Characterization of the recruitment of intimal smooth muscle cells in vascular disease

Jang, Sunyoung

https://hdl.handle.net/2144/14329

Boston University
CHARACTERIZATION OF THE RECRUITMENT OF INTIMAL SMOOTH MUSCLE CELLS IN VASCULAR DISEASE

by

SUNYOUNG JANG

B.S., University of Massachusetts, Amherst, 2008

Submitted in partial fulfillment of the requirements for the degree of Master of Arts

2014
Approved by

First Reader
Chris Andry, M.Phil., Ph.D.
Associate Professor of Pathology and Laboratory Medicine

Second Reader
Richard Mitchell, M.D., Ph.D.
Lawrence J. Henderson Professor of Pathology and HST
Harvard Medical School
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Mitchell, for his guidance and support throughout the research. His patient and continued support led me to the right way.

I would like to extend my appreciation to my advisor at BU, Dr. Andry for his advice during my research and my sincere appreciation is extended to Nir Grabie and current coworkers in Vascular Biology Division at BWH for their supervision and help throughout this process.

Above all, my deepest expression of appreciation goes to my family. Their love, support, and motivational talks have made it possible for me to pursue this degree.
CHARACTERIZATION OF THE RECRUITMENT OF INTIMAL SMOOTH MUSCLE CELLS IN VASCULAR DISEASE

SUNYOUNG JANG

ABSTRACT

Intimal hyperplasia occurs as a response to a variety of vascular insults and results in vascular stenosis, and organ ischemia. This process represents a fundamental component of atherosclerosis, venous graft stenosis, and allograft arteriopathy in solid organ transplants. Smooth muscle cells (SMCs), and their associated extracellular matrix (ECM) form major components of intimal hyperplasia. We hypothesize that chemokines play a critical role in SMC migration into such intimal lesions. A number of chemokine-chemokine receptor interactions that mediate inflammatory cell recruitment have been characterized. However, the specific chemokine-chemokine receptor pathways that contribute to SMC recruitment are not known. The aims of this study are to examine the expression of C-C chemokine receptor 1 (CCR1) on medial SMCs (MSMCs), and to test its functionality in SMC recruitment. SMCs were derived from murine aortas; cultures were >95% SMC as demonstrated by the expression of smooth muscle α-actin (SMA), calponin, smooth muscle myosin heavy chain (SM-MHC). Interferon-gamma (IFN-γ) and Tumor necrosis factor- alpha (TNF-α) – stimulated MSMCs express CCR1 with a peak expression between 30 h and 48 h after cytokine stimulation. The functionality of receptors was initially demonstrated by agonist-induced calcium mobilization: the
addition of CCR1 ligands, Regulated on activation, Normal T cell expressed and secreted (RANTES) and Macrophage inflammatory protein -1α (MIP-1α) to MSMCs caused an increase in intracellular Ca\(^{2+}\) concentration. Blockade of CCR1 by BX471, a CCR1 antagonist, inhibited the Ca\(^{2+}\) mobilization induced by RANTES and MIP-1α. The results suggest that up-regulation of CCR1 expression on cytokine-stimulated SMCs may facilitate recruitment into intimal lesions through endothelial-derived chemokine expression.
TABLE OF CONTENTS

Title Page..........................................................................................................................i
Copyright Page..................................................................................................................ii
Reader's Approval Page.......................................................................................................iii
Acknowledgements...........................................................................................................iv
Abstract..............................................................................................................................v
Table of Contents.............................................................................................................vii
List of Tables....................................................................................................................ix
List of Figures..................................................................................................................x
List of Abbreviations.........................................................................................................xi

1. INTRODUCTION ......................................................................................................... 1

2. MATERIALS & METHODS .......................................................................................... 5
   2.1 Mice ....................................................................................................................... 5
   2.2 Reagents ................................................................................................................ 5
   2.3 Primary smooth muscle cells (SMCs) isolation and culture................................. 5
   2.4 Flow cytometry analysis......................................................................................... 6
   2.5 Immunofluorescence of CCR1 in MSMCs.............................................................. 7
   2.6 Intracellular Ca^{2+} flux measurement.................................................................... 7

3. RESULTS ...................................................................................................................... 9
   3.1 MSMCs express typical SMC markers................................................................. 9
   3.2 Expression of CCR1 on MSMCs............................................................................ 12
   3.3 CCR1 expression increases at 30 h and 48 h......................................................... 15
3.4 Addition of RANTES and MIP-1α induce calcium mobilization in MSMCs........ 17

4. DISCUSSION ................................................................................................................. 20

LIST OF JOURNAL ABBREVIATIONS............................................................................ 23

REFERENCES .................................................................................................................. 24

VITA ...................................................................................................................................... 27
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chemokine receptors and their cognate ligands potentially involved in SMC activation and recruitment</td>
<td>4</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flow cytometry analysis of specific SMC markers on MSMCs</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Flow cytometry analysis of CCR1 expression by unstimulated and cytokine-stimulated MSMCs</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Immunofluorescent of staining of CCR1 in MSMCs</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Time course analysis of CCR1 surface expression on MSMCs</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>Measurement of $\text{Ca}^{2+}$ flux in cultured MSMCs in response to MIP-1$\alpha$ and RANTES</td>
<td>18</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AB..................................................................................................Antibody
α-SMA..................................................................................Smooth muscle α-actin
CCR1......................................................................................C-C chemokine receptor - 1
DMEM..................................................................................Dulbecco’s modified eagle medium
EC.........................................................................................Endothelial cells
ECM.....................................................................................Extracellular matrix
FBS..........................................................................................Fetal bovine serum
HBSS....................................................................................Hanks’ balanced salt solution
IFN-γ......................................................................................Interferon - gamma
IP-10......................................................................................Interferon gamma-induced protein 10
iSMC......................................................................................Intimal smooth muscle cells
ITAC...................................................................................Interferon-inducible T cell-chemoattractant
mAB........................................................................................Monoclonal antibody
MCP-1..................................................................................Monocyte chemotactic protein-1
MIP-1α................................................................................Macrophage inflammatory proteins -1 alpha
MSMC.....................................................................................Medial smooth muscle cells
PBS..........................................................................................Phosphate buffered saline
PDGF-B...............................................................................Platelet-derived growth factor - BB
PECAM-1...............................................................................Platelet endothelial cell adhesion molecule
RANTES………………..Regulated on activation, Normal T cell expressed and secreted
SM-MHC……………………………………………..Smooth muscle myosin heavy chain
TNF-α …………………………………………………….Tumor necrosis factor - alpha
INTRODUCTION

Intimal hyperplasia, which occurs in response to vascular injury, represents a fundamental component of vasculoproliferative disorders such as atherosclerosis, venous graft stenosis, and allograft arteriopathy in solid organ transplants. Smooth muscle cells (SMCs), and their associated extracellular matrix (ECM) are major components of intimal hyperplasia. We hypothesized that chemokines play a critical role in SMC migration into such intimal lesions. A number of chemokine-chemokine receptor interactions have been characterized for inflammatory cell recruitment. However, the specific chemokine-chemokine receptor pathways that contribute to SMC recruitment are not known.

The earliest response to vascular injury takes place within the endothelium, resulting in endothelial dysfunction. Endothelial cell (EC) injury promotes adhesion molecule expression, which in turn increases the attachment and activation of circulating inflammatory cells. A variety of recruited cell types, including activated ECs, platelets, inflammatory cells, and SMCs subsequently secrete inflammatory mediators, such as cytokines and growth factors that drive lesion progression. Although the exact molecular mechanisms are incompletely defined, inflammatory events cause SMC activation and proliferation within the intima, and the expanded intima subsequently leads to vascular occlusion.

SMCs are the most abundant cell type in a normal vessel and also are the major cellular constituent in intimal hyperplastic lesions. Notably, a number of studies have
shown phenotypic differences between MSMCs and those found within intimal lesions\(^1\),\(^{20, 22}\). MSMCs exhibit a non-proliferative, non-synthetic, and contractile phenotype, whereas intimal SMCs (iSMCs) are less-differentiated with non-contractile, proliferative, and synthetic characteristics. Contractile MSMCs regulate vascular tone, blood pressure, and lumen size, and generally produce relatively little ECM. In response to injury, these SMCs undergo a phenotypic switch from the contractile phenotype to the active, synthetic phenotype. In this state, iSMCs proliferate and migrate to the intima where markedly increase ECM synthesis\(^1, 20, 22\). However, although iSMCs share some similarities with MSMCs, definitive evidence of phenotypic interconversion is lacking. In addition, the distinct functional differences suggest that MSMCs may have a different cell of origin than intimal SMCs\(^9, 11\).

Given the functional and phenotypic differences between MSMCs and iSMCs, the source of SMCs in intimal lesions has been subject of considerable research. It was initially hypothesized that mSMCs migrate from the media into the intima, where they subsequently proliferate and synthesize ECM\(^20\). However, more recent studies suggest that other sources of iSMCs may exist, including bone marrow-derived cells\(^10, 13\). A growing body of evidence supports the notion that SMCs in intimal lesions derive from multiple sources, and that bone marrow precursors account for up to 10-15% of the total of intimal SMC population\(^13\).

The recruitment of SMCs to the intima is an essential step in most vasculoproliferative disorders. We hypothesized that the migratory and proliferative activities of SMCs are regulated through the interactions of chemokines with their
corresponding receptors. Chemokines are small proteins which play a critical role in inflammatory events by regulating inflammatory cell trafficking and inducing the activation and migration of smooth muscle cells to site of vascular injury.\textsuperscript{21, 23, 25} Chemokines can be classified into four subfamilies based on the number and the arrangement of conserved cysteine residues: C, CC, CXC, and C(X)3C. The cellular effects of chemokines are mediated through binding to specific seven transmembrane-G-protein-coupled receptors. Table 1 lists a summary of the major chemokines and their associated receptors that are potentially involved in SMC recruitment and activation.

Multiple chemokines can bind to CCR1, CCR2, or CXCR3 receptors, and are expressed at high levels in intimal lesions.\textsuperscript{16} Modulation of such chemokines or blockade of their receptors can ameliorate intimal hyperplasia.\textsuperscript{14} An elegant study by Shimizu et al. suggested that MSMCs and iSMCs have distinct chemokine receptor expression, and that MSMCs do not express chemokine receptors that facilitate intimal recruitment. Moreover, the authors of this study demonstrated that stimulation of CCR1 by its ligand, RANTES, specifically promotes iSMCs proliferation. In addition, they showed that intimal hyperplasia was attenuated in CCR1-deficient animal models.\textsuperscript{11} We hypothesized that EC are a potential source of the chemokines responsible for the activation and recruitment of these MSMCs. The specific chemokine-chemokine receptor pathways that regulate MSMC recruitment remain unclear. We tested our hypothesis by treating quiescent MSMCs with the pro-inflammatory cytokines, IFN-\(\gamma\) and TNF-\(\alpha\) to mimic vascular injury and then evaluated whether these cytokines can activate and modulate the phenotypic
switch in MSMCs and eventually induce migration and proliferation through the CCR1-regulated pathway.

The aim of this study was to examine the capacity of MSMCs to express relevant chemokine receptors. We specifically investigated the expression of CCR1 in MSMCs, comparing CCR1 expression in the presence and absence of IFN-γ and TNF-α. We also verified that CCR1 expression was functional by evaluating responsiveness to CCR1 ligands. To assess the functionality of CCR1 to its corresponding chemokine, we tested the effect of RANTES and MIP-1α on MSMC function. Our results suggest that MSMCs can up-regulate the expression of functional CCR1 after cytokine stimulation.

<table>
<thead>
<tr>
<th>Chemokine Receptors</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC receptors (CXCR1 to CXCR6)</td>
<td>Mig, IP-10, I-TAC</td>
</tr>
<tr>
<td>CXCR3</td>
<td></td>
</tr>
<tr>
<td>CXCR4</td>
<td>SDF-1α, 1β; PBSF</td>
</tr>
<tr>
<td>CC receptors (CCR1 to CCR11)</td>
<td>MIP-1α, RANTES, MCP-1</td>
</tr>
<tr>
<td>CCR1</td>
<td>MCP-1, MCP-2, MCP-3, MCP-4, HCC4</td>
</tr>
<tr>
<td>CCR2</td>
<td>Eotaxin, Eotaxin-2, MCP-3, RANTES, MCP-2, MCP-4</td>
</tr>
<tr>
<td>CCR3</td>
<td>TARC, MDC</td>
</tr>
<tr>
<td>CCR4</td>
<td>MIP-1α, 1β; RANTES, Eotaxin, HCC1, HCC4</td>
</tr>
</tbody>
</table>

Adapted from Mitchell et al., 2007
MATERIALS & METHODS

2.1 Mice

C57BL/6 mice (8 weeks) were purchased from The Charles River Laboratories (Wilmington, MA).

2.2 Reagents

Recombinant mouse interferon gamma (IFN-γ), Recombinant mouse tumor necrosis factor alpha (TNF-α), and Monoclonal antibody (mAB) to CCR1 were purchased from R&D systems (Minneapolis, MN). Monoclonal rat anti-mouse CD31-Phycoerythrin (PE) antibody was purchased from BD biosciences (San Jose, California). Recombinant RANTES (CCL5), MIP-1α (CCL3), and Recombinant rat platelet-derived growth factor (PDGF) – BB were purchased from R&D systems (Minneapolis, MN). Fluo-4-AM was purchased from Life Technologies (Carlsbad, CA) and Ionomycin, BX471(R-N-[5-chloro-2-[2-[4-[(4-fluorophenyl) methyl] - 2-methyl-1-piperazinyl]-2-oxoethoxy] phenyl] urea hydrochloric acid salt), and Monoclonal anti-α smooth muscle-FITC was purchased from Sigma-Aldrich (St. Louis, MO). Anti-calponin antibody and Anti-myosin smooth muscle heavy chain 1 and 2 antibody were purchased from Abcam (Cambridge, MA).

2.3 Primary smooth muscle cells (SMC) isolation and culture

Aortas were harvested from 8 week-old WT B6 mice with sterile dissecting scissors. Adipose tissue was carefully removed, and aortas were placed in a 60-mm Petri dish containing Dulbecco’s modified eagle medium (DMEM) with 5mg/ml Collagenase
type II and incubated for 20 minutes at 37°C, before being placed in cold DMEM to remove adventitia. The aortas were then transferred to Collagenase type I and Elastase type III mixture in a 10 cm Petri dish, cut into pieces and incubate for 30 to 40 minutes at 37°C for a second round of digestion. Digested tissues and medium were transferred to fresh SMC culture medium (DMEM with 5% FBS, 1% penicillin/streptomycin; 2% non-essential amino acids, 1% L-glutamine; Life Technologies) and plated in a T75 flask (Corning, Fisher scientific) and grown at 37°C. The purity of SMC cultures was >95%, as determined by flow cytometry with anti-SM α-actin(SMA), calponin, and smooth muscle myosin heavy chain (SM-MHC) antibodies. SMCs of passages 4 to 13 were used for the study.

2.4 Flow cytometry analysis

For quantitative analysis of CCR1 expression on the cell surface, C57bl6 medial smooth muscle cells were cultured in medium alone or in the presence of recombinant mouse IFN-γ (10ng/ml; R&D Systems) and TNF-α (10ng/ml; R&D Systems) for 48 hours at 37°C. Cells were then harvested with trypsin (0.25% trypsin-EDTA; Invitrogen) for one minute at 37°C and then washed with SMC culture medium (DMEM with 5% FBS, 1% penicillin/streptomycin; 2% non-essential amino acids, 1% L-glutamine; Invitrogen). Cells were then washed once with 1% fetal bovine serum (FBS) in Phosphate buffered saline (PBS) and incubated with Allophycocyanin (APC) -conjugated anti-CCR1 (R&D Systems) or control isotype (rat IgG2A; BD Biosciences) on ice for 30 minutes; after washing in 1% fetal bovine serum in PBS, cells were analyzed by flow
cytometer (FACScalibur, BD Biosciences, San Jose, CA), and the data were collected (logarithmic scale) and expressed as the percentage of positive cells. The fluorescence produced by isotype-matched control antibody was considered background.

2.5 Immunofluorescence of CCR1 in medial smooth muscle cells

C57BL/6 medial smooth muscle cells were cultured under adherent conditions in 12-well plates in the presence of recombinant mouse IFN-γ (10 ng/ml; R&D Systems) and TNF-α (10 ng/ml; R&D Systems) for 48 hours at 37°C. Cells were then washed once with 1% fetal bovine serum in PBS, and then incubated for 30 min on ice with either a mAB anti-mouse CCR1 (R&D Systems) or the control isotype antibody (BD biosciences) at 1:100 dilution in 1% fetal bovine serum in PBS. After a final wash, cells were imaged on an Olympus microscope and analyzed using MetaMorph software (Molecular Devices Corp).

2.6 Intracellular Ca²⁺ influx measurement

For calcium measurements, C57bl6 medial smooth muscle cells were cultured in medium alone or stimulated with recombinant mouse IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) for 48 hours at 37°C, then trypsinized and resuspended in pre-warmed (37°C) Hanks’ balanced salt solution (HBSS) containing the calcium-sensitive dye Fluo-4 AM ester (2 μM, Life technologies). The cell suspension was then incubated at 37°C for 15 minutes. After washing twice in HBSS, Fluo-4-loaded cells were equilibrated at room temperature for one hour. Intracellular free Ca²⁺ was first recorded for 30 seconds to
establish a baseline. The cells were then stimulated by adding Ionophore (2 µM; A23187; Sigma-Aldrich), MIP-1α (100 ng/ml; R&D Systems), or RANTES (100 ng/ml; R&D Systems). After stimulation with different agonists, cells were screened continuously in a FACScan for 2 minutes to assess the increase in mean fluorescence induced by each agonists and were related to the fluorescence obtained by baseline. For the CCR1 blocking experiment, medial SMCs were treated with BX471 (250 nM, Sigma Aldrich), a CCR1 antagonist, for 15 minutes before the addition of agonists. The effect of the Ca^{2+} ionophore (A23187, Life technologies) and PDGF-B (R&D System) were used as positive controls for Ca^{2+} flux.
RESULTS

3.1 MSMCs express typical SMC markers

MSMCs were derived from uninjured aortas and cultures were first analyzed for typical SMC markers. Previously proven markers used for SMC lineage indications are: a) α-SMA (smooth muscle α-actin), an actin isoform typical of SMC and constitutive elements in SMC; b) calponin, a calcium-binding protein that normally functions to inhibit SMC contraction; and c) SM-MHC (smooth muscle myosin heavy chain), a myosin contractile protein specific for the SMC lineage. At confluence, MSMCs were harvested; fixed and permeabilized, then stained with typical SMC markers indicated above. Flow cytometry analysis showed high expression levels of all markers in MSMCs, although SM-MHC was expressed in MSMCs to a lesser extent than for the other two markers (Fig 1B-D). Furthermore, the purity of MSMCs was evaluated by staining for cell surface expression of endothelial cell-specific marker, platelet endothelial cell adhesion molecule (PECAM-1, also known as CD31). Flow cytometry analysis showed that the MSMCs were simultaneously negative for CD31, which excludes endothelial cells contamination (Fig 1F).
Figure 1. Flow cytometry analysis of specific SMC markers on MSMCs. A. Forward (size) vs. side (granularity) scatter and the enclosed area was the population analyzed in B, C, and D. The smaller cells in the lowest forward (FSC) and side scatter (SSC) represent dead cells which were excluded from analysis. B-D. The histograms show the relative fluorescence intensity of the MSMCs stained with specific SMC markers,
including SMA, calponin, and SM-MHC11 (blue line). The red line represents the isotype-matched control antibody. MSMCs are positive for SMA (A), calponin (B) and SM-MHC11 (C). E-F. Purity of MSMCs, as measured by CD31-negative cells. E. Cells were gated on FSC and SSC to exclude dead cells. F. The additional histogram for MSMCs shows the negative CD31 expression (F, orange line) compared to the positive control (F, blue line). The red line (F) indicates staining with isotype-matched control antibody.
3.2 Expression of CCR1 on MSMCs.

We performed flow cytometry analysis to characterize the effects of the addition of both IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) on MSMCs surface expression of CCR1. At confluence, cells were cultured in the absence of cytokines or in the presence of cytokines for 48h at 37°C. Cells were then examined to determine the percentage of cells that expressed CCR1, as well as the mean fluorescence intensity of this expression. Flow cytometry analysis revealed that addition of both IFN-γ (10 ng/ml) and TNF-α (10 ng/ml) caused higher mean fluorescence intensity of CCR1 expression (Fig 2C) than in the absence of cytokines (Fig 2B). In Fig 2, an APC-labeled Ab against CCR1 was used. CCR1 expression on MSMCs was further analyzed by immunofluorescent staining. Immunofluorescence showed that cytokine-stimulated MSMCs displayed a high percentage of cells stained positive for CCR1 (Fig 2E), confirming the previous results obtained by flow cytometry.
Figure 2. Flow cytometry analysis of CCR1 expression by unstimulated and cytokine-stimulated MSMCs. A. MSMCs were gated on FSC and SSC to exclude dead cells. B-C. The histograms show the relative fluorescence intensity of the cells stained with anti-CCR1 antibody (blue line) compared with the corresponding isotype control (red line). Confluent MSMCs (passages 4-13) were cultured in the absence of cytokines (B) or in the presence of cytokines for 48 hours (C) and analyzed by flow cytometry for cell surface expression of CCR1. Higher CCR1 expression is seen on cytokine-stimulated MSMCs (C) than for unstimulated cells (B).
Figure 2D-E. Immunofluorescent staining of CCR1 in MSMCs. Immunofluorescent analysis of CCR1 (red) expression in MSMCs. MSMCs were stimulated with or without IFN-γ (10 ng/ml) and TNF-α (10 ng/ml). Cytokine-stimulated MSMCs showed a higher percentage of cells stained positive for CCR1 (E) than for unstimulated cells (D). Magnification is 20X.
3.3 CCR1 expression increases at 30 h and 48 h

In order to characterize the time course of maximal CCR1 expression, CCR1 expression on the cell surface was also determined during a longer time course. Time course analysis of CCR1 expression was determined 6, 12, 18, 24, 30, 36, 42, and 48 hours following treatment of MSMCs with both IFN-\(\gamma\) (10ng/ml) and TNF-\(\alpha\) (10ng/ml). The percentage of CCR1 expressing cells, defined as CCR1- positive cells, showed peak expression at 30 h to 48 h after treatment (Fig 3).
**Figure 3. Time course analysis of CCR1 surface expression on MSMCs.** A. MSMCs were gated on FSC and SSC to exclude dead cells. B. Cytokine- stimulated MSMCs were stained with anti-CCR1-APC after the indicated time in culture. IFN-γ and TNF-α – stimulated MSMCs express CCR1 with a peak expression between 30 h and 48 h after cytokine stimulation.
3.4 Addition of RANTES and MIP-1α induce calcium mobilization in MSMCs

Receptor expression alone does not prove functionality. Chemokine binding to chemokine receptors should induce several intracellular changes, including mobilization of intracellular calcium\textsuperscript{21}. Therefore calcium mobilization studies were performed to further characterize whether the chemokine receptor expressed on MSMCs was functionally active. MSMCs were stimulated with cytokines for 48 h at 37°C, and then allowed to take up Fluo-4 (2 µM) at 37°C for 10 min. These cells were then treated with 100 ng/ml MIP-1α or RANTES. Treatment of cytokine-stimulated MSMCs with RANTES (Fig 4D) and MIP-1α (Fig 4E) caused an increase in intracellular Ca\textsuperscript{2+} concentration. To establish the maximal Ca\textsuperscript{2+} flux, MSMCs were stimulated with 2 µM ionophore (Fig B). Platelet-derived growth factor-B (PDGF at 50 ng/ml; Fig C) was used as a positive control. The FACS analysis indicated that the CCR1 expressed in MSMCs was functional based on response to ligands, RANTES and MIP-1α.

In addition, we examined whether the induction of Ca\textsuperscript{2+} mobilization by RANTES and MIP-1α occurred through CCR1. Anders et al. demonstrated that blockade of CCR1 by BX471, a CCR1 antagonist, reduced leukocyte infiltration and renal fibrosis after unilateral ureter obstruction (UUO)\textsuperscript{16}. Therefore, Fluo-4 preloaded MSMCs were treated with BX471 (250 nM) to test its ability to inhibit agonist-induced Ca\textsuperscript{2+} mobilization in CCR1 expressing MSMCs. Preliminary data showed that BX471 inhibited Ca\textsuperscript{2+} mobilization induced by RANTES (Fig 4G) and MIP-1α (Fig 4H), demonstrating that BX471 is a CCR1 specific antagonist. When administered alone, BX471 did not induce Ca\textsuperscript{2+} mobilization, indicating that it has no internal agonistic activity (Fig 4F).
Figure 4. Measurement of Ca^{2+} in cultured MSMCs in response to MIP-1α and RANTES. A. Forward (size) vs. side (granularity) scatter and the enclosed area was the population analyzed in B, C, D, and E. B-E. The effect of different agonists on intracellular Ca^{2+} concentration in Fluo-4 preloaded MSMCs was studied by flow cytometry. Cytokine-stimulated MSMCs were treated with either RANTES (100 ng/ml)
or MIP-1α (100 ng/ml), and intracellular Ca$^{2+}$ was measured for at least 2 minutes.

Ionophore (2 µM) and PDGF-B (50 ng/ml) were used as positive controls. RANTES (D) and MIP-1α (E) induced Ca$^{2+}$ mobilization in MSMCs as well as the positive controls, ionophore (A) and PDGF-B (B). F-H Fluo-4 preloaded MSMCs were pretreated with BX471 for 15 minutes then treated with CCR1 chemokines, MIP-1α (100 ng/ml) and RANTES (100 ng/ml). BX471 inhibited the ability of CCR1 chemokines, RANTES (G) and MIP-1α (H) to increase intracellular Ca$^{2+}$ concentration. BX471 alone did not induce Ca$^{2+}$ mobilization (F). Data are shown as representative experiment (n=1).
DISCUSSION

Intimal hyperplasia in response to vascular insults is the fundamental pathological event in most vascular diseases\textsuperscript{12}. SMC and BM-derived cells can migrate into vessel intima following injury, resulting in vascular stenosis\textsuperscript{13,20}. The specific chemokines that coordinate these events and the molecular mechanisms that underlie the recruitment of SMCs to the intima are largely unknown. In this study, we aimed to identify chemokine receptors on MSMCs that are induced by inflammatory stimuli to drive their recruitment to intima.

Using primary MSMCs cultures from uninjured aortas, the key findings of this study include: a) MSMCs can be cultured at >95% homogeneity as determined by SMC marker expression; b) pro-inflammatory cytokines, IFN-\(\gamma\) and TNF-\(\alpha\), significantly up-regulate CCR1 expression on MSMCs as shown by flow cytometry; c) among the known CCR1 chemokines, RANTES and MIP-1\(\alpha\) cause an increase in intracellular Ca\(^{2+}\) on cytokine-stimulated SMCs, suggesting that CCR1 expressed on MSMCs in response to IFN-\(\gamma\) and TNF-\(\alpha\) is functional; and d) blockade of CCR1 by BX471 abolished the ability of RANTES and MIP-1\(\alpha\) to induce Ca\(^{2+}\) mobilization. Taken together, the results suggest that functional CCR1 is expressed on cytokine-stimulated MSMCs.

The markers that are used to identify SMCs are well established\textsuperscript{10}. MSMC cultures derived from uninjured aortas were >95% SMC as demonstrated by specific SMC markers, including \(\alpha\)-SMA, and calponin, although SM-MHC was expressed in MSMCs to a lesser extent than the other two markers. To investigate whether CCR1 is
expressed on MSMCs, we stimulated MSMCs with IFN-γ and TNF-α; these two pro-inflammatory cytokines were found to effectively induce CCR1 expression on cultured MSMCs.

Chemokines are important chemoattractant that regulate inflammatory cell trafficking and promote SMC migration and proliferation\textsuperscript{21, 23, 25}. Previous studies demonstrated that a high level of chemokines including RANTES, MCP-1 (Monocyte chemotactic protein-1), MIP-1α, IP-10 (Interferon gamma-induced protein 10), and I-TAC (Interferon-inducible T cell-chemoattractant) are present in intimal lesions and could conceivably regulate SMC recruitment to the intima\textsuperscript{11}. Shimizu et al. found that RANTES served as a chemoattractant to direct iSMC migration and proliferation in intimal lesions through the activation of CCR1. RANTES can be produced by activated ECs, T cells, macrophages, and platelets\textsuperscript{11}. In order to investigate whether CCR1 expression on MSMCs was functional, an agonist-induced Ca\textsuperscript{2+} mobilization assay was performed. Preliminary data demonstrated that both RANTES and MIP-1α caused increases in intracellular Ca\textsuperscript{2+} concentration. This signaling was abrogated by BX471 (CCR1 antagonist) blockade; our results, not yet confirmed, showed that BX471 inhibited Ca\textsuperscript{2+} mobilization induced by RANTES and MIP-1α. Experiments are under way to demonstrate that the RANTES and MIP-1α (CCR1 ligands) are responsible for the Ca\textsuperscript{2+} mobilization. Additional data (not shown) demonstrated that cytokine-stimulated MSMCs, also exhibited increased chemotaxis to stable gradients of cultured supernatants from activated endothelial cells. The preliminary data suggests that cytokine-stimulated SMCs may be induced to migrate via a CCR1-regulated pathway.
Shimizu et al. examined the expression of CCR1 on both MSMCs and iSMCs and reported it to be absent on activated MSMCs\(^{11}\). A possible explanation of this apparently discrepant result is that the number of passages or the cellular confluence at the time of testing was different. Our work shows that the cellular density has a significant effect on the CCR1 expression in the setting of IFN-\(\gamma\) and TNF-\(\alpha\) stimulation. In addition, Shimizu et al. stimulated MSMCs with IFN-\(\gamma\) and TNF-\(\alpha\) for only 24 hours. However, our timecourse study revealed that peak expression of CCR1 in MSMCs -stimulated with cytokines occurs between 30 h and 48 h (Fig 3).

Although CCR1 expression was present on nearly all cytokine-stimulated MSMCs, mobilization of intracellular calcium after ligand binding (RANTES and MIP-1\(\alpha\)), was restricted to a subpopulation in MSMCs (Fig 4A), likely reflecting cell viability. We hypothesize that in addition to CCR1 expression, cytokine stimulation of MSMC induces a pro-apoptotic state. If these activated MSMCs do not bind to CCR1 ligands, they undergo a programmed cell death. Consequently, the CCR1 expression level and binding of RANTES and MIP-1\(\alpha\) to CCR1 do not correlate with calcium flux.

Our results suggest that MSMCs can be stimulated to express the chemokine receptors, CCR1. This up-regulation of functional CCR1 expression on cytokine-stimulated MSMCs may facilitate recruitment into intimal lesions. Further work will be required to demonstrate chemotaxis on activated MSMCs to stable gradients of CCR1 ligands. In addition, siRNA knock down of selected endothelial-derived chemokines will allow the identification of the relevant mediators.
### LIST OF JOURNAL ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acta Med Indones-Indones</td>
<td>The Indonesian Journal of Internal Medicine</td>
</tr>
<tr>
<td>Annu Rev Immunol</td>
<td>Annual Review of Immunology</td>
</tr>
<tr>
<td>Annu Rev Path</td>
<td>Annual Review of Pathology</td>
</tr>
<tr>
<td>Arterioscler Thromb Vasc biol</td>
<td>Arteriosclerosis, Thrombosis, and Vascular Biology</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Atherosclerosis Journal</td>
</tr>
<tr>
<td>Bio Pharm</td>
<td>Biochemical Pharmacology</td>
</tr>
<tr>
<td>Can, J. Physiol. Pharmacol</td>
<td>Canadian Journal of Physiology and Pharmacology</td>
</tr>
<tr>
<td>Circ Res</td>
<td>Circulation Research</td>
</tr>
<tr>
<td>Curr Opin Organ</td>
<td>Current Opinion in Organ Transplantation</td>
</tr>
<tr>
<td>Curr Atheroscler Rep</td>
<td>Current Atherosclerosis Reports Journal</td>
</tr>
<tr>
<td>FASEB Journal</td>
<td>The Journal of the Federation of American Societies for Experimental Biology</td>
</tr>
<tr>
<td>Histo Histopathol</td>
<td>Histology and Histopathology</td>
</tr>
<tr>
<td>Immunity</td>
<td>Immunity</td>
</tr>
<tr>
<td>J Clin Invest</td>
<td>Journal of Clinical Investigation</td>
</tr>
<tr>
<td>Nature</td>
<td>Nature</td>
</tr>
<tr>
<td>Physiol Rev</td>
<td>Physiological Reviews</td>
</tr>
<tr>
<td>Transplant Proc</td>
<td>Transplantation Proceedings</td>
</tr>
</tbody>
</table>
REFERENCES


VITA

Sunyoung Jang

Division of Vascular Biology
Brigham and Women's Hospital
77 Ave Louis Pasteur. NRB 754
Boston, MA 02115
Tel: (617) 525-4994

263 Harvard st., Apt 8
Cambridge, MA 02139

1983.................................................Born-Seoul, Republic of Korea

February 2008.................................Bachelor of Science, Department of
Biology, University of Massachusetts,
Amherst, MA

September 2012 – May 2014.......................Graduate student, Pathology and
Laboratory Medicine, Boston University
School of Medicine, Boston, MA

February 2008 – Present.............................Research assistant, Department of
Pathology, Brigham and Women’s
Hospital, Boston, MA