resolution in vivo imaging of monocyte trafficking to atherosclerotic lesions. We demonstrate the feasibility of detection of the recruitment of monocytes labeled with [indium 111] oxinoline (\(^{111}\)In-oxine) to existing atherosclerotic lesions of apolipoprotein E–deficient (Apoe\(^{-/-}\)) mice by micro–single-photon emission computed tomography (micro-SPECT/CT). The long half-life of \(^{111}\)In (2.8 days) enabled the detection of monocytes for up to 7 days after adoptive transfer, and the high-resolution anatomic data derived from CT allowed localization of hotspots of monocyte infiltration in a submillimeter range. Furthermore, we demonstrate that micro-SPECT/CT imaging allows for assessment of the effect of an intervention (statin drugs) on monocyte recruitment to plaques in vivo. This technique may open new avenues to investigate the mechanisms of atherogenesis and other inflammatory diseases noninvasively and to evaluate new antiinflammatory therapies effectively. Importantly, with both human SPECT/CT scanners and the Food and Drug Administration–approved \(^{111}\)In-oxine in clinical use, the results of the present study support the potential to conduct similar studies in humans in the future.

**Methods**

**Materials**

RPMI 1640, HBSS, and FBS were purchased from Cellgro (Hemdon, Va). PBS was obtained from BioWhittaker (Walkersville, Md). RPMI 1640, HBSS, and FBS were purchased from Cellgro (Hemdon, Md). PBS was obtained from BioWhittaker (Walkersville, Md). \(^{111}\)In-oxine was purchased from Amersham-GE (Piscataway, NJ). PBS was obtained from BioWhittaker (Walkersville, Md). Dexamethasone (Sigma, St Louis, Mo) negative immunomagnetic depletion to isolate murine monocytes from the peripheral blood, as described by Swirski et al, with minor modifications. In brief, peripheral blood (1 to 1.3 mL) was drawn from C57BL/6 mice (typically \(n=15\) mice/isolation) via cardiac puncture, diluted 5:1 with HBSS, and subjected to density centrifugation (30 minutes, 1600 rpm, room temperature) over Histopaque-1083 (Sigma). Theuffy coat containing peripheral blood mononuclear cells (PBMCs) was aspirated, washed 3 times in HBSS, and subsequently washed once in MACS buffer (PBS containing 0.1% [wt/vol] BSA and 0.5 mmol/L EDTA). T cells, B cells, and natural killer cells were depleted from the PBMC population with a cocktail of immunomagnetic beads (anti-CD90, anti-CD45R, and anti-CD49b beads; Miltenyi Biotec, Auburn, Calif). Monocyte purity was determined by morphological analysis of cells prepared by cytospin and Wright-Giemsa staining, as well as independently via fluorescence-activated cell sorter analysis.

**Antibodies**

The primary polyclonal anti-GFP (green fluorescent protein) antibody was purchased from Chemicon International (Temecula, Calif; catalog No. AB3080).

**Animals**

Apoe\(^{-/-}\) mice (B6.129P2-Apopem1tm1Kircher1), enhanced GFP (EGFP)-expressing mice [C57BL/6-Tg(Actb-EGFP)1Osb/J], and C57BL/6 mice (6 to 8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, Me). The Apoe\(^{-/-}\) animals had been backcrossed to the C57BL/6 background for at least 10 generations. Mice were cared for according to the institution’s animal facility guidelines. At 4 weeks of age, Apoe\(^{-/-}\) animals were placed on a Western diet (42% adjusted caloric diet, 0.2% cholesterol, Harlan Teklad, Madison, Wis) and fed until used for experiments. The remaining animals remained on a regular chow diet. All protocols were approved by the Animal Review Committee.

**Statin Treatment**

Apoe\(^{-/-}\) animals in the treatment group received simvastatin (oral, 0.57 mg · kg\(^{-1}\) · d\(^{-1}\), Zocor, Merck, Whitehouse Station, NJ) and atorvastatin (oral, 0.57 mg · kg\(^{-1}\) · d\(^{-1}\); Lipitor, Pfizer, New York, NY) dissolved in 100 mL of PBS via intragastric instillation for 3 days by mouth (0.57 mg/kg corresponds to a 40-mg dose used clinically in humans [70 kg body weight]; recommended atorvastatin dose in humans is 10 to 80 mg/d). Mevastatin (Sigma, St Louis, Mo) was administered subcutaneously (20 mg · kg\(^{-1}\) · d\(^{-1}\); according to Budzyn et al13) dissolved in 20 mL of PBS. Control animals received PBS only via intragastric or subcutaneous instillation, respectively.

**Measurement of Cholesterol Levels**

Total blood cholesterol levels of atorvastatin-treated mice and nontreated control mice were measured with a Raichem (San Diego, Calif) cholesterol reagent kit according to the protocol of the manufacturer.

**Monocyte Isolation**

We used a 2-step combination of density gradient separation and negative immunomagnetic depletion to isolate murine monocytes from the peripheral blood, as described by Swirski et al, with minor modifications. In brief, peripheral blood (1 to 1.3 mL) was drawn from C57BL/6 mice (typically \(n=15\) mice/isolation) via cardiac puncture, diluted 5:1 with HBSS, and subjected to density centrifugation (30 minutes, 1600 rpm, room temperature) over Histopaque-1083 (Sigma). Theuffy coat containing peripheral blood mononuclear cells (PBMCs) was aspirated, washed 3 times in HBSS, and subsequently washed once in MACS buffer (PBS containing 0.1% [wt/vol] BSA and 0.5 mmol/L EDTA). T cells, B cells, and natural killer cells were depleted from the PBMC population with a cocktail of immunomagnetic beads (anti-CD90, anti-CD45R, and anti-CD49b beads; Miltenyi Biotec, Auburn, Calif). Monocyte purity was determined by morphological analysis of cells prepared by cytospin and Wright-Giemsa staining, as well as independently via fluorescence-activated cell sorter analysis.

**Monocyte Labeling, Viability, and Function**

**Labeling**

Purified monocytes were labeled with \(^{111}\)In-oxine according to the manufacturer’s protocol (Amersham Health Medi-Physics, Arlington Heights, Ill). Briefly, cells were washed with HBSS, spun, and resuspended in \(^{111}\)In-oxine for 20 minutes at 37°C, pH 6.5 to 7.5. The cells were then washed 3 times with HBSS.

**Viability**

Potential cytotoxic effects of \(^{111}\)In-oxine on monocyte viability were assessed by Trypan blue exclusion staining.

**Function**

We used a model of thioglycolate-induced sterile peritonitis modified after that of Segal et al.15 Sterile thioglycolate medium (1 mL) was injected intraperitoneally into C57BL/6 mice (\(n=3\)). After 24 hours, 3·10\(^{6}\) monocytes were isolated as described and divided into 2 batches. Batch 1 was labeled with \(^{111}\)In-oxine (5 pCi/cell) and subsequently colabeled with the indocyanine dye DiO (Molecular Probes, Carlsbad, Calif). Batch 2 was incubated with PBS only and subsequently labeled with the dye DiD (Molecular Probes). Batches 1 and 2 were then mixed together, and 10\(^6\) cells of the mixture were injected via tail vein into each of the 3 recipient mice with peritonitis. Again 24 hours later, animals were euthanized and peritoneal lavages performed. The recovered cells were analyzed via fluorescence-activated cell sorter analysis in the FL1 channel (to detect DiO/\(^{111}\)In-oxine–labeled monocytes) and the FL4 channel (to detect DiD/mock-labeled monocytes).

**Micro-SPECT/CT Imaging and Postprocessing**

For in vivo SPECT/CT imaging, monocytes were isolated as described and labeled with \(^{111}\)In-oxine. For selected experiments, PBMCs or PBMCs without monocytes were also used for experiments. Recipient Apoe\(^{-/-}\) or C57BL/6 wild-type mice, respectively, were placed under general isoflurane inhalation anesthesia, and 3·10\(^3\) cells were injected via tail vein. The total amount of activity injected into each animal was measured with a radioisotope calibrator (Capintec, Ramsey, NJ) immediately after injection and before imaging time points to account for differences in injected/excreted activity. SPECT/CT imaging was performed with an integrated high-resolution micro-SPECT/CT imaging system (X-SPECT; Gamma Medica Inc, Northridge, Calif), which combines SPECT and
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Results

Monocyte Isolation and Effect of $^{111}$In-Oxine Labeling on Monocyte Function

We isolated total murine monocytes using a recently described 2-step negative-depletion method.

Autoradiography

Mice were euthanized and aortas excised and exposed on a Phosphorimager SI (Molecular Dynamics, Sunnyvale, Calif) for 24 hours. $^{111}$In-oxine standards were exposed together with the specimen on the same plate, which allowed for calibration and quantitative data analysis by region-of-interest analysis with ImageJ software (National Institutes of Health, Bethesda, Md) as described previously.

Immunohistochemistry

Monocytes were isolated from EGFP-expressing mice (The Jackson Laboratory) and adoptively transferred into 20-week-old ApoE$^{-/-}$ mice. EGFP was used for its antigenic rather than its fluorescent properties. Five days after transfer, animals were euthanized and aortas excised, frozen in OCT compound, and cut into 5-μm sections in a cryostat. After sections were dried at room temperature for 20 minutes, they were treated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity and were incubated for 30 minutes in a cryostat. After sections were dried at room temperature for 20 minutes, conditions were used with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity and were incubated for 30 minutes in blocking solution. Adjacent sections were then incubated with primary polyclonal anti-EGFP (Chemicon, catalog No. AB3080) or rat anti-mouse Mac-3 antibodies diluted in PBS supplemented with 4% of the species-respective normal serum (GFP 1:25; Mac-3 1:50). After sections were washed with PBS, species-appropriate secondary antibodies were applied, followed by avidin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif). Slides were rinsed in PBS after each incubation step. The reaction was visualized with 3-amino-9-ethyl carbazole substrate (AEC; Sigma). Sections were counterstained with Gill’s hematoxylin solution (Sigma) and mounted.

Statistical Methods

To test for differences between treatment groups, 1-way ANOVA with Tukey multiple-comparison test was computed with GraphPad Prism software (GraphPad Software, San Diego, Calif). With small sample sizes and an unbalanced design, the data may not meet ANOVA assumptions, and therefore, caution is required in interpretation. To test for correlation between monocyte numbers and SPECT signal, Pearson’s product-moment correlation coefficient was calculated with Excel (Microsoft, Redmond, Wash). The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

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show substantial monocyte recruitment. Autoradiography of excised aortas verified the localized recruitment to the ascending aorta (Figure 2C). In contrast, neither PBMCs depleted of monocytes (which consisted mainly of T and B lymphocytes) injected into ApoE−/− mice nor monocytes injected into C57BL/6 wild-type mice demonstrated substantial recruitment to the aorta. Magnified views and a 3D rendering of the 2D SPECT/CT data set of monocyte transfer into ApoE−/− mice are shown in Figure 3 and in Movie I in the online-only Data Supplement.

To investigate the relationship between the in vivo SPECT signal and the actual numbers of 111In-labeled monocytes in the aortas, we estimated monocyte numbers on the basis of autoradiography data of excised aortas obtained after performance of in vivo SPECT imaging. The analysis showed a strong linear correlation between the in vivo SPECT signal and the actual numbers of 111In-labeled monocytes in the aortas.
and monocyte content, with an $R^2$ of 0.87 (online-only Data Supplement Figure 1).

**Statin Treatment of ApoE$^{-/-}$ Mice Rapidly Reduces Acute Monocyte Recruitment to Existing Plaque**

Having established SPECT/CT imaging as a tool to assess cellular recruitment to atherosclerotic lesions, we extended our studies to investigate whether a therapeutic intervention could modulate the imaging signal in vivo. Statin treatment can reduce the total number of macrophages in murine plaques after 6 months of treatment; however, the static nature of the study did not address the question of mechanism of action and whether the statin directly affected monocyte recruitment. Thus, further experiments evaluated in vivo the effect of mevastatin, simvastatin, or atorvastatin on monocyte recruitment. We report here that monocyte recruitment to plaques can be assessed by micro-SPECT/CT imaging and thus can be visualized in a noninvasive, dynamic, 3D, and quantitative fashion. The long half-life of radiotracer isotopes available for SPECT imaging enables repetitive imaging of monocyte biodistribution in the same animal, tracking its migration from circulation to atherosclerotic lesions.

Previous reports have indicated that lesion macrophage content may be gauged by magnetic resonance imaging, optical coherence tomography, or CT; however, these methods can only describe the total number of resident macrophages or overall metabolic activity, respectively, in a plaque at a given time. Because atherogenesis is a chronic and dynamic process, the number of macrophages present in a plaque at a given time is the combined result of monocyte recruitment, differentiation, death, and efflux from the plaque. The same limitation holds true for positron-emission tomography studies with fluorodeoxyglucose, which have shown promise in estimating the overall degree of vascular inflammation. In contrast to these techniques, in vivo SPECT/CT imaging now enables assessment of monocyte accumulation serially in the same animal, which allows a much more direct study of factors that modulate recruitment.

The method we describe here has several limitations. The resolution of the present approach is lower than that of certain high-resolution modalities such as magnetic resonance imaging. Coregistration with CT (as in the present study) and, in the future, with magnetic resonance imaging can reduce this shortcoming by providing greater anatomic detail and hence better spatial localization of the tracer signal.

SPECT/CT imaging of adoptively transferred cells measures the total number of $\gamma$-photons in a defined 3D space and assumes an equal number of photons emitted from each cell. The method may underestimate the number of recruited cells.
as they undergo apoptosis, which may lead to loss of tracer. The known progressive loss of $^{111}$In over time in healthy cells is relatively constant, although some variations may occur in vivo in different disease models. Indium that has lost its association with cells does not significantly influence the local cell-bound activity, because our prior studies have shown that free $^{111}$In is excreted rapidly through the kidneys.\textsuperscript{14}

A confounding variable in the interpretation of the results is the possibility that monocytes turn over in atherosclerotic plaques because of continued recruitment and emigration. However, data from Llodra et al\textsuperscript{24} have shown that although this is true for wild-type mice, only minimal emigration of monocytes from plaques in ApoE\textsuperscript{-/-} mice occurs. On the basis of these data, we believe that the signal detected in lesions in the present study reflects the accumulation of monocytes over the 5-day period from adoptive transfer until the imaging end point. We understand that the number of adaptively transferred monocytes ($3 \times 10^6$) exceeded the number of circulating monocytes in the recipient animal, which may affect the rate of monocyte trafficking, particularly in the initial hours or days.

Several technical factors were crucial to the development of a robust imaging technique. Because monocytes occur in relatively small numbers in the peripheral blood of mice and do not express a known unique surface marker, their isolation represents a challenge. Here, we used a 2-step method, recently described and characterized in more detail,\textsuperscript{14} that allows high-purity isolation while preserving cell viability and functionality.

Prerequisites for a suitable radioactive tracer used to label monocytes include ready availability, negligible cytotoxic effects, sufficient labeling efficiency, and a long half-life. The radiotracer compound $^{111}$In-oxine used here is available in a form ready for coincubation with cells and is approved by the Food and Drug Administration for labeling autologous leukocytes. Despite extensive testing in vitro and in vivo, we did not find adverse effects on viability or function of monocytes. $^{111}$In-oxine has a physical half-life ($t_{1/2} = 2.8$ days) that is significantly longer than that of isotopes available for positron emission tomography imaging, such as $^{64}$Cu (12.7 hours) or $^{18}$F (110 minutes).\textsuperscript{25} This translated to our ability to visualize monocyte trafficking by micro-SPECT/CT for up to 7 to 10 days, whereas the longest cell-tracking periods possible with state-of-the-art positron emission tomography imaging have been reported to be 24 to 36 hours,\textsuperscript{25} except for herpes simplex virus thymidine kinase (HSV-tk) techniques, which require genetic modification of cells. This property has particular importance to the present study, because we were able to use a delayed imaging time point (5 days) for the readout, thus avoiding detection of false-positive signal derived from circulating monocytes (we had recently determined the half-life of circulating blood monocytes in mice to
be 43.5 ± 7.9 hours, which corresponds to 95% monocyte clearance from the circulation at 5 days after injection.44

The SPECT signal was localized predominantly in regions of the ascending aorta, the site of the largest plaques in the majority of animals, whereas certain small lesions did not emit detectable signal. This observation may result from lesion heterogeneity, ie, some lesions may be more active than others. This method may therefore be used to identify lesions that are recruiting more monocytes. Alternatively, the total number of cells in smaller lesions is below the SPECT detection threshold. Further improvement of labeling efficiency and improvement in SPECT technology should increase sensitivity in the future.

With an in vivo imaging system at hand, we could then use it to efficiently assess the effect of modulating factors on monocyte recruitment, thus avoiding labor-intensive ex vivo procedures. All 3 tested statins had an immediate effect on monocyte recruitment that was rapid and independent of significant effects on plasma cholesterol levels. To the best of our knowledge, this is the first time that such immediate statin effects have been reported. Prospective clinical trials have convincingly demonstrated that statins can effectively lower the incidence of cardiovascular events in primary and secondary prevention.26 An increasing body of evidence suggests that statins cause these clinical benefits in part through antiinflammatory effects not directly related to the lowering of low-density lipoprotein cholesterol, a hypothesis supported by the present observations. Statin-induced alterations in arterial biology that may not depend on cholesterol lowering.30,31 The relevance of the by the present observations. Statin-induced alterations in arterial biology that may not depend on cholesterol lowering include reduction in the expression of factors involved in the recruitment of inflammatory cells, such as monocyte chemotactic protein-1,17,27 intercellular adhesion molecule-1,26 interleukin-6 and -8,28 tumor necrosis factor-α,17 nuclear factor-κB activity27 and monocytes.7 Several reviews point to uncertainty about the in vivo relevance of such “pleiotropic” effects.26,29 Recent analyses of clinical trials, however, support the concept that an important component of reduction by statins of recurrent cardiovascular events does not depend on low-density lipoprotein lowering.30,31 The relevance of the low-density lipoprotein–independent effects of statins also remains unclear, because many in vitro studies used statin concentrations too high to have clinical relevance.29 Most of the studies in mice used dosages between 10 and 30 mg/kg,32,33 which is at least an order of magnitude higher than the clinically prescribed range of 10 to 80 mg per person per day (0.14 to 1.14 mg/kg for a standard weight of 70 kg). The atorvastatin dosage used in the present study (0.57 mg/kg) corresponds to a 40-mg dose in humans. The present study, using a low dose, showed that atorvastatin causes an immediate and substantial reduction in monocyte recruitment to atherosclerotic plaques. After treatment of ApoE−/− mice with only 3 statin doses, monocyte recruitment to plaques fell 5-fold in vivo. This finding has considerable importance because it highlights the potential of statins to suppress a key step in atherogenesis via an effect independent of low-density lipoprotein cholesterol lowering. Furthermore, it supports the relevance of previous in vitro reports on the effect of statins on mediators of monocyte attachment to and transmigration through the vascular endothelium. Reciprocal experiments treated the donor mice (and therefore exposed the monocytes to atorvastatin but not the vessel wall of the recipients) and showed no significant reduction in monocyte recruitment. This finding suggests that atorvastatin reduces recruitment by acting at the level of the arterial wall rather than on the monocytes themselves. However, the possibility remains that the statin effect on monocytes in vivo may have been lost during monocyte isolation and no longer operates on adoptive transfer. Therefore, we cannot conclude that statins affect the vascular wall exclusively under these conditions.

In summary, we present novel mechanistic insight into monocyte accumulation in atheromata and the antiinflammatory action of statins using a new tool that permits noninvasive tracking of cells to the vascular wall in vivo. Using monocytes in the context of atherogenesis, we exemplify how this technique can be applied to elucidate important biological events. Because the key components of the technique are in clinical use, its application to human patients may be within reach.

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Disclosures
Dr Libby is the recipient of a research grant from the National Heart, Lung, and Blood Institute and serves as a consultant/advisory board member of GE Medical Systems. Dr Allport has an ownership interest in Novartis Pharmaceuticals. Dr Weissleder has an ownership interest in VisEn Medical. The remaining authors report no conflicts.

References


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**CLINICAL PERSPECTIVE**

Inflammation drives many aspects of atherogenesis and lesion progression and complication. Monocytes, the most numerous inflammatory cells in plaques, function as major effectors of this inflammatory response. Traditional imaging techniques generally visualize the structure of atherosclerotic lesions but do not report the cellular events that critically control the clinical consequences of the disease. The present study describes a technique that enables noninvasive in vivo imaging of monocyte trafficking to atheromata, with the use of Food and Drug Administration–approved components. Its application could provide novel biological insights, aid the evaluation of antiatherogenic drugs, and sharpen the risk stratification of selected patients.
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$y = 1.96x + 23.73$

$R^2 = 0.87$

Supplementary Figure