Exogenous Microparticles of Iron Oxide Bind to Activated Endothelial Cells but, Unlike Monocytes, Do Not Trigger an Endothelial Response

Andrew Jefferson, Neil Ruparelia and Robin P Choudhury

Department of Cardiovascular Medicine and Oxford Acute Vascular Imaging Centre, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom.

Corresponding author: Professor Robin P. Choudhury, Department of Cardiovascular Medicine, Level 6, West Wing, John Radcliffe Hospital, Oxford. OX3 9DU. Tel: +44-1865-234664 Fax: +44-1865-234667 E-mail: robin.choudhury@cardiov.ox.ac.uk.

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Abstract

Targeting particles to sites of inflammation is of considerable interest for applications relating to molecular imaging and drug delivery. We and others have described micron-sized particles of iron oxide (MPIO) that can be directed using specific ligands (e.g. antibodies, peptides and oligosaccharides) to bind to mediators of vascular inflammation in vivo. Since leukocyte binding to these molecules can induce changes in the target cell, an outstanding question has been whether the binding of imaging particles to these mediators induces biologically significant changes in the endothelial cells, potentially initiating or propagating inflammation. Here, we address these questions by looking for changes in endothelial cells following binding of contrast agent. Specifically, we have quantified calcium flux, rearrangement of the actin cytoskeleton, production of reactive oxygen species (ROS), apoptosis and potential secondary changes, such as changes in gene and protein expression following binding events to primary endothelial cells in vitro.

Although leukocytes induced changes to endothelial cell function, we did not see any significant changes to endothelial calcium flux, cytoskeletal organisation, production of ROS or induction of apoptosis in response to antibody-MPIO binding. Furthermore, there were no changes to gene expression monitored via real-time RT-PCR or presentation of protein on the cell surface measured using flow cytometry.

Our experiments demonstrate that whilst antibody-targeted microparticles mimic the binding capability of leukocytes to inflamed endothelium, they do not trigger the same cellular responses and do not appear to initiate or compound inflammation. These properties are desirable for targeted therapeutic and diagnostic agents.

Key words: Molecular Imaging, Inflammation, Microparticles

Introduction

Since the original description of McAteer et al.[1], targeted micron-size range particles of iron oxide have been applied for molecular imaging in experimental models of important human disease including multiple sclerosis[2, 3], cerebral metastases[4], epilepsy[5], ischemia reperfusion injury[6, 7] and atherosclerosis.[8, 9] Targeted contrast agents typically consist of a contrast element that imparts sensitivity for detection by a particular imaging modality and a targeting element, that is often an antibody or other molecule that confers specificity and binding affinity for the target of choice. Furthermore, particle-based
approaches have been proposed for the delivery of therapeutic agents, including drugs and miRNAs.[10, 11]

For the purposes outlined above, particles have been designed to bind to molecules that are differentially upregulated on activate endothelium, such as E-selectin, P-selectin and VCAM-1. These agents have been termed leukocyte-mimetic since their localisation relies on the binding to molecules that are crucial in leukocyte recruitment.

However, particle binding to functional, molecules expressed on the vascular endothelium has the potential to trigger cell signalling pathways and could even induce or compound inflammation at a tissue level. For instance, intercellular adhesion molecule-1 (ICAM-1), which is highly expressed in inflamed tissue in vivo,[12] plays a key role in endothelial cell response to leukocyte binding. Interactions between ICAM-1 and the leukocyte antigens αM/β2 (Mac-1) and α1/β2 (LFA-1) are responsible for leukocyte adhesion and spreading on the endothelium.[13, 14] ICAM-1 clustering as a result of leukocyte binding promotes release of intracellular calcium,[15], activation of RhoA[16] and the formation of actin stress filaments.[17] Stress filaments form docking structures at the site of leukocyte binding and have also been reported to form a cup structure around 5 μm beads bound to the surface of an activated endothelial cell.[18] Less is known about signalling pathways originating via interaction of VCAM-1 with VLA-4, although it has been postulated that ligation of VCAM-1 with specific antibodies may induce similar cytoskeletal rearrangements to that seen when cross-linking ICAM-1.[17] Cross-linking of VCAM-1 by means of specific antibody-labelled 9.9 μm beads activated endothelial cell NADPH oxidase to generate reactive oxygen species (ROS).[19] In turn, ROS oxidise and stimulate protein kinase C (PKC) activity.[19] E-selectin mediated responses appear to be via protein clustering and localisation to components of the actin cytoskeleton, including α-actinin, filamin, vinculin, paxillin and functional adhesion kinase.[20] The role of E-selectin in endothelial signalling has been long-established, with anti-E-selectin antibodies capable of inducing changes to human umbilical vein endothelial cells (HUVEC)[21]; later work revealing E-selectin mediated phosphorylation and activation of MAP Kinase.[22]

As targeted microparticles are used more widely and have been proposed for human use[23], it is important to determine their effects on endothelial function. Accordingly, we report the effects of antibody-targeted microparticles (directed against endothelial E-selectin and VCAM-1)[24]) on endothelial cells in vitro. Specifically, we have investigated whether particle binding induces changes to cellular calcium flux, production of reactive oxygen species, cytoskeletal rearrangement, gene expression levels and surface expression of molecular markers of inflammation. We compare the effects of MPIO vs. leukocyte binding on these processes.

**Methods**

**Cell culture**

Primary human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) (Invitrogen, Paisley, UK) were cultured in Medium 200 supplemented with Low-Serum Growth Supplement (LSGS) (Invitrogen). Cells were used between passages 2 and 4 and, when required, stimulated with recombinant human tumour necrosis factor-α (TNF-α, 10 ng mL⁻¹) (Invitrogen) The human acute monocyte leukemia cell line (THP-1) was used as a positive biological control; cells were grown in RPMI-1640 medium supplemented with 10% FBS Gold (PAA Laboratories, Yeovil, UK) and 2 mM L-glutamine. When used as a positive control, THP-1 cells were centrifuged to remove culture medium, washed briefly in 1 x PBS and resuspended in LSGS-supplemented Medium 200 prior to adding to endothelial cells at a concentration of 1 x 10⁵ cells mL⁻¹ in all experiments.

**Antibody-MPIO conjugation and handling**

Mouse anti-human monoclonal antibodies against VCAM-1 (Clone 4B2), E-selectin (5D11) and ICAM-1 (BBIG-I1) (R&D Systems, Abingdon, UK) (25 μg of E-selectin and VCAM-1 antibody for E+V-MPIO, and 50 μg of ICAM-1 antibody for ICAM-1-MPIO) were covalently conjugated to ~1.25 x 10⁹, 1 μm-diameter tosyl-activated Dynalbeads (MPIO) (Invitrogen) as previously described.[1] The efficiency of the conjugation reaction is estimated to be >90% based on protein quantification assays taken before and after labelling. Antibody-MPIO were added to endothelial cells in all experiments at a concentration of 10 pg mL⁻¹ antibody; ~ 2.5 x 10⁸ MPIO mL⁻¹. Prior to adding to endothelial cells, antibody-MPIO suspensions were thoroughly vortexed and briefly sonicated to ensure a mono-dispersed suspension of particles and to limit clumping. Antibody-MPIO bind to cells either as separate, individual particles or small clumps which are limited to no more than approximately 5 particles.

**Calcium imaging**

HAEC grown in 35 mm culture dishes were stimulated with TNF-α for 7 hours and loaded with the fluorescent calcium indicator Fluo-4 (Invitrogen) as per the manufacturer’s instructions for 1 hour at 37°C. Calcium responses to antibody-MPIO binding
under shear stress was performed by mounting cul-
ture dishes on a parallel-plate flow chamber (Gly-
coTech, Gaithersburg, USA) fitted with gasket B (0.25
cm x 0.025 cm) and connected to a syringe infusion
pump (Pump 22; Harvard Apparatus, Cambridge,
USA) and set to generate a shear stress over the cells
of 1 dyne cm⁻². MPIO and THP-1 cells were also flu-
 orescently labelled to allow differential detection;
MPIO were labelled in suspension by incubating with
10 μg mL⁻¹ goat anti-mouse Alexa Fluor 594 (Invitro-
gen) antibody for 30 minutes at 37°C and THP-1 cells
were stained with the nuclear stain Hoechst 33342
(Invitrogen) at 2 μg mL⁻¹ for 30 minutes then washed
and resuspended in Medium 200 prior to running
through the flow chamber. An Olympus IX-71 in-
verted microscope fitted with a 20x, 0.4 NA objective
(Olympus UK, Southend-on-Sea, UK), and a QICAM
cooled monochrome CCD camera (QImaging, Surrey,
Canada) driven using ImagePro-Plus (Media Cyber-
etics, Bethesda, USA) were used for imaging. Fluoresc-
tence intensity was monitored for 3 minutes post-MPIO binding to a cell.

Reactive oxygen species assay

TNF-α stimulated and basal HAEC and HUVEC
were incubated with 0.05% (wt/vol) nitroblue tetra-
zolium for 15 minutes prior to adding E+V-MPIO,
ICAM-1-MPIO or THP-1 cells. Cells were briefly
washed with 1 x PBS and then fixed for 5 minutes in
methanol at room temperature. The production of
reactive oxygen species was assessed by looking for
the formation of intracellular formazan precipitate
using a 40x 0.6 NA objective. One hundred cells
for each experiment were scored for the presence or ab-
sence of formazan.

Cytoskeletal rearrangements

HAEC and HUVEC grown on poly-d-lysine
coated glass were stimulated with TNF-α for 6 hours,
and incubated with E+V-MPIO, ICAM-1-MPIO or
THP-1 cells for a further 2 hours. Cells were washed
with PBS and fixed in methanol-free formaldehyde 4
% for 10 minutes at room temperature. Phal-
lloidin-TRITC (Invitrogen) was used to stain F-actin.
After a final wash in PBS, coverslips were mounted on
a standard microscope slide in SlowFade Gold antifulu-
secent reagent with 4',6-diamidino-2-phenylindole (Invitro-
gen). Images were taken using a 100x 1.3 NA oil im-
mersion objective fitted to the microscope detailed
above.

RNA Extraction and RT-PCR

Quantitative real-time RT-PCR was used to mea-
sure expression of VCAM-1 (CD106), E-selectin
(CD62E) and ICAM-1 (CD54) in HAEC and HUVEC
under basal and stimulated conditions following
binding of E+V-MPIO, ICAM-1-MPIO or THP-1 cells.
GAPDH was used as a normalization gene. Following
stimulation with TNF-α for 2 hours, targeted MPIO
and THP-1 cells were added and incubated for a fur-
ther 2 hours. RNA was extracted using an RNeasy
Mini Kit (Qiagen, Crawley, UK) and cDNA was syn-
thesised using a QuantiTect reverse transcription kit
(Qiagen). TaqMan™ primers for VCAM-1, E-selectin,
PECAM-1 and GAPDH were used to amplify cDNA
on a StepOne PCR system (Applied Biosystems, Warr-
rington, UK). Relative quantities of mRNA expressed
in arbitrary units were calculated using the 2ΔΔCt
method.[25]

Flow cytometry to establish relative ligand abundance

HAEC and HUVEC were stimulated with TNF-α
for 6 hours, and incubated with E+V-MPIO,
ICAM-1-MPIO or THP-1 cells for a further 2 hours.
Following washing with PBS cells were detained us-
ing non-enzymatic cell dissociation solution (Sigma,
Poole, UK). The cells were centrifuged (1200 rpm;
5 minutes) and resuspended in 100 μL PBS. Mouse an-
ti-human E-Selectin antibody was added at a final
concentration of 10 μg mL⁻¹. Goat anti-mouse Alexa
Fluor 488 (Invitrogen) was added at a concentration of
10 μg mL⁻¹. Flow cytometry experiments were per-
formed on a CyAn flow cytometer and the data ana-
lysed using FlowJO software, version 7.6.3 (Tree Star
Inc., Ashland, USA), gates of interest were set using
unstained and IgG stained samples to control for
background and autofluorescence.

Apoptosis assay

The effect of antibody-MPIO on cell viability and
induction of apoptosis in endothelial cells was deter-
mined by staining with a FITC-conjugated Annexin-V
antibody and Propidium Iodide (PI) to identify
apoptotic and/or dead cells respectively, using an
Annexin-V/Dead Cell Apoptosis Kit (Invitrogen). The
kit was used in accordance with the manufacturer’s
protocol and cells analysed immediately after staining
on a CyAn flow-cytometer as described above.
Apoptosis was induced in HAEC and HUVEC as a
positive control by serum starving cells for 4 hours
prior to staining. To allow differentiation between
endothelial and THP-1 cells in the relevant samples,
THP-1 cells were loaded with Hoechst 33342 as de-
scribed above, then washed prior to incubating with
endothelial cells.

Results

Binding of THP-1 cells, but not E+V-MPIO in-
duce calcium flux in aortic endothelial cells

Fluo-4 intensity was tracked over a 3 minute pe-
period following MPIO or THP-1 binding to TNF-α-stimulated HAEC. Binding of dual-targeted E+V-MPIO to cells (Figure 1A) did not result in changes to calcium concentration within the cells observed. Calcium flux was observed upon binding of THP-1 cells (Figure 1B); following binding, a slow increase in Fluo-4 intensity which peaked at around 60 seconds post-binding was observed before fluorescence normalised to baseline (Figure 1C).

**Targeted MPIO binding to endothelial cells does not result in the production of reactive oxygen species**

The production of reactive oxygen species (ROS) in endothelial cells in response to antibody-MPIO binding was investigated using nitroblue tetrazolium (NBT), which in the presence of ROS is reduced to formazan, a black, insoluble intracellular precipitate. Cells incubated with NBT were then incubated with E+V-MPIO and ICAM-1-MPIO, and a positive control of ascorbic acid. Representative images taken without phase contrast for clarity of MPIO and formazan precipitate are shown for HAEC (Figure 2A-D) and HUVEC (Figure 2E-H). Scoring cells for presence or absence of formazan (Figure 2I) revealed a low percentage of positive HAEC and HUVEC when incubated with E+V- and ICAM-1-MPIO and negative controls, none of which were statistically different (P > 0.05 in all instances), but a significantly higher count in the ascorbic acid positive control (P = 0.0001). Incubating HAEC and HUVEC with the corresponding antibody-only controls also resulted in a low percentage of formazan-positive cells, with no significant difference from the negative control (P > 0.05 in all instances) (data not shown).

**Endothelial cytoskeleton is reorganised upon exposure to THP-1 cells but not with targeted MPIO**

Confocal analysis of phalloidin-stained endothelial cells revealed no difference between cells incubated with E+V-MPIO and ICAM-1-MPIO, and negative controls, with a fine network of stress fibres running through the cells, as is characteristic for cells cultured on glass. However, when incubating endothelial cells with THP-1 macrophages, F-actin filaments were bundled at the site of binding. Z-stacks were obtained and flat projections generated for HAEC incubated with E+V-MPIO (Figure 3A), negative control (Figure 3B) and THP-1 (Figure 3C). Inserts show a side-on view generated with a 3D projection of image slices. Transverse line profiles (shown on images) reveal a characteristic pattern in E+V-MPIO and negative cells, with a clearly different profile - representing peripheral bundling - observed in THP-1-treated cells. Classifying F-actin organisation as either “fine filaments” or “bundled filaments” further corroborates the image findings, with a large proportion of HAEC and HUVEC having bundled filaments when treated with THP-1 cells, but not with E+V-MPIO, ICAM-1-MPIO or antibody-alone controls (Figure 3D&E).
Fig 2. The production of ROS following binding events was assessed by loading cells with nitroblue tetrazolium and looking for the reduction product formazan via light microscopy. In HAEC (A-C) and HUVEC (E-G) treated with E+V-MPIO and ICAM-1-MPIO, and THP-1 macrophages. In all stimulated samples, there was no statistically significant difference in expression of any of the genes tested between samples incubated with the antibody-MPIO, THP-1 cells or negative (vehicle only) controls (P > 0.05 for all comparisons) (Figure 4A-F).

In the basal (unstimulated) samples, minimal expression of all genes was measured in the antibody-MPIO samples and negative samples. Upregulation of all genes was observed in basal endothelial cells treated with THP-1 macrophages. Contribution of THP-1 RNA towards expression data was accounted for by extracting RNA from THP-1 cells in isolation and testing for expression of E-selectin, VCAM-1 and ICAM-1; no gene expression was detected (data not shown).

Antibody-MPIO binding does not induce changes to gene expression of inflammatory markers

Expression of E-selectin, VCAM-1 and ICAM-1 mRNA was assessed in TNF-α stimulated and basal HAEC and HUVEC incubated with E+V-MPIO and ICAM-1-MPIO, and THP-1 macrophages. In all stimulated samples, there was no statistically significant difference in expression of any of the genes tested between samples incubated with the antibody-MPIO, THP-1 cells or negative (vehicle only) controls (P > 0.05 for all comparisons) (Figure 4A-F).

In the basal (unstimulated) samples, minimal expression of all genes was measured in the antibody-MPIO samples and negative samples. Upregulation of all genes was observed in basal endothelial cells treated with THP-1 macrophages. Contribution of THP-1 RNA towards expression data was accounted for by extracting RNA from THP-1 cells in isolation and testing for expression of E-selectin, VCAM-1 and ICAM-1; no gene expression was detected (data not shown).

Antibody-MPIO binding does not induce changes to the surface density of inflammatory markers on endothelial cells

The surface density of E-selectin, as a representative surface marker of inflammation, was assessed in stimulated and basal HAEC and HUVEC incubated with antibody-MPIO and THP-1 cells using flow cytometry. No significant differences were observed in the geometric mean fluorescence intensity of cells incubated with E+V-MPIO and ICAM-1-MPIO when compared with negative controls (P > 0.05 for all comparisons) (Figure 5A & B).

Antibody-MPIO binding does not induce apoptosis or affect endothelial cell viability

Annexin-V and PI staining of endothelial cells was used to look for induction of apoptosis and cell death following antibody-MPIO binding. Apoptosis was not induced in stimulated and basal HAEC and HUVEC incubated with MPIO and THP-1 cells, with no significant differences observed in the fold-change in numbers of apoptotic (Annexin V+/PI-) cells when compared with negative controls (P > 0.05 for all comparisons) (Figure 6A, B & C). A significant difference was observed however when comparing experimental conditions in stimulated HAEC and HUVEC with the positive controls of serum starvation to induce apoptosis (P ≤ 0.05 for all comparisons). Furthermore, no significant difference in the percentage of dead cells (Annexin V+/PI+) was observed between MPIO, THP-1 and negative controls (P > 0.05 for all comparisons) (data not shown).
Fig 3. Phalloidin-TRITC was used to stain F-actin filaments to assess the morphology of the endothelial cytoskeleton via laser scanning confocal microscopy. Images are single-plane projections generated by taking Z-stacks, with side-on projections also presented as inlays. No large-scale changes to actin organisation were seen when E+V-MPIO bound to stimulated HAEC, nor in negative controls (A & B). Upon binding of THP-1 to stimulated HAEC, F-actin becomes bundled and is redistributed at the edges of cells and at sites of THP-1 binding (C). Line profiles drawn across cells demonstrate different cross sectional staining in cells treated with THP-1 cells. Classification of F-actin filaments as “fine” or “bundled” shows no difference between cells with MPIO and antibody-only bound and negatives, but a marked difference when THP-1 cells have bound (D & E).
Fig 4. Real-time RT-PCR was used to assess expression of E-selectin (A & D), VCAM-1 (B & E) and ICAM-1 (C & F) in stimulated (black columns) and basal (white columns) HAEC and HUVEC respectively incubated with E+V- and ICAM-1-MPIO, and THP-1 cells. In all stimulated cells, there were no differences between any of the treatments (P>0.05 in all comparisons). In basal HAEC and HUVEC, binding of THP-1 cells induced expression of all genes tested.

Fig 5. Flow cytometry to assess the relative surface density of E-selectin on stimulated (+) and basal (-) HAEC and HUVEC treated with MPIO and THP-1 cells. E-selectin was expressed on the surface of stimulated HAEC (A) and HUVEC (B), with no significant difference between MPIO-treated and non-treated cells (P>0.05). The apparent reduction in E-selectin density in cells incubated with THP-1 may be due to masking of E-selectin by bound THP-1 cells.
Fig 6. Flow cytometry of Annexin-V/Propidium Iodide-stained endothelial cells was used to assess induction of apoptosis following binding of antibody-MPIO. The flow cytometry plot (A) divides cells into 3 main groups; live cells (Annexin-V-/PI-) are represented in Q3, live but apoptotic cells (Annexin-V+/PI-) in Q4 and apoptotic dead cells (Annexin-V+/PI+) in Q2. When looking at fold change in apoptosis, binding of E+V-MPIO and ICAM-1-MPIO did not induce apoptosis in stimulated and basal HAEC and HUVEC (B & C).

Discussion

Targeted particles that bind to sites of endothelial activation are of considerable interest as imaging contrast agents[1-6, 9, 26] and for the local delivery of drugs[10] and genes.[11] Leukocyte binding to activated endothelium provokes a variety of responses in the endothelial cell. Therefore, an important consideration for the development of endothelium-targeted agents towards clinically usable products is whether their binding induces potentially detrimental effects in the target cell.

Here, we show that, in contrast to the response to monocyte binding, antibody-targeted MPIO binding to primary endothelial cells in vitro has no measurable effect on a range of biologically important processes. Specifically, particle binding did not promote calcium flux, production of ROS or cytoskeletal rearrangement - all of which occur shortly after leukocyte binding. These results indicate that, whilst mimicking some of the binding properties of leukocytes, which is desirable for localising to sites of disease, antibody targeted contrast agents recognising either E-selectin or VCAM-1 do not trigger endothelial signalling events that may activate or exacerbate inflammation. Furthermore, we demonstrate that our contrast agent does not induce apoptosis in the target endothelial cells.

We also examined what might be considered secondary changes to endothelial cell phenotype such as the expression of mRNA for the inflammatory mediators E-selectin, VCAM-1 and ICAM-1 and surface density of these markers. We did not identify any significant changes to these parameters with antibody-MPIO binding.

The inclusion of both basal-state and TNF-α stimulated cells was designed to identify whether our targeted contrast agent 1) stimulated basal cells and 2) compounded a pre-existing inflammatory stimulus. In neither case was there a significant change in endothelial cells treated with contrast agent. These findings have important ramifications for the use of such contrast agents in vivo, where switching-on and/or compounding inflammation would be undesirable consequences that would confound the molecular imaging and potentially modify / exacerbate the disease process.

The response of endothelial cells to leukocyte binding appears to be driven in part by endothelial ligand cross linking; in this instance, molecules far apart on the endothelial cell surface may be brought into close contact by the bound cell, thus inducing cellular responses.[15-17] Similar effects have been noted when using larger particles not dissimilar in size to that of cells to mimic the binding of leukocytes.[18, 19] However, in direct contrast to these studies, our choice of using smaller, 1 μm particles may be responsible for the absence of a detectable response. The smaller diameter of our particles might minimise the spatial effect of molecular cross-linking...
of E-selectin, VCAM-1 and ICAM-1, which could account for the lack of cell response. Endothelial cell endocytosis of antibody-labelled MPIO does not appear to occur,[9] and thus the interaction between cells and particles is due to antibody-receptor interaction at the cell surface.

Conclusions

Antibody-based targeted microparticles of iron oxide do not appear to trigger signalling mechanisms in endothelial cells resulting in the phenotypic changes that are characterised upon the binding of leukocytes.

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Competing Interests

The authors have declared that no competing interest exists.

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