Protein Kinase C-Delta Mediates Adventitial Cell Migration Through Regulation of Monocyte Chemoattractant Protein-1 Expression in a Rat Angioplasty Model

Yi Si, Jun Ren, Pu Wang, Debra L. Rateri, Alan Daugherty, Xu-Dong Shi, K. Craig Kent, Bo Liu

Objective—The adventitia is increasingly recognized as an important player during the development of intimal hyperplasia. However, the mechanism of adventitial cell recruitment to the subintimal space remains largely undefined. We have shown previously that gene transfer of protein kinase C-delta (PKCδ) increases apoptosis of smooth muscle cells following balloon injury. In the current study, we investigated a potential role of PKCδ in regulating the recruitment of adventitial cells.

Methods and Results—Conditioned media from PKCδ-overexpressing smooth muscle cells stimulated migration and CCR2 expression of adventitial fibroblasts through a MCP-1 dependent mechanism. Following balloon injury of rat carotid arteries, overexpression of PKCδ in smooth muscle cells significantly increased MCP-1 and CCR2 expression and the number of adventitia-originated cells detected in the neointima. Administration of an anti-MCP–1 antibody markedly diminished the recruitment of adventitial cells. Combined PKCδ overexpression and anti-MCP–1 inhibited intimal hyperplasia more effectively than either approach alone.

Conclusion—Our data suggest that PKCδ regulates recruitment of adventitial cells to the neointima via a mechanism involving upregulation of the MCP-1/CCR2 signaling axis in injured arteries. Blockage of MCP-1 while enhancing apoptosis may serve as a potential therapeutic strategy to attenuate intimal hyperplasia. (Arterioscler Thromb Vasc Biol. 2012;32:943-954.)

Key Words: apoptosis ■ carotid arteries ■ restenosis ■ vascular biology ■ intimal hyperplasia

Intimal hyperplasia is a primary cause of restenosis after vascular interventions, including balloon angioplasty, stenting, endarterectomy, and bypass.1 Neointimal formation is a complex process, triggered by injury to endothelial cells as well as vascular smooth muscle cells (SMCs). Sensing the intimal and medial injury, the tunica adventitia responds with robust cell proliferation, matrix remodeling, and elaboration of inflammatory cells and cytokines.2,3 In addition to fibroblasts/myofibroblasts and resident immune cells, stem cells or progenitor cells are now believed to reside within the adventitia.4-6 Evidence supporting the potential migration of adventitial cells through the media and into the neointima is derived mostly from animal studies involving selective labeling of adventitial cells or transplanting cultured adventitial cells onto the adventitial side and monitoring movement of these cells following arterial injury. Although several cell types residing within the adventitia have the potential to directly contribute to the intimal lesion through migration, adventitial fibroblasts have been investigated most extensively in this content. Substantial experimental data suggest that adventitial fibroblasts may respond to arterial injury by transforming to a migratory SMC-like myofibroblast phenotype.7-9 However, the mechanism of adventitial fibroblasts sensing endothelial injury and subsequently initiating migration is poorly understood. Because adventitial fibroblasts do not have direct cell–cell contact with endothelial or SMCs, it is plausible to propose that fibroblasts are recruited into the subintimal space by soluble factors released by injured endothelium or/and SMCs.

We have shown previously that protein kinase C (PKCδ), a member of the PKC family of serine-threonine kinases, plays a critical role in mediating vascular injury response. PKCδ expression is upregulated in human restenotic lesions where it colocalizes with apoptotic SMCs. Mice that lack PKCδ develop exacerbated intimal hyperplasia associated with diminished medial SMC apoptosis when subjected to vein grafting or carotid ligation.10,11 Furthermore, gene transfer of PKCδ in the rat carotid angioplasty model inhibits intimal hyperplasia, which is associated with a profound upregulation of apoptotic activity within medial SMCs.10

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Recent data from our laboratory suggest that PKCδ also regulates the production of monocyte chemoattractant protein-1 (MCP-1) in vitro. Being able to mediate both apoptosis and chemokine expression, PKCδ is poised as a potential mechanism underlying the recruitment of adventitial cells by injured medial SMCs.

MCP-1 is a member of the small inducible gene family. Increased MCP-1 expression has been reported in injured arteries. Blocking MCP-1 signaling through the use of neutralizing antibodies or gene deletion of the MCP-1 receptor CCR2 attenuates intimal hyperplasia in experimental artery injury models. Interestingly, inhibition of the MCP-1 signaling pathway was found to reduce intimal hyperplasia without altering macrophage infiltration, although the MCP-1/CCR2 axis is mostly known for its role in the recruitment of monocytes and other types of inflammatory cells. Similarly, we did not detect any significant alterations in inflammatory infiltration following PKCδ gene transfer and arterial injury. This observation further motivated the present study in which we examined whether PKCδ through MCP-1 regulates the cell--cell communication between SMCs and fibroblasts in vitro and in vivo. In addition, we investigated whether inhibition of the MCP-1 signaling attenuates migration of the adventitial cells through the media and into the subintimal space.

Materials and Methods

Animal Model

After induction of anesthesia with 2.5% isoflurane, arterial injury was induced in male Sprague-Dawley rats (2~3 months, ~350g) by means of carotid balloon angioplasty as described before. Gene transfer to medial SMCs was achieved by intraluminal perfusion with adenoviruses that expresses PKCδ (AdPKCδ) or empty vector (AdNull) as described previously. Cells in the perivascular zone or adventitia were labeled with an adenovirus expressing LacZ (Ad-LacZ) administered via pluronic gel on the adventitial surface prior to wound closure as described elsewhere. A sham group underwent surgery without balloon angioplasty/intraluminal viral infection. In a subset of studies, two sequential survival surgery procedures were performed. During the first procedure, AdLacZ was administered to the adventitial surface of left common carotid arteries. Three days later, the second procedure was performed to administer angioplasty balloon injury to the AdLacZ treated arterial segment. In the antibody administration study, mice receiving either AdNull or AdPKCδ perfusion were assigned randomly to administration of either an anti-MCP-1 antibody or an IgG control group. Anti-MCP-1 (Biolegend, CA, 1 mg/kg) or equivalent amounts of nonimmunized hamster IgG (Biolegend, CA, 1 mg/kg) were injected via tail vein 30 minutes before arterial injury, followed by 2 additional injections 12 and 24 hours, as described by Furukawa et al. Arteries were harvested on days 3, 7, and 14 by perfusion fixation with 4% paraformaldehyde at physiological pressure (90 mmHg).

X-gal staining was performed by using X-gal staining assay kit (Genlantis, CA). Unless it stated otherwise, 3 animals were used for each group. All experimental protocols were approved by the Institute Animal Care and Use Committee at University of Wisconsin Madison (#MO02285) and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH Publication No. 85 to 23, 1996 revision.

Statistical Analyses

Values were expressed as mean±SEM derived from at least 3 independent experiments unless stated otherwise. Differences between two groups were analyzed by 2-tailed Student t test since all data conformed to the constraints of parametric analyses. For comparisons between more than two sets of experimental conditions, one-way analysis of variance (ANOVA) was performed with post hoc testing using a Fisher least significant difference test. Probability values less than 0.05 were considered as statistically significant.

Other methods are detailed in the Materials and Methods section in the online-only Data Supplement.

Results

PKCδ-Expressing SMCs Attracted Adventitial Fibroblasts Through MCP-1

To determine the molecular mechanism underlying cell--cell communication between medial SMCs and adventitial cells, we isolated SMCs and fibroblasts from the media and adventitia of rat carotid arteries. Isolated arterial SMCs were characterized by immunostaining of smooth muscle-specific α-actin (SMA) and calponin (Figure I in the online-only Data Supplement). Adventitial fibroblasts were positive for the fibroblast marker ER-TR7 and mesenchymal marker thy1.1 but negative for calponin (Figure I). A large portion of cultured fibroblasts expressed SMA, albeit to a lesser degree compared to SMCs (Figure I), indicating spontaneous transformation to myofibroblasts during in vitro manipulations. However, expression of CD68, a macrophage marker, was undetectable in either SMC or fibroblast cultures (Figure I).

A transwell chemotaxis assay was used to study fibroblast migration in vitro. Compared to control media (DMEM supplemented with 0.5% FBS), media conditioned by SMCs significantly increased the number of adventitial fibroblasts migrated through a porous membrane (7.4±3.1 versus 22.3±6.9). To mimic high PKCδ expression in SMCs of injured arteries, we infected SMCs with AdNull (empty viral vector), AdPKCδ, or a kinase dead mutant of PKCδ. Compared to AdNull-infected SMCs, SMCs infected with AdPKCδ but not its kinase dead form stimulated migration of fibroblasts by 80% (Figure 1A). We further activated exogenous PKCδ with 1 nmol/L PMA. At this concentration, PMA, by itself, had minimum effects on SMCs but further increased PKC-associated chemoattractant properties (Figure 1A). In parallel, AdPKCδ-infected SMCs had substantially increased abundance of MCP-1 protein and mRNA compared to the AdNull controls (Figure 1B and 1C). We also analyzed mRNA expression of several other chemokines and cytokines including stromal cell-derived factor-1 (CXCL12), RANTES (CCL5), TNF-α, PDGF-BB, IFN-γ, IL-6, and IL-1β that are known to be associated with vascular injury and remodeling. In this small panel of factors, only IL-6 mRNA was upregulated significantly by PKCδ (Figure II in the online-only supplement). However, the magnitude of IL-6 induction was much less than that of MCP-1.

Next, we determined the role of MCP-1 in communication between SMCs and fibroblasts by determining whether purified MCP-1 is capable of attracting fibroblasts. As shown in Figure 1D, recombinant MCP-1 concentrations-dependently stimulated migration of fibroblasts. To further validate that migration of fibroblasts is indeed caused by MCP-1, we repeated the migration assay in the presence of a neutralizing antibody specific to MCP-1. Neutralizing MCP-1 greatly diminished the ability of recombinant MCP-1 or SMCs to attract fibroblasts (Figure 1E and 1F).
Figure 1. PKCδ-expressing SMCs stimulated adventitial fibroblasts migration via MCP-1. A, B, Medial SMCs were infected by AdNull (empty viral vector), AdPKCδ, or AdPKCδKD (a kinase dead mutant of PKC) followed by PMA treatment (1 nM, 12 h). Media conditioned by the control and virus-incubated SMCs were harvested to induce migration of adventitial fibroblasts (A) or to determine MCP-1 protein.
We have shown previously that overexpression of PKCδ causes SMC apoptosis. To determine whether MCP-1 is produced by apoptotic SMCs or their surviving neighbors, we coimmunostained cultured SMCs for MCP-1 and TUNEL. Confocal analysis showed that overexpression of PKCδ increased MCP-1 expression in both TUNEL positive and negative cells (Figure 1G).

To exclude the possibility that conditioned media may contain significant amount of adenovirus lingered from infection of SMCs, we evaluated the expression of exogenous PKCδ in fibroblasts using an antibody against the FLAG tag that is engineered in AdPKCδ. No FLAG-tagged PKCδ band was detected in fibroblasts, although it was abundantly present in AdPKCδ-infected SMCs (Figure III in the online-only Data Supplement). These results indicated that the effects of PKCδ on fibroblasts migration, at least in our transwell assay, is indirect and is achieved through secretion of chemoattractant molecules by SMCs.

**MCP-1 Attracted Fibroblasts Through Induction of CCR2**

The chemoattractant effect of MCP-1 has been extensively studied in monocytes and macrophages and is believed to be mediated predominantly by its receptor CCR2. We therefore examined CCR2 expression in migrating fibroblasts collected from the bottom wells at the end of the transwell assay. As shown in Figure IV in the online-only Data Supplement, migrated fibroblasts were positive for CCR2, SMA, and thy.1 but negative for calponin and CD68. However, CCR2 protein was undetectable in the naïve or general population of adventitial fibroblasts (Figure IV). We then determined whether exposure to MCP-1 during the chemotaxis assay induces CCR2 expression in adventitial fibroblasts. As shown in Figure 2A, incubating with recombinant MCP-1 triggered a rapid and transient increase in CCR2 mRNA abundance. This induction was blocked by incubation with an anti-MCP–1 neutralizing antibody (Figure 2B). Conditioned media derived from AdPKCδ-infected SMCs produced a similar effect on CCR2 mRNA abundance in fibroblasts (Figure 2C). Finally, siRNA knocking down of CCR2 mRNA abundance in fibroblasts markedly reduced MCP-1 induced migration (Figure 2D).

In contrast, similar amounts of SMA were detected in fibroblasts incubated with conditioned media made by SMCs infected with AdNull, AdPKCδ, or kinase dead mutant of PKCδ (Figure V in the online-only Data Supplement), suggesting the stimulatory effect of PKCδ-expressing SMCs on CCR2 mRNA abundance was likely to be specific, and not secondary to phenotypic transformation.

**MCP-1/CCR2 Signaling Axis Was Upregulated by PKCδ in Injured Arteries**

After having established a role of PKCδ-mediated MCP-1 expression in cell–cell communication between medial SMCs and adventitial fibroblasts, we determined whether the MCP-1/CCR2 signaling axis was upregulated by PKCδ in injured arteries. To this end, we administered AdPKCδ or AdNull through luminal infusion following carotid balloon injury, a method that has been demonstrated previously to transfer genes predominantly in the medial layer of the carotid artery wall. Indeed, elevated PKCδ expression produced by AdPKCδ luminal infusion was predominantly in the tunica media (Figure VIA and VIB in the online-only Data Supplement). Consistent with our previous report, AdPKCδ reduced the I/M ratio by 33.8% and caused extensive SMC apoptosis (Figure VIC and VID). After validating the effectiveness of PKCδ gene transfer, we evaluated whether PKCδ overexpression upregulated the MCP-1/CCR2 axis following arterial injury. Noninjured arteries (sham) contained undetectable MCP-1 or CCR2 protein (Figure 3A and 3B), which was confirmed by qPCR (data not shown). Both MCP-1 and CCR2 protein became clearly noticeable after injury (Figure 3A and 3B). Additionally, PKCδ gene transfer further increased abundance of MCP-1 and CCR2 protein by 4- and 2.4-fold, respectively. The MCP-1 upregulation peaked around 7 days and subsided by 14 days postinjury (Figure 3A and D). The presence of MCP-1 was detected predominantly in the media, although a few MCP-1 positive cells were detectable in adventitia. Confocal imaging confirmed the spatial association between MCP-1 and PKCδ-overexpressing cells (Figure 3C). Interestingly, CCR2 induction showed different kinetics. Temporally, CCR2 induction persisted to at least 14 days after injury (Figure 3B and 3E). Spatially, the CCR2-positive cells first accumulated in the adventitia (Figure 3B). As the arterial injury response proceeded, significant number of CCR2-positive cells became detectable in the media-intima region (Figure 3B), presumably due to migration of adventitial cells toward the luminal side. To quantify the CCR2 induction, we harvested the injured carotid arteries and analyzed CCR2 mRNA abundance by quantitative RT-PCR. As shown in Figure 3F, AdPKCδ increased the CCR2 mRNA abundance by 3.5-fold.

We next determined whether adventitial myofibroblasts of injured arteries were a source of CCR2 stimulated by injury and PKCδ gene transfer. Arterial sections harvested 7 days after the carotid injury were costained with antibodies specific to CCR2 and SMA. Although abundant in vascular SMCs, SMA is also expressed in myofibroblasts in the adventitia and has been widely used as a marker for this.
population of fibroblasts. Confocal microscopic analyses showed that significant SMA/CCR2 double positive cells were present in the adventitial of arteries (Figure 4A). As expected, infiltrated CD68+ macrophages expressed CCR2 as well (Figure 4B). Pearson’s coefficient analyses indicated that colocalization between CCR2 and SMA was significantly higher in AdPKCδ/Arteries compared to AdNull group (Figure 4C). However, PKCδ gene transfer had no effect on macrophage infiltration or CCR2/CD68 colocalization (Figure 4D and Figure VII in the online-only Data Supplement).

**MCP-1/CCR2 Axis Mediated Adventitial Cell Migration and Contributed to Intimal Hyperplasia**

To study the migration of adventitial cells in injured arteries, we labeled adventitial cells by applying AdLacZ to the adventitial surface of balloon injured arteries as described previously by Siow and colleagues. In agreement with this report, we detected LacZ positive cells only in the adventitia of noninjured (sham) arteries (Figure 5A and 5B), suggesting that perivascular administration of AdLacZ did not lead to significant gene transfer in medial SMCs or endothelial cells. In contrast, LacZ positive cells were readily recognized throughout the arterial wall of injured arteries presumably due to active migration of adventitial cells such as myofibroblasts (Figure 5A and 5B). To rule out the possibility that AdLacZ may also migrate from the adventitia to inner side through increased vasa vasorum of injured arteries and directly infect medial SMCs, we performed balloon injury 3 days after viral infection when excessive viral particles have been cleared or become inactivated. A similar intimal/medial LacZ expression was primarily resulted from migration of adventitial cells rather than adenoviruses.
Figure 3. Expression of MCP-1 and CCR2 was upregulated in injured carotid A, B, Representative arterial sections harvested 3, 7 and 14 days after angioplasty from sham, injured and AdNull-treated, or injured and AdPKCδ-infected rats were immunostained for MCP-1 (A) or CCR2 (B). Nuclei were indicated by positive stains to DAPI (blue). Internal and external elastic laminas are indicated by arrows and arrow heads, respectively. Scale bar=50 mm. C, Representative confocal images of arterial sections harvested 3 days after angioplasty from sham, injured and AdNull-treated, or injured and AdPKCδ-treated rats. Tissue sections were immunostained for MCP-1 (red), PKCδ (green) and nuclei (DAPI blue). Internal and external elastic lamina are indicated by arrows and arrow heads, respectively. Scale bar=50 μm. D, E, Quantification of MCP-1 and CCR2 immunostaining. F, Levels of CCR2 mRNA in injured carotid arteries were determined by qPCR. Total RNA were extracted from injured carotid arteries 14 days after luminal perfusion of AdNull or AdPKCδ. n=3; *P <0.01.

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Figure 4. CCR2 positive myofibroblasts, but not macrophages, were increased in PKC-overexpressed arteries. A, B, Representative confocal microscopic images of arterial sections co-immunostained for CCR2 (red), SMA (green in A) or CD68 (green in B). Injured arteries were harvested 7 days after luminal perfusion of AdNull or AdPKCδ following balloon injury. DAPI (blue) indicates nuclei. External elastic lamina is indicated by arrows and double positive cells are indicated by arrow heads. Scale bar = 50 μm. C, D, Pearson’s coefficient analysis for colocalization between CCR2 and SMA (C) or CD68 (D). n=3; *P<0.05.
Figure 5. A, Representative images of X-gal stained carotid arterial sections 3, 7 and 14 days after extraluminal administration of AdLacZ via pluri-
	ronlic gel. In two of the three rat groups, arteries were injured with balloon catheters followed by luminal perfusion with AdNull or AdPKCδ. Scale 

bar=200 μm. B, Magnified images of X-gal stained carotid arterial sections at 14 days. Scale bar=50 mm. Internal and external elastic laminas are 

indicated by arrows and arrow heads, respectively. C, D, Quantification of LacZ-positive cells in indicated layers of injured arteries at 3 and 7 days 

(C) or 14 days (D). n=3. *P <0.01.
Figure 6. Migration of adventitial cells depended on MCP-1/MCP-1/CCR2 signaling. A, Representative images of X-gal stained carotid arterial sections 14 days balloon injury and adenovirus infection. Rats were randomly assigned to 2 groups, each received tail vein injection of either a neutralizing antibody to MCP-1 (1 mg/kg) or nonimmunized hamster IgG (1 mg/kg) 30 minutes before injury, followed by two additional injections administered 12 and 24 h later. Internal and external elastic laminas are indicated by arrows and arrow heads, respectively. Scale bar = 50 μm, and 200 μm in 40× magnification. B, Quantification of LacZ-positive cells in the neointima/media region. n = 3. *P < 0.05. C, The intimal to medial area ratio or I/M ratio measured 14 days after injury. *P < 0.01 as compared to the AdNull/IgG group; †P < 0.01 as compared to the AdNull/Anti-MCP-1 or AdPKCδ/IgG group. D, Representative images of arterial sections immunostained for CCR2 and DAPI 14 days after injury. Scale bar = 50 μm.
Using LacZ as a marker for adventitial originated cells, we determined the effects of PKCδ gene transfer on adventitial cell migration 3, 7, and 14 days following balloon angioplasty. PKCδ gene transfer to medial SMCs markedly increased the number of adventitia-originated cells in the medial-intimal region (Figure 5A–5D). At day 7, the number of LacZ+ cells detected in the medial-intimal area of AdPKCδ-infected arteries was over 7-fold higher than that of the control virus (AdNull)-infected arteries (X-gal positive areas in pixels: 763±178 AdPKCδ versus 90±31 AdLacZ, P<0.01, n=3) (Figure 5A and 5C). By day 14, the majority of LacZ+ or adventitia-originated cells were found to accumulate in the neointima (Figure 5A–D).

To test whether PKCδ-expressing SMCs recruited adventitial myofibroblasts through the MCP-1/CCR2 axis, we administrated a MCP-1 neutralizing antibody before and after carotid injury and adenosinenvascular infection. Compared to the group administered control IgG, the anti-MCP–1 antibody profoundly reduced the number of adventitia-originated cells detected in the medial/intimal region of injured arteries (Figure 6A and 6B), indicating a role of MCP-1 in regulation of adventitial cell migration. Additionally, neutralizing MCP-1 caused further reduction of intimal hyperplasia. The combination of PKCδ gene transfer and anti-MCP–1 inhibited the I/M ratio by 62% as compared to a 36.3% reduction produced by AdPKCδ alone (P<0.01, n=3) (Figure 6C). Of note, inhibition of MCP-1, without PKCδ overexpression, reduced I/M ratio by 30.8%. Finally, CCR2 expression was also diminished by the anti-MCP–1 antibody (Figure 6D), further supporting the notion that arterial expression of CCR-2 was MCP-1 dependent.

**Discussion**

The importance of the adventitia in intimal hyperplasia and vascular remodeling has been illustrated by experimental data accumulated through the past 10 years. Adventitial fibroblasts, as well as resident progenitor cells, have been shown to migrate through the media to neointima in experimental models of intimal hyperplasia. However, other than the involvement of MMPs, molecular mechanisms underlying adventitial cell activation and migration in response to injuries of endothelium and SMCs remain to be delineated. To this end, we examined the effect of overexpressing the proapoptotic factor, PKCδ, on the migration of adventitial cells using a rat carotid balloon injury model. Our results provide evidence that adventitial cells are recruited to the neointima, at least in part, through a PKCδ-MCP-1-CCR2 pathway. As a key mediator of apoptosis, PKCδ has been shown to be upregulated in apoptotic arterial SMCs following balloon injury as well as human restenotic lesion. Manipulation of PKCδ, either through gene deletion or adenosinemediated overexpression, altered the prevalence of apoptosis in the injury vessel wall thus the size of neointimal lesion. The current study demonstrated a new function of PKCδ, which is to mediate the recruitment of adventitial cells through MCP-1. As a result of this dual function, gene transfer of PKCδ to medial SMCs following balloon injury increased not only SMC apoptosis but also the migration of adventitial cells. With an anti-MCP–1 antibody, we separated PKCδ’s proapoptotic function from its role in the recruitment of adventitial cells. In the presence of a MCP-1 neutralizing antibody, gene transfer of PKCδ promoted SMC apoptosis without trigging migration of adventitial cells and therefore produced a significantly greater inhibitory effect on neointimal development as compared to PKCδ gene therapy alone.

We were surprised by the lack of alteration in CD68+ macrophage infiltration in PKCδ-gene transferred arteries because the upregulation of MCP-1 in these arteries was profound. MCP-1 is a well-established inflammatory chemokine that is expressed by SMCs, macrophages, endothelial cells, and fibroblasts. The primary receptor of MCP-1 is CCR2, a member of the family of G-protein coupled chemokine receptors. The MCP-1/CCR2 axis plays a critical role in chemotaxis of monocytes and other inflammatory cells and has been implicated in a variety of inflammatory disease processes including rheumatoid arthritis. MCP-1 is induced in medial SMCs during early atherosclerotic lesion development and in animal models of arterial injury. Mice deficient of MCP-1 or CCR2 developed smaller intimal lesions, albeit the effect of CCR2 deficiency appears to be more dramatic. Additionally, blocking the MCP-1 signaling with a neutralizing antibody or a dominant negative mutant inhibits intimal hyperplasia in various models of arterial injuries, including balloon or wire injury in rodent arteries, vein graft, cardiac allografts and in-stent stenosis. However, whether blocking the MCP-1 signaling affects vascular inflammation appears to differ among these early studies. Inhibition of the MCP-1/CCR2 axis generally does not alter the inflammatory response in vascular injury associated balloon angioplasty of rat carotid and wire injury of C57B6 mice. It is possible that the MCP-1/CCR2 axis contributes to intimal hyperplasia by stimulating SMC proliferation as suggested by several in vitro studies. However, the mitogenic effect of MCP-1 has not been demonstrated in vivo. Using a rat carotid balloon injury model, Furukawa showed that an anti-MCP–1 antibody did not alter intimal medial proliferation although the number of neointimal cells was reduced. These authors also reported that recombinant rat MCP-1 failed to stimulate chemotactic activities of culture SMCs. Using a mouse wire injury model, Kim and colleagues found that MCP-1 deficiency inhibited intimal hyperplasia without affecting medial DNA synthesis. Here, we provide a novel mechanism in which MCP-1, secreted by high PKCδ-expressing SMCs, prompts adventitial cells migration to the neointima. This theory was based on the following observations: (1) recombinant MCP-1 initiated migration of adventitial fibroblasts in vitro; (2) SMCs, through a PKCδ- and MCP1/CCR2-dependent mechanism, caused migration of adventitial fibroblasts in vitro; (3) both MCP-1 and CCR2 were upregulated in PKCδ-overexpressing arteries following carotid balloon injury; and (4) neutralization of MCP-1 with an antibody attenuated the transmedia migration of adventitial cells in injured carotid arteries.

Our immunostaining and qPCR analyses revealed no detectable levels of CCR2 protein and mRNA in adventitial fibroblasts isolated from the carotid arteries of healthy adult rats, which is in agreement with previously published data on
adventitia of young normal blood vessels. However, when adventitial fibroblasts were incubated with either recombinant MCP-1 or MCP-1-rich media conditioned by PKCδ overexpressing SMCs, CCR2 mRNA expression was induced rapidly. In support of this in vitro finding, blocking MCP-1 in vivo with a neutralizing antibody diminished CCR2 protein abundance in PKCδ-overexpressing arteries. The migration of fibroblasts toward MCP-1 or SMC-conditioned media was sensitive to siRNA knockdown of CCR2, suggesting that CCR2 is the primary receptor that transmits the extracellular chemotactic signal into adventitial fibroblasts. The critical role of adventitial CCR2 in intimal hyperplasia has been previously demonstrated by Efthig and colleagues who showed that perivascular overexpression of short hairpin RNA against CCR2 inhibits vein graft thickening in hypercholesterolemic apolipoprotein E3-Leiden mice.

In addition to MCP-1, several other chemokines and their receptors are implicated in the recruitment of progenitor cells during arterial remodeling. Most noticeably, CXC-chemokine stromal cell-derived factor-1α is upregulated in injured arteries and contribute to intimal hyperplasia through a CXCR4-dependent recruitment of smooth muscle progenitor cells. However, our qPCR analysis suggests that PKCδ did not alter stromal cell-derived factor-1α mRNA expression in SMCs, at least in vitro.

One limitation of our study is that perivascular application of AdLacZ nondistinguishably labels cells residing in the adventitia. Additionally, we cannot rule out the possibility that bone marrow-derived progenitor cells become infected by AdLacZ on entering the adventitia. Of note, we observed a subpopulation of CCR2+ cells in the adventitia that are negative for SMA or CD68. The MCP-1/CCR2 axis has been implicated in the adhesion of bone marrow-derived endothelial progenitor cells during re-endothelialization following carotid angioplasty. Moors et al also reported that the MCP-1/CCR2 axis is important for recruitment of bone marrow-derived fibrocytes to the alveolar space after pulmonary fibrotic injury. Furthermore, we have shown in vitro that MCP-1 stimulates migration of mesenchymal stem cells. Although we observed some adventitia-derived cells in the intima that were also positive for CD31 (data not shown), future studies using cell lineage-specific labeling and tracing methods are necessary to prove the role of adventitial cells in endothelial regeneration.

In conclusion, we have demonstrated that PKCδ plays a dual function in arterial injury response. Upregulated in medial SMCs following injury, PKCδ stimulates apoptosis of SMCs and increases MCP-1 expression. Whereas the PKCδ-mediated SMC apoptosis results in diminished intimal hyperplasia, PKCδ-induction of MCP-1 promotes the repair mechanism by activating the CCR2-mediated migration of myofibroblasts and possibly progenitors from the adventitia to the neointima. These findings reiterate the complexity of arterial injury response. Stimulating apoptosis of SMCs may be a logical approach to reduce intimal hyperplasia; however, considerations should be given to potential repair mechanisms including the recruitment of progenitor cells or/myofibroblasts evoked by proapoptotic genes/factors. Future studies aimed to delineate the molecular link between cell injury and repair are necessary for designing effective therapeutic strategies to treat intimal hyperplasia.

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Disclosures

None.

References


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General Material

Fetal Bovine Serum (FBS), Dulbecco’s Modified Eagles Medium (DMEM) and cell culture reagents were from Gibco BRL Life Technologies (Carlsbad, CA). Phorbol 12-myristate 13-acetate (PMA, activator of PKC) was purchased from Enzo Lifesciences (Farmingdale, NY). All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO).

Adenoviral Vectors

Adenoviral vectors expressing PKCδ (AdPKCδ), or PKCδ-kinase dead (AdPKCδKD) were constructed as described previously.1, 2 Virus was amplified in HEK-293 cells (ATCC, VA) and purified by CsCl gradient centrifuging as described previously.3

Cell Culture

Rat carotid arterial smooth muscle cells (SMC) and adventitial fibroblasts were isolated according to a method described previously.4 Both types of cells were maintained in DMEM containing 10% FBS at 37°C with 5% CO2. For experiments using conditioned media, rat SMCs (2×10^5 cells/well in 6-well plates) were infected with adenovirus (30,000 particles/cell) in DMEM containing 2% FBS for 4 hours, followed by starvation in DMEM contained 0.5% FBS for 48 hours. The cells were then incubated with PMA (1nM) or solvent (DMSO) for indicated period of time. Media was collected and used for ELISA or chemotaxis assays as described below.
Immunocytometry for Cell Characterization

SMCs and adventitial fibroblasts were plated in 8-well culture slides (BD Biosciences, San Diego, NJ) at a density of $3 \times 10^4$/ml in low glucose DMEM containing 10% FBS. Immunofluorescent staining was performed with mouse monoclonal anti-α-actin (Abcam, Cambridge, MA, 1:1000); mouse monoclonal anti-calponin (Abcam, Cambridge, MA, 1:1000); rat monoclonal ER-TR7 (Abcam, Cambridge, MA, 1:100), mouse monoclonal thy1.1 (Abcam, Cambridge, MA, 1:500); anti-CD68 (AbD Serotec, Kidlington, UK, 1:250), and donkey anti-mouse Alexa 488 (Invitrogen, Carlsbad, CA, 1:300), donkey anti-rat Alexa 555 (Invitrogen, Carlsbad, CA, 1:300) or donkey anti-goat Alexa 555 (Invitrogen, Carlsbad, CA, 1:300). DAPI was used to identify nuclei. TUNEL (Roche, Indianapolis, IN) was used to identify apoptotic cells following the manufacturer’s instructions. Staining was visualized with a Nikon Eclipse E800 upright microscope or Nikon A1RSi Confocal system. Digital images were acquired using a RetigaEXi CCD digital camera.

Western Blot Analyses

Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 10 µg/ml aprotinin). Thirty micrograms of protein from each sample were separated on 10 % SDS-PAGE gels. The protein samples were then transferred to nitrocellulose membranes. Protein expression was confirmed by immunoblotting with the following antibodies: mouse monoclonal anti-flag M2 antibody, mouse monoclonal anti-α-actin, mouse anti-β-actin and rabbit α/β tubulin (Cell Signaling, Boston, MA). After incubation with the appropriate primary and horseradish peroxidase-conjugated
secondary antibodies (Bio-Rad, Hercules, CA) the membranes were developed with enhanced chemiluminescence reagent (Thermo Fisher Scientific, Rockford, IL).

**Cell Migration Assay**

Fibroblasts were placed in the upper chamber \(2 \times 10^4/\text{well}\) of Costar 24-well transwell plates with 8 \(\mu\)m pore filters (Corning, Inc., Corning, NY). Conditioned media or recombinant MCP-1 (R&D Systems, Minneapolis, MN) diluted in PBS as previously described\(^3\) was placed into the lower chambers. After 4 hours incubation at 37°C, migrated cells accumulated on the bottom of filters were counted. In some experiments, migration was carried out for 12 hours and migrated cells accumulated in lower chambers were harvested and characterized by immunostaining.

**ELISA**

Enzyme-linked immunosorbent assay was used to detect MCP-1 secreted by rat carotid SMC using rat MCP-1 ELISA kit (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions.\(^3\)

**RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)**

Total RNA was isolated from fibroblasts or injured carotid arteries using RNaseasy Plus Mini Kit (Qiagen, Valencia, CA). Potential contaminating genomic DNA was removed by using gDNA Eliminator columns provided in the kit. Stabilization of RNA harvested from arteries was performed directly by using the RNAlater RNA stabilization Reagent (Qiagen, Valencia, CA) following the manufacturer’s instructions. RNA (2 \(\mu\)g) was used
for the first-strand cDNA synthesis (Applied Biosystems, Carlsbad, CA). To confirm the absence of DNA contamination in RNA samples, control reverse transcription was performed in the absence of reverse transcriptase. qRT-PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Each cDNA template was amplified in triplicate using SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) with gene specific primers. Primer for rat MCP-1, SDF-1 (CXCL12), RANTES (CCL5), TNF-α, PDGF-BB, IFN-γ, IL-6, and IL-1β were purchased from Qiagen (Valencia, CA). CCR2 primers were 5’-CTCTGACCTGCTCTTCCTGC-3’ and 5’- CCAATGTGATAAAGCCCTGTG -3’, and GAPDH primers were 5’- CGGCAAGTTCAACGGCACAG -3’ and 5’- GACGCCAGTAGACTCCACGACAT -3’. All samples were analyzed by melting curve analyses, and sample concentrations were determined by standard curves method.

**Morphometric Analysis and Immunohistochemistry**

OCT embedded arteries were cut into 5 μm sections. X-gal staining was performed following the protocol provided by the manufacturer (Genlantis, San Diego, CA). Morphometric analyses were carried out as described previously. For each artery, 10 non-consecutive sections were selected for analysis using Image J 1.42q and Photoshop CS4. Immunofluorescent staining was performed with anti-MCP-1 (Santa Cruz, CA, 1:50), goat anti-CCR2 (Santa Cruz, CA, 1:50), anti-CD68 (AbD Serotec, Kidlington, UK, 1:250), mouse anti-α-actin (1:500), and donkey anti-mouse Alexa 488 (Invitrogen, Carlsbad, CA, 1:300) or donkey anti-goat Alexa 555 (Invitrogen, Carlsbad, CA, 1:300). DAPI was used to identify nuclei. Staining was visualized with a Nikon Eclipse E800.
upright microscope and digital image were acquired using a RetigaEXi CCD digital camera. Double staining areas were analyzed by using NIS Elements Advanced software. Confocal images were taken with Nikon A1RSi Confocal system with the appropriate argon beam laser. Pearson’s coefficient analysis was done by using NIH image software (ImageJ 1.42q). Pearson coefficient values range from -1 to 1, where the value close to 1 means a perfect correlation and a high value (>0.7) indicate reliable colocalization. Each point in the graph represents a Pearson correlation coefficient for each sample.
Supplemental Figure III

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Western blots showing expression of FLAG and β-actin.
Supplemental Figure IV

A

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B

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Supplemental Figure V

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Supplemental Figure VII
Supplemental Figure VIII

Sham

Injury
Supplemental Figure Legends

**Supplemental Figure I. Characterization of isolated fibroblasts and SMCs.**
Representative images of isolated fibroblast and smooth muscle cell from carotid artery stained for α-actin, thy1.1, ER-TR7, calponin or CD68. A spleen section of wild type rat stained for CD68 was shown as a control.

**Supplemental Figure II. Cytokines and chemokines mRNA expression in PKCδ-overexpressing SMCs**
Rat carotid SMCs were infected with either AdNull or AdPKCδ followed by incubation with PMA (1nM, 4 hours). mRNA abundance of selected chemokines or cytokines were evaluated by qPCR analyses. Values were expressed as fold changes compared to an AdNull group. n=3. #, p <0.05; *, p <0.01.

**Supplemental Figure III. Evaluation of exogenous PKCδ in fibroblasts cultured with conditioned-media.**
Cell lysates from SMCs infected by AdPKCδ or AdNull, or from fibroblasts incubated with SMC conditioned-media were analyzed by Western blotting using antibody against the FLAG-tag. Different exposure time was applied as 5 mins and 30 mins, respectively.

**Supplemental Figure IV. SMC-conditioned media induces CCR2 expression in fibroblasts.**
A. Representative images of immunostaining. Naïve fibroblasts (naïve FB) and fibroblasts that have migrated into the lower chamber (migrated FB) during the migration assay using SMC-
conditioned media were co-stained for CCR2 with α-actin, thy1.1, calponin or CD68. Scale bar =50μm. B. Representative immunostaining images of spleen sections stained for CCR2. CCR2 KO: CCR2 knockout mouse, WT: wild type mouse. Scale bar =50μm.

Supplemental Figure V. Conditioned media from PKCδ-overexpressing SMCs did not affect α-actin expression in fibroblasts.
Conditioned media was harvested from SMCs infected with AdNull, AdPKCδ, or AdPKCδKD in the presence or absence of PMA (1nM, 12 h) as described in methods. After culturing in conditioned media for 4 hours, fibroblasts were lysed. Cell lysates from fibroblasts were analyzed by Western blotting using antibody against α-actin or tubulin.

Supplemental Figure VI. Overexpressing-PKCδ in medial SMC of injured artery moderately inhibited intimal hyperplasia.
A. Representative images of arterial section immunostained for PKCδ (green) and DAPI (blue). Injured arteries were harvested 7d after luminal perfusion of AdPKCδ or empty vector (AdNull) following balloon injury. Scale bar =50μm. B. Quantification of PKCδ immunostaining. C. The intimal to medial area ratio or I/M ratio measured 14 days after injury. n=3. #, p <0.05; *, p <0.01.
D. Representative confocal images of arterial sections harvested 3 days after angioplasty from sham, injured and AdNull-treated, or injured and AdPKCδ-infected rats were immunostained for TUNEL (red), PKCδ (green) and nuclei (DAPI blue). Internal and external elastic lamina are indicated by arrows and arrow heads, respectively. Scale bar =50μm.
Supplemental Figure VII. Overexpression of PKCδ did not affect macrophage infiltration following arterial injury.

Representative images of carotid sections co-stained for CCR2 (red) and CD68 (green). Injured arteries were harvested 7d after luminal perfusion of AdPKCδ or empty vector (AdNull) following balloon injury. DAPI (blue) was stained for nucleus. Scale bar =50 μm.

Supplemental Figure VIII. Perivascular AdLacZ administration served as a method to label adventitial cells.

Representative images of X-gal stained carotid arterial sections harvested 7 days after angioplasty. AdLacZ via pluronic gel were administered to the adventitial surface of the left common carotid during the first of the two sequential surgical procedures. Three days later, the second procedure was performed to administer angioplasty balloon injury to the AdLacZ treated arterial segment. Scale bar =50μm.

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