Transforming Growth Factor-beta Promotes Recruitment of Bone Marrow Cells and Bone Marrow-derived Mesenchymal Stem Cells through Stimulation of MCP-1 Production in Vascular Smooth Muscle Cells

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Running title: TGF-β induces MCP-1, a MSC chemoattractant.

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Bone marrow derived progenitor cells have recently been shown to be involved in the development of intimal hyperplasia after vascular injury. TGF-β has profound stimulatory effects on intimal hyperplasia, but it is unknown whether these effects involve progenitor cell recruitment. In this study we found that although TGF-β had no direct effect on progenitor cell recruitment, conditioned media derived from vascular smooth muscle cells (VSMCs) stimulated with TGF-β induced migration of both total bone marrow (BM) cells and BM-mesenchymal stem cells (MSC), and also induced MSC differentiation into smooth muscle like cells. Furthermore, overexpression of the signaling molecule Smad3 in VSMCs via adenovirus mediated gene transfer (AdSmad3) enhanced TGF-β’s chemotactic effect. Microarray analysis of VSMCs stimulated by TGF-β/AdSmad3 revealed monocyte chemoattractant protein-1 (MCP-1) as a likely factor responsible for progenitor cell recruitment. We then demonstrated that TGF-β through Smad3 phosphorylation induced a robust expression of MCP-1 in VSMCs. Recombinant MCP-1 mimicked the stimulatory effect of conditioned media on BM and MSC migration. In the rat carotid injury model, Smad3 overexpression significantly increased MCP-1 expression after vascular injury, consistent with our in vitro results. Interestingly, TGF-β/Smad3-induced MCP-1 was completely blocked by both Ro-32-0432 and rotterlin suggesting PKC-δ may play a role in TGF-β/Smad3 induced MCP-1 expression. In summary, our data demonstrate that TGF-β, through Smad3 and PKC-δ, stimulates VSMC production of MCP-1, which is a chemoattractant for bone marrow derived cells, specifically MSCs. Manipulation of this signaling system may provide a novel approach to inhibition of intimal hyperplasia.

Key words: TGF-β, MCP-1, VSMCs, mesenchymal stem cells.

INTRODUCTION

Intimal hyperplasia after vascular injury is a complex process involving vascular smooth muscle cell proliferation, migration, and extracellular matrix deposition. It is believed that vascular injuries transform vascular smooth muscle cells (VSMCs) from a quiescent to a synthetic and proliferative phenotype (1). These “activated” VSMCs migrate to the subintimal space where they continue to proliferate thus form the neointimal lesion (2). Recent evidence, however, suggests that bone marrow derived progenitor cells (BMPCs) are recruited to sites of vascular injury and may also play an essential role in the development of intimal hyperplasia (3-7).
The mechanisms underlying BMPC recruitment to the site of arterial injury are now being elucidated. The following three steps are believed to occur with BMPCs in response to arterial injury: 1) mobilization of cells from the bone marrow, 2) recruitment of these cells to the site of injury, and 3) differentiation of progenitor cells into cells of the arterial wall (8). A number of chemokines are responsible for the second phase of this process and proteins that have been implicated include but are not limited to stromal derived factor-1α (SDF-1α) (3,4), c-kit (5), and monocyte chemoattractant protein 3 (MCP-3) (9,10). We and others have recently found that some of these chemokine receptors can be released by VSMCs at the time of arterial injury, although the signals that stimulate their release have not been elucidated.

Mesenchymal stem cells (MSCs) are present in bone-marrow stroma and capable of differentiating into multiple cell types, including smooth muscle cells, endothelial cells, and cardiomyoblast progenitors. MSCs are believed to contribute to the repair of injured myocardial and nerve tissue (9,10). Furthermore, it has recently been shown in a mouse femoral artery wire injury model that MSCs are recruited to the neointimal lesion (11).

Transforming growth factor-beta (TGF-β) has been repeatedly demonstrated to be a provocative factor in the development of intimal hyperplasia. Studies have shown that TGF-β mRNA and protein levels are elevated during progressive neointimal thickening (12,13). Furthermore, administration of recombinant TGF-β after injury has been shown to enhance intimal hyperplasia (14), while blockade of TGF-β signaling reduces this process (15). Active TGF-β signals by binding to a specific serine/threonine kinase type II receptor, which then recruits and phosphorylates the type I receptor. Receptor activation by TGF-β leads to phosphorylation of Smads 2 and 3, both which heterodimerize with Smad4. The resulting complex enters the cell nucleus to directly regulate transcriptional activation of target genes (16,17). We have previously shown that TGF-β through Smad3 signaling increases the expression of fibronectin in VSMCs, an extracellular matrix protein that has been implicated in intimal hyperplasia (2).

Monocyte chemoattractant protein-1 (MCP-1) is a member of the C-C chemokine family, which is classically known to play a major role in inducing monocyte migration to sites of inflammation and injury. Recently it has been reported that MCP-1, acting through its receptor CCR2, plays a key role in the recruitment of monocytes from the peripheral circulation into early atherosclerotic and restenotic lesions (18). Furthermore, suppression of MCP-1 by administration of a neutralizing MCP-1 antibody or gene transfer of mutant MCP-1 inhibited neointimal hyperplasia in injured rat carotid arteries (19) (20). Although these studies of MCP-1 have focused on the recruitment of monocytes and macrophages to the arterial wall, the possibility that MCP-1 might also enhance intimal hyperplasia through the recruitment of stem or progenitor cells has not been addressed. Suggesting this as a possibility is recent evidence that mesenchymal stem cells express the MCP-1 receptor, CCR2 (21,22). Additionally, the related cytokine MCP-3 has been shown to play an essential role in recruiting mesenchymal stem cells to injured myocardial tissue (9). Together, these findings suggest that MCP-1 may be involved in the pathogenesis of intimal hyperplasia by inducing progenitor cell recruitment in a manner similar to its known effects on mononuclear cells.

In the present study, we hypothesize that TGF-β through Smad3 signaling induces VSMC secretion of MCP-1, which acts as a bone marrow derived progenitor cell or mesenchymal stem cell chemoattractant. These findings raise the possibility that the stimulatory effect of TGF-β on intimal hyperplasia includes at least in part, the recruitment of bone marrow progenitor cells to the neointima.

MATERIALS AND METHODS

General materials- Recombinant TGFβ1 and rat MCP-1 were obtained from R & D Systems. Dulbecco’s Modified Eagles Medium (DMEM) and cell culture reagents were from Invitrogen. Other reagents, if not specified, were purchased from Sigma Chemical Co.

Smooth muscle cell culture- Rat aortic SMCs were isolated from the thoracic aorta of male...
Sprague-Dawley rats based on a protocol described by Clowes et al (23) and maintained in DMEM containing 10% fetal FBS at 37°C with 5% CO2. Cell viability was assayed by the trypan blue exclusion method, which indicated that <5% of the cells took up the dye both before and after the infection of adenoviral vectors or treatment with recombinant TGF-β1 or inhibitors. For experiments using conditioned media, RSMCs (2x10^5 cells/well in 6-well plates) were infected with adenovirus (30,000 particles/cell in DMEM containing 2% FBS for 4 hours at 37°C), followed by starvation in DMEM containing 0.5% FBS for 48 hours. The cells were then treated with recombinant TGF-β1 (5ng/ml) or solvent (4mM HCl with 2% bovine serum albumin) for 6 hours. Media was collected and used for ELISA or chemotaxis assays as described below.

**Rat mesenchymal stem cell culture-** Rat Mesenchymal Stem Cells (MSC) isolated from rat bone marrow were obtained from Cell Applications Inc. (San Diego, CA). The cells were maintained through the use of Mesenchymal Stem Cell Growth Medium Kit (Cell Applications Inc.). MSC populations were passaged 1 to 4 times. Cells were harvested from plates using a solution containing 0.25% trypsin and 1 mM EDTA (Invitrogen Corp). Confirmation of MSCs was made through the presence of cell surface markers such as CD90, CD44, and CD54 and the absence of CD31 and CD45. For cell differentiation experiments, MSCs up to passage 4 were plated in MSC growth medium (non-differentiating).

**Isolation of rat bone marrow-** Bone marrow from Sprague-Dawley rats was isolated by flushing the long bones with Dulbecco's PBS containing 2% bovine serum albumin (BSA), and heparin (1000 U/ml). Cells from individual animals were pooled. Bone marrow was dissociated by aspiration with a Pasteur pipette. Red blood cells were excluded using Ficoll Hypaque (Pharmacia, Piscataway, NJ) density centrifugation. The remaining cells were then rinsed twice in PBS followed by centrifugation.

**Construction of Adenoviral Vectors and Infection-** Adenoviral vectors expressing Smad3 (AdSmad3), PKCθ (AdPKCθ), and GFP (AdGFP) were constructed as previously described (2).

**Cell Migration Assay-** 2 x 10^5 bone marrow cells or 2 x 10^5 MSCs were placed in the upper chamber of Costar 24-well transwell plates with 5-μm pore filters (Corning, Inc., Corning, NY). Cultured conditioned medium was placed into the lower chambers or wells. After incubating plates for 6 hours at 37 °C, migrated cells were collected from the lower chambers and counted.

**siRNA Knockdown of MCP-1 and Smad3-** RSMCs were plated at 50–60% confluence in DMEM culture medium in 6-well plates and incubated for 24 h. Cells were then transfected in Opti-MEM I medium with 20 nmoles siRNA for rat MCP-1 (Santa Cruz Biotechnology), 100 pmoles siRNA for Smad3 (Invitrogen), or control siRNA using RNAiMax transfection reagent (Invitrogen), as described by the manufacturer's protocol. After 6 hours, the Opti-MEM I medium was replaced by DMEM containing 0.5% FBS and cells were starved for 48 hours. Cells that were infected with adenovirus were infected the day following transfection, then starved for the remaining 48 hours. The cells were then stimulated with recombinant TGF-β1 (5ng/ml) or solvent for 6 hours, after which conditioned media was collected for chemotaxis assays and MCP-1 ELISA, and cells were lysed for protein used in Western blotting.

**Western Blot Analysis-** Cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 10 μg/ml aprotinin). Fifty 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: rabbit anti-Smad3 (AbCam), rabbit anti-pSmad3 (AbCam), and mouse anti-β-actin (Sigma). After incubation with the appropriate primary and horseradish peroxidase-conjugated secondary antibodies, the membranes were developed with enhanced
chemiluminescence reagent (Amersham Biosciences).

Microarray analysis- RSMCs were infected with AdSmad3 or AdGFP followed by starvation for 48 hours in DMEM containing 0.5% FBS, then treated with recombinant TGF-β1 (5ng/ml) or solvent for 6 hours. Cells were harvested and total RNA was extracted by RNeasy Mini Kit (Qiagen, Valencia, CA). This RNA was then processed according to the protocol recommended by Affymetrix, using the Superscript Choice kit (Invitrogen) for double-stranded cDNA synthesis and the Enzo Bioarray kit for in vitro transcription and labeling of cRNA. 15-μg samples of fragmented cRNA were hybridized for 16 h at 45 °C to Rat 230. 2.0 arrays (Affymetrix). Data analysis was performed using GeneSpring™ software 5.1 (Silicon Genetics) for normalization, fold change calculations, and clustering.

RT-PCR- Expression of the mRNA of MCP-1 was detected by RT-PCR. RNA was isolated from rat SMCs using a kit from Qiagen Corp. (Valencia, CA). A segment of the cDNA was detected using RT-PCR for MCP-1 using MCP-1 primers, which were obtained from Santa Cruz (Santa Cruz Biotechnology).

ELISA for MCP-1- ELISA to detect MCP-1 secreted by RSMC was performed using rat MCP-1 ELISA kit (BD Biosciences). RSMC were cultured at a density of 1 x 10^5/ml in 1 ml of complete medium in the presence or absence of different stimuli in 6-well plates (Costar). After incubation for specified periods of time at 37°C, cell-free culture supernatants were obtained. The concentrations of MCP-1 were then measured according to the manufacturer’s instructions.

Rat Balloon injury model and In Vivo gene delivery- Male Sprague-Dawley rats (450-500g) underwent balloon injury of the left common carotid artery as described elsewhere in accordance with institutional guidelines and approval (24,25). Briefly, following induction of anesthesia with isoflurane, a 2-Fr balloon catheter was inserted through the left external carotid artery into the common carotid and insufflated with 1 mL of air three times. Recombinant adenoviral vectors were constructed to express Smad3 as previously described; an empty viral vector was used as a control (26). Following injury, animals received intraluminal administration of adenoviral vectors as indicated (2.5 x 10^9 pfu in 200μl of serum free media over 20 min). The external carotid artery was then ligated and flow was re-established through the common carotid and internal carotid arteries. Rats were sacrificed 5 days after injury and perfusion fixed with 4% PFA diluted in phosphate buffered saline (PBS) at a pressure of 100 mm Hg for 15 minutes. The left and right carotid arteries were then harvested and fixed in 4% PFA for an additional 30 minutes. The arteries were then paraffin embedded and cut into 7 μm sections for histological analysis.

Bone marrow labeling in vitro and implantation- Bone marrow cells were extracted from rat long bones and labeled with fluorescent dyes using a PKH26 red fluorescent cell linker kit (Sigma-Aldrich, St. Louis, MO). After left carotid balloon angioplasty was performed, a 24G angiocath was inserted into the external carotid arteriotomy and secured. 5 x 10^7 BM suspended in 1 ml PBS were injected systemically through the arteriotomy. The external carotid artery was then ligated and flow re-established from the common carotid artery to the internal carotid artery. Rats were sacrificed 48 hours after BM cell injection and the tissue frozen into blocks with OCT media.

Immunohistochemistry- Immunostaining for MCP-1 using monoclonal anti-MCP-1 antibody (Santa Cruz) was performed as described previously (24). Slides were visualized with a Nikon Eclipse E800 upright microscope. Digital images were acquired using a RetigaExi CCD digital camera and processed and analyzed using IPLab software. Semi-quantitative immunohistochemical analysis was performed using Adobe Photoshop 7.0 and NIH imaging software (ImageJ) (24).

Statistical analysis- Data are expressed as mean ± SE. Unpaired Student’s t test was used to evaluate the statistical differences between control and treated groups. In cases of multiple groups, differences were first analyzed with one-way analysis of variance (ANOVA. Values of p < 0.05 were considered significant. All experiments were repeated at least in triplicate.
RESULTS

TGF-β induces VSMCs to secrete bone marrow and progenitor cell chemoattractants.

To begin testing our hypothesis that TGF-β stimulates intimal hyperplasia through the recruitment of BMPC, we first established a bone marrow cell chemotaxis assay using SDF-1α as a positive control (Fig. 1A). We then evaluated whether recombinant TGF-β could directly stimulate either total bone marrow cell or mesenchymal stem cell (MSC) migration. TGF-β alone had no direct effect on progenitor cell migration (Fig. 1A and 1B). We then asked whether TGF-β might stimulate VSMCs to produce progenitor cell chemoattractants since it is well established that TGF-β levels are elevated after vascular injury. As shown in Fig. 1C and 1D, conditioned media derived from rat aortic VSMCs treated with TGF-β significantly increased both total bone marrow cell as well as isolated rat MSC chemotaxis. These findings suggest that TGF-β activates VSMCs, which in turn produce a secreted factor that is responsible for recruitment or chemotaxis of bone marrow progenitor cells.

The effect of TGF-β on progenitor cell chemotaxis is mediated by Smad3.

Smad3 is a major signaling molecule for TGF-β that we and others have shown to be important in mediating many of TGF-β’s effects on VSMCs. We therefore hypothesized that Smad3 might mediate the effect of TGF-β on progenitor cell recruitment. To examine whether Smad3 plays a role in TGF-β-induced secretion of BMPC chemoattractants by VSMCs, we infected rat aortic VSMCs with adenoviral vectors expressing Smad3 (AdSmad3) and then stimulated cells with recombinant TGF-β1, thus enhancing TGF-β/Smad3 signaling. As shown in Fig. 2, conditioned media from VSMCs treated with AdSmad3 and TGF-β significantly increased both total bone marrow cell as well as isolated rat MSC migration, compared to conditioned media from VSMCs treated with TGF-β alone.

To further study the role of Smad3 in this process, we examined the effect of inhibition of Smad3 signaling on BMPC recruitment. VMSCs were pre-treated with SB431542 (10µg/ml), a TGF-β receptor kinase inhibitor that, at this concentration, is specific for Smad2/3 phosphorylation. As shown in Fig. 3A, treatment with SB431542 effectively inhibited TGF-β induced phosphorylation of both endogenous and adenovirus-derived Smad3, but did not affect total expression of Smad3. VMSCs treated with TGF-β or TGF-β/AdSmad3 were pretreated with SB431542 and the effect of this conditioned media on bone marrow cell and MSC migration was evaluated. As shown in Fig. 3B and 3C, pre-treatment of VMSCs with SB431542 followed by stimulation with TGF-β or TGF-β/AdSmad3 resulted in a significant decrease in the chemotaxis of both bone marrow cells and MSCs in response to conditioned media derived from VMSCs. These data confirm that phosphorylation of Smad3 is an essential step in TGF-β mediated recruitment of progenitor cells by vascular SMCs.

To more specifically inhibit Smad3 function we developed a siRNA to rat Smad3. As shown in Fig. 3D, transient transfection of VMSCs with Smad3 siRNA effectively downregulated Smad3 expression. Furthermore, conditioned media derived from VMSCs transiently transfected with Smad3 siRNA followed by stimulation with TGF-β produced significantly decreased total BM and MSC migration compared to conditioned media from VMSCs transfected with scrambled siRNA (Fig. 3E and 3F). Taken together, these results demonstrate that Smad3 is the signaling protein through which TGF-β mediates its effect on progenitor cell recruitment.

Identification of the progenitor cell chemoattractant released by VSMCs in response to TGF-β and Smad3.

Our next goal was to determine the identity of the progenitor cell chemoattractant released by VMSCs in response to TGF-β. To explore the possibilities we performed a microarray analysis of TGF-β/AdSmad3 stimulated VMSCs. Treatment with TGF-β/AdSmad3 increased by more than 4-fold the expression of 566 genes and decreased expression by at least 4 fold of 284 genes. The selected genes were clustered into groups that
influence cell differentiation or cell growth using GeneSpring™ software. We were able to identify a number of known progenitor cell chemoattractants stimulated by TGF-β in Smad3 overexpressing cells, as summarized in Table I. One of the most highly induced genes was that of monocyte-chemoattractant protein-1 (MCP-1), with a greater than 80 fold induction. Since MCP-1 has been shown to attract neural progenitors cells to areas of tissue injury (27), we hypothesized that MCP-1 may be the critical factor that allows TGF-β-stimulated vascular SMCs to function as a chemoattractant of bone marrow progenitor cells to sites of arterial injury.

**TGF-β induces VSMC production of MCP-1 through Smad3.**

As shown in Figure 4A, TGF-β stimulated the production of MCP-1 in RSMCs in a dose-dependent manner. Smad3 overexpression via adenovirus mediated gene transfer also increased the production of MCP-1, with further enhancement when Smad3 overexpressing cells were stimulated with TGF-β (Fig. 4B). To demonstrate that the increase in protein level was secondary to an increase in gene transcription, RT-PCR confirmed that TGF-β and Smad3 overexpression both increased expression of MCP-1 mRNA in VSMCs (Fig. 4C).

We next investigated the impact of inhibiting Smad3 signaling on TGF-β-induced MCP-1. As shown in Fig. 4D, inhibition of Smad2/3 phosphorylation using SB431542 (10μg/ml) significantly blocked TGF-β-induced MCP-1 in both control RSMCs and RSMCs overexpressing Smad3. Furthermore, Smad3 siRNA also significantly decreased TGF-β induced MCP-1 expression in RSMCs (Fig. 4E).

MCP-1 is an important chemokine for the recruitment of bone marrow derived cells.

To determine the role of MCP-1 in the recruitment of bone marrow cells and MSCs, we first tested the effect of recombinant MCP-1 on total bone marrow cell and MSC migration. Fig. 5A and 5B show that recombinant MCP-1 markedly induced both total bone marrow cell and MSC chemotaxis. To further examine whether MCP-1 is necessary for the recruitment of bone marrow cells by VSMCs treated with TGF-β and AdSmad3, we used a siRNA to silence MCP-1 expression in these cells. As shown in Fig. 5C, MCP-1-specific siRNA significantly reduced the amount of MCP-1 released by SMCs treated with TGF-β, whereas scrambled siRNA had no significant effect. Inhibition of MCP-1 gene expression by the siRNA was also confirmed by RT-PCR (Fig. 5D). Transfection of MCP-1 siRNA into RSMCs followed by stimulation of these cells with TGF-β/AdSmad3 significantly decreased the chemoattractive property of the conditioned media to both bone marrow cells and MSCs, in comparison to conditioned media from cells treated with scramble siRNA. (Fig. 5E and 5F) These findings were confirmed by repeating identical studies with conditioned media from VSMCs pretreated with an MCP-1 blocking antibody (Fig. 5G and 5H). Specific inhibition of MCP-1 by MCP-1 antibody resulted in a 26 % and 21% reduction in the chemoattractive properties of VSMC conditioned media for total bone marrow cells and MSCs, respectively, thus suggesting that MCP-1 is not the only progenitor cell chemoattractant induced by TGF-β/Smad3. This, however, is consistent with our gene array data, which shows that TGF-β/Smad3 induces several other potential progenitor cell chemoattractants, including VEGF, FGF-2, and c-kit ligand (Stem cell factor). Together, these data provide convincing evidence that MCP-1 is one of the critical factors released by SMCs that mediates TGF-β induced chemoattraction of bone marrow progenitor cells.

**VSMC conditioned media induces MSC to express smooth muscle cell markers**

In addition to a small percentage of progenitor cells, total bone marrow contains many cells that differentiate to the leukocyte and monocyte lineage and are expected to be recruited by MCP-1. To show that MCP-1 not only induces progenitor cell chemotaxis but can also induce progenitor cell differentiation into smooth muscle-like cells, MSC were cultured with conditioned media derived from VSMCs.
treated with or without TGF-β or AdSmad3. After 3 days of exposure to conditioned media, the morphology of treated MSCs changed significantly to resemble SMCs (Fig. 6A-C). Cellular protein levels of α-SMA, calponin and desmin were determined by Western blotting. As shown in Fig. 6D, the expression levels of α-SMA, calponin and desmin in MSC were significantly increased in response to treatment with conditioned media derived from VSMCs treated with TGF-β and AdSmad3. TGF-β itself did not have a significant effect on VSMC morphology or on expression of α-SMA or calponin (Fig. 6E).

Smad3 overexpression in vivo increases MCP-1 expression by VSMCs after injury.

We next turned to the rat carotid balloon injury model to test our hypothesis in vivo, that TGF-β through Smad3 signaling stimulates SMC production of MCP-1. Smad3 was overexpressed, via adenovirus mediated gene transfer, in rat carotid arteries following balloon injury, and MCP-1 expression was evaluated immunohistochemically. We have previously shown that overexpression of Smad3 after arterial injury produces an intense neointimal response. We found that MCP-1 expression was significantly increased at day 5 after injury in arteries infected with AdSmad3 as compared to those infected with AdNull. (Fig. 7A -C) This in vivo finding confirms our in vitro results showing that Smad3 overexpression stimulates production of MCP-1 by VSMCs.

Bone marrow cells migrate to areas of arterial wall injury

We then examined whether arterial injury induces migration of BM cells into carotid arteries in vivo after injury. Bone marrow cells were labeled and injected systemically at the time of injury as described in the Methods. As shown in Fig. 7 D and E, few labeled bone marrow cells were observed in injured arteries but not in uninjured arteries.

PKCδ signaling pathway is necessary for TGF-β stimulation of MCP-1

Although Smad3 is a critical signaling protein for TGF-β, several studies have previously shown that TGF-β can also signal through the ubiquitous kinase, protein kinase C-delta (PKC-δ) (28-30). Moreover, our previous data suggest that there may be crosstalk between the PKC-δ and Smad3 pathways (2). To test whether PKC-δ plays a role in TGF-β-induced MCP-1 expression, we first examined the effect of a general inhibitor of PKC, Ro 32-0432, on TGF-β induced MCP-1 production. At a concentration of 1-10 μM, Ro 32-0432 has been shown to block PKC-dependent activities in whole cells (31). As shown in Fig. 8A and B, Ro 32-0432 prevented both TGF-β- and TGF-β/AdSmad3-induced MCP-1 production in a dose-dependent manner, with complete inhibition at 5-10 μM. VSMCs were then treated with TGF-β and AdSmad3 in the absence and presence of the selective PKC-δ inhibitor, rottelin that has an IC50 of 3-6 μM for PKCδ (32). As shown in Fig. 8C, pretreatment with rottelin prevented TGF-β/AdSmad3- induction of MCP-1. To further confirm the involvement of PKC-δ in the generation of MCP-1, we infected RSMC with an adenovirus expressing PKC-δ (AdPKCδ) or AdNull and found that overexpression of PKC-δ significantly induced MCP-1 production (Fig. 8D).

To determine if PKC-δ is necessary for TGF-β/AdSmad3 induced recruitment of bone marrow progenitor cells, we pretreated AdSmad3-infected VSMCs with rottelin, followed by stimulation with TGF-β. Conditioned media from SMCs pretreated with rottelin induced significantly less BM cell or MSC migration compared to control conditioned media (Fig. 8E and 8F). Taken together, these data suggest that PKCδ signal transduction is necessary for TGF-β/Smad3 induced MCP-1 expression as well as the recruitment of bone marrow progenitor cells by TGF-β.

DISCUSSION

Our findings suggest a potential mechanism for the profound effect of TGF-β on intimal hyperplasia following arterial injury. TGF-β is known as a pro-fibrotic cytokine; consequently, induction of extracellular matrix and collagen deposition by TGF-β has been
proposed as one of the mechanisms to explain the enhancing effect of TGF-β on intimal hyperplasia. Contradicting this hypothesis, it has been shown repeatedly that the neointimal lesion in animal models is cellular and composed primarily of SMCs rather than extracellular matrix (33-35). We have recently reported that TGF-β, in the presence of Smad3 upregulation that occurs in VSMCs following arterial injury, is a stimulant of SMC proliferation. This may partially account for the accumulation of VSMCs following vascular injury. Recently, an important role for progenitor cells in the development of the neointimal lesion has also been proposed. We therefore explored whether the stimulatory effect of TGF-β on intimal hyperplasia might also be mediated through the recruitment of progenitor cells to the site of arterial injury.

In this study, we have made three novel findings. First, we have found that TGF-β, through a direct action on VSMCs, is able to stimulate bone marrow or mesenchymal stem cell chemotaxis. We have also shown that this effect is mediated at least in part through Smad3 signaling, and the release of MCP-1 from VSMCs. Furthermore, we have demonstrated that the PKC-δ signaling pathway is also necessary for TGF-β/Smad3 induced MCP-1 production, suggesting crosstalk between Smad3 and PKC-δ.

The role of MCP-1 as a key mediator of neointimal hyperplasia has previously been described. However, the proposed mechanism by which MCP-1 produces this effect is believed to be through the recruitment of leukocytes to the site of injury. Studies have shown that arterial injury is associated with a rapid but transient adherence of leukocytes to the arterial wall. However, in normolipemic animals, leukocytes do not accumulate in the arterial wall after vascular injury (33). Moreover, previous studies using rodent models of balloon arterial injury have shown that there is no significant accumulation of macrophages in the arterial wall and that the majority of cells comprising the neointima are smooth muscle cells (19,33). Despite this, blocking MCP-1 has still been described to inhibit intimal hyperplasia. Genetic deletion of CCR2, the receptor for MCP-1, has been reported to reduce intimal hyperplasia by 61.4% after femoral artery wire injury in a mouse (33). Interestingly, this was ascribed to a decrease in early SMC proliferation in CCR2 -/- mice and not to a decrease in the number of macrophages present in the neointimal lesion. Even at very early time points after injury (1 hour up to 5 days), there were essentially no macrophages (<2/section) in both CCR2 +/- or CCR2 -/- mice. The forgoing suggest an important role for MCP-1 in the development of intimal hyperplasia, however the mechanism for this effect is unclear and perhaps not related to chemotraction of leukocytes. Our studies provide an alternative hypothesis for the action of MCP-1. The neointimal response produced by MCP-1 may be the consequence of chemotraction of bone marrow progenitor cells into the vascular lesion that ultimately differentiate into SMCs.

The contribution of BMPC to the neointimal lesion has been well described (3-5,33), however characterization of the progenitor cells is still controversial. Initial studies suggested that hematopoietic stem cells (HSCs) may differentiate into SMCs or endothelial cells, however later studies have suggested that transdifferentiation of HSCs into non-hematopoetic cells is a rare event (6,36-39). On the other hand, bone marrow-derived MSCs in both in vivo and in vitro models (40,41) have been reported to migrate to sites of tissue damage (42) and differentiate into smooth muscle cells. Our results also suggest that factors secreted by SMCs can induce MSC differentiation into SMCs (Fig. 6). Since TGF-β stimulates the production of multiple factors by SMCs (Table 1), it is yet to be determined whether MCP-1 specifically is the factor that drives MSC differentiation. However, our results do emphasize the effect of TGF-β on SMCs: not only does TGF-β stimulate SMCs to secrete progenitor cell chemoattractants, but also factors that drive progenitor cell differentiation into more SMCs.

Other studies have also demonstrated that MSCs specifically are attracted to sites of tissue injury. Shenck et al reported that MSCs transiently home to the myocardium after acute myocardial infarction thereby improving cardiac remodeling, and that MCP-3 acts as a MSC homing factor (9). Moreover, MCP-1 and
MCP-3 both bind to the same receptor CCR2 (43), and MSCs express this receptor. Therefore, it is not surprising that we now find that MCP-1, like MCP-3, has the ability to attract bone marrow MSCs. Previous studies in other systems have evaluated the role of MCP-1 as a chemoattractant of MSCs. MSCs have been shown to migrate towards ischemic rat cerebral tissue extracts; migration was inhibited when the tissue extracts were pre-incubated with an MCP-1 blocking antibody (10). Furthermore, breast cancer cells have also been shown to secrete MCP-1, which in turn attracts MSCs to tumors (44). Contradicting these findings are those of Ringe et al. who reported that MSCs did not migrate toward recombinant MCP-1 over a wide range of concentrations (45). Our current findings suggest that MCP-1, at least in the vascular system, can function as a potent chemoattractant of mesenchymal stem cells. TGF-β appears to mediate its effect on MCP-1 through Smad3. This is evident from our in vitro studies; as well, in vivo levels of MCP-1 are increased in the arterial wall of Smad3-overexpressing animals after injury. Although this mechanism has not been previously described in SMCs, in human endothelial cells, TGF-β has also been shown to induce MCP-1 through Smad3 signaling (46). In these studies, promoter analysis revealed the presence of a Smad-binding element in the MCP-1 promoter that was required for endothelial cells to respond to TGF-β. Conversely, some authors have found that TGF-β, through Smad3 signaling, inhibits MCP-1 expression. In a mouse macrophage cell line (47), examination of the MCP-1 promoter failed to reveal a Smad3 binding site. However Smad3 was found to bind to c-jun, a member of the AP-1 family, prevent c-jun binding to the MCP-1 promoter, and consequently inhibit promoter activation. These results suggest that the effects of TGF-β on MCP-1 gene expression are cell specific.

Conditioned media derived from rat VSMCs treated with TGF-β significantly enhances bone marrow and mesenchymal cell migration albeit the effect is only approximately 30%. This effect is further enhanced in SMC overexpressing Smad3. It is always difficult to translate in vitro findings to in vivo. Local concentrations of TGF-β or MCP-1 might be greater in vivo, further enhancing our observed effect on migration. Alternatively concentrations of both proteins might be less in vivo and the observations made in these experiments may be physiologically irrelevant. Interestingly, bone marrow or mesenchymal stem cells, even if few in number have the potential to differentiate into multiple cell types in vivo, such as endothelial or smooth muscle cells, which in turn can lead to a cascade of events that do have physiologic significance. Moreover, assay methods of cell migration may underestimate the chemotactic effect of conditioned media, since control media itself also induces some degree of spontaneous cell migration. Therefore, we believe that while conditioned media derived from VSMC treated in vitro with TGF-β did not induce robust changes in bone marrow cell migration, this effect may have significant biological consequences in vivo.

Of note, downregulation of Smad3 using an siRNA to Smad3 caused a partial but significant decrease in the chemoattractive properties of conditioned media from SMCs treated with TGF-β, but a much more dramatic decrease in MCP-1 production. As shown in our gene array data (Table 1), stimulation of RSMCs with TGF-β/AdSmad3 affected the expression of numerous genes; whereas the genes for many cytokines and pro-angiogenic factors were induced, many others were inhibited, including the gene for SDF-1α. It is possible that concurrent with the induction in MCP-1 gene expression, stimulation with TGF-β resulted in a decrease in other progenitor cell chemoattractants, such as SDF-1α, that partially opposed the effect of MCP-1. The partial effect of Smad3 siRNA could also be caused by an incomplete “knock-down” of Smad3 expression which is often associated with this approach. Alternatively, this result could suggest that TGF-β signals through additional pathways within SMCs, such as Smad2 or a Smad-independent pathway. Having shown that Smad3 is a critical
mediator of TGF-β induced MCP-1, it was therefore surprising to find that inhibition of PKC-δ could completely block TGF-β induced MCP-1 expression as well as progenitor cell chemotaxis. These findings suggest that PKC-δ may play a key role in MCP-1 expression. Moreover, PKC-δ may act as a critical downstream component of TGF-β/Smad3 signaling in VSMCs. We have recently found that PKC-δ is required for induced MCP-1 expression in macrophages. Likewise, PKC-δ may be necessary for MCP-1 expression in VSMCs, independent of TGF-β/Smad3 signaling. Alternatively, we and others have previously described crosstalk between PKC-δ and Smad3 in VSMCs (2,28). It has been reported that phosphorylation of serine residues in the MH1 domain of Smad3 by PKC-δ alters the DNA binding properties of Smad3 (48). Therefore, it is possible that PKC-δ mediated phosphorylation of Smad3 is required for TGF-β/Smad3 induced MCP-1 expression in SMCs. This hypothesis will be explored in future studies.

In conclusion, our data suggest a novel mechanism through which TGF-β stimulates production of MCP-1 in VSMC. We have demonstrated that TGF-β, acting on VSMCs, induces progenitor cell chemotaxis and progenitor cell differentiation into smooth muscle-like cells. Furthermore, we have shown that the pathway through which TGF-β produces this effect involves Smad3, PCK-δ and MCP-1. Taken together, these results provide new insight into the intricacies and consequences of TGF-β and its effects on the arterial wall. Manipulation of this signaling system and its effects on progenitor cell chemotaxis may provide a novel approach to the inhibition of intimal hyperplasia.

REFERENCES

ACKNOWLEDGEMENTS AND SOURCES OF FUNDING

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FIGURE LEGENDS

Fig. 1. **TGF-β stimulates SMCs to express factors that attract bone marrow progenitor cells.** Rat bone marrow cell chemotaxis assays. Recombinant TGF-β (5 ng/ml) alone had no effect on total bone marrow cell migration as compared to positive control SDF-1α (100 ng/ml) (A), or on isolated rat MSC migration (B). However, conditioned media from VSMCs stimulated with TGF-β was found to induce both (C) total bone marrow cell migration and (D) isolated rat MSC migration. (n = 3). *, p < 0.05 compared to control.

Fig. 2. **Smad3 overexpression enhances the stimulatory effect of TGF-β on SMCs.** VSMCs were infected with control virus (AdGFP) or AdSmad3 and stimulated with TGF-β. Conditioned media was
then used in chemotaxis assays. Treatment with AdSmad3 in addition to TGF-β further induced VSMC secretion of factors that attract both (A) total bone marrow cells and (B) isolated MSCs. (n = 3). *, p < 0.05, compared to control; # p<0.05 compared to TGF-β.

**Fig. 3. TGF-β stimulates BMPC migration through Smad3 signaling.** (A) Western blot showing inhibition of phosphorylation of both endogenous and adenovirus-derived Smad3 by SB431542 (10μg/ml). Chemotaxis assay using whole bone marrow cells (B) or isolated rat MSCs (C) and conditioned media from VSMCs pre-treated with SB431542 and TGF-β/AdSmad3. Data expressed as fold change compared to conditioned media from VSMCs infected with AdGFP only. (D) Western blot showing downregulation of total Smad3 in cells treated with Smad3 siRNA. Chemotaxis assay using total bone marrow (E) and isolated rat MSCs (F) and conditioned media from VSMCs transfected with Smad3 siRNA and treated with TGF-β. Data expressed as fold change compared to conditioned media from VSMCs transfected only with lipofectamine. n = 3, * p<0.05.

**Fig. 4. TGF-β, through Smad3 signaling, induces VSMC production of MCP-1.** (A) TGF-β induced VSMC production of MCP-1 in a dose dependent manner, as shown by ELISA. (B) Enhancement of Smad3 signaling by infection with AdSmad3 also induced MCP-1 production, and enhanced the stimulatory effect of TGF-β, as shown by both ELISA and RT-PCR (C). (D) Blockade of Smad3 phosphorylation using SB431542 (10 μg/ml) inhibited TGF-β induced MCP-1 production as measured by ELISA. (E) Knockdown of Smad3 using a Smad3 siRNA also inhibited TGF-β induced MCP-1. (n=4; *, p < 0.05 compared with control)

**Fig. 5. MCP-1 mediates TGF-β induced bone marrow and progenitor cell migration.** Recombinant MCP-1 (20ng/ml) induced both (A) total bone marrow cell and (B) rat MSC migration in chemotaxis assays. MCP-1 gene silencing using a MCP-1 siRNA inhibited TGF-β/Smad3 induced MCP-1 production both on MCP-1 ELISA (C) and RT-PCR (D). Conditioned media from VSMCs treated first with MCP-1 siRNA, then AdSmad3 and TGF-β attracted fewer total bone marrow cells (E) and rat MSCs (F) when compared to conditioned media from cells treated with control siRNA or no siRNA. Addition of an MCP-1 blocking antibody to conditioned media from VSMCs treated with AdSmad3 and TGF-β also attracted fewer total bone marrow cells (G) and rat MSCs (H) when compared to conditioned media from VSMCs treated with only AdSmad3 and TGF-β. n = 3; *p<0.05 compared with TGF-β or TGF-β/AdSmad3.

**Fig. 6. Conditioned media from VSMCs stimulated with TGF-β induces MSC differentiation into SMCs.** MSCs were cultured in conditioned media from RSMCs (A), VSMCs treated with TGF-β (B), or VSMCs treated with TGF-β/AdSmad3 (C) for 3 days. (D) MSCs were treated with conditioned media from RSMCs for 3 days. Protein lysates were analyzed by Western blotting using antibodies against α-SMA, calponin and desmin. Tubulin served as a loading control. (E) MSCs were treated with or without TGF-β (5 μg/ml) for 3 days, protein lysates were analyzed by Western blotting using antibodies against α-SMA, calponin and β-tubulin.

**Fig. 7. Upregulation of Smad3 in vivo increases MCP-1 expression and injury of arteries induce migration of BM in the arterial wall.** (A-B) MCP-1 IHC in rat carotid arteries 5 days after balloon injury and infection with AdNull or AdSmad3. (C) Semiquantitative immunohistochemistry showing increased MCP-1 expression in arteries infected with AdSmad3. * p<0.05. (D and E) After implantation of PKH26-labelled BM in rat circulation for 24 hours, PKH26-labelled BM (white arrowheads) are observed in uninjured arteries (C) but not in injured arteries (D).
Fig. 8. Inhibition of PKC prevents TGF-β/Smad3 induced MCP-1 expression by RSMCs. (A) MCP-1 ELISA showing inhibition of TGF-β/Smad3 induced MCP-1 after pre-treatment with the PKC inhibitor Ro-32-0432 (5μM). (B) The effect of Ro-32-0432 was dose dependent (concentration in μM). (C) MCP-1 ELISA showing inhibition of TGF-β/Smad3 induced MCP-1 by the PKC-δ specific inhibitor rotterlin (concentration in μM). (D) Conversely, overexpression of PKC-δ alone induces MCP-1 expression in RSMCs. Chemotaxis assays using conditioned media from VSMCs pre-treated with rotterlin (2μM) then stimulated with TGF-β/AdSmad3 showed that inhibition of PKC-δ decreased the chemoattractive properties of conditioned media for both (E) total bone marrow cells and (F) rat MSCs. (n = 3). *p<0.05.
Table I.
Potential progenitor cell chemoattractants (fold change)

<table>
<thead>
<tr>
<th>Growth Factors</th>
<th>Chemokines</th>
<th>Other molecules</th>
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<tbody>
<tr>
<td>VEGF (7.7)</td>
<td>MCP-1 (83)</td>
<td>Sphingosine kinase (3.2)</td>
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<tr>
<td>VEGF-R (5.5)</td>
<td>MCP-3 (37)</td>
<td>Sphingosine-1 phosphate (1.4)</td>
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<td>IL-6 (8.5)</td>
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<tr>
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<td>RANTES (3)</td>
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<tr>
<td>SCF (3.3)</td>
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</tbody>
</table>
Fig. 1

A. BM migration

B. MSC migration

C. BM migration

D. MSC migration

Control TGF-β SDF-1α

Control TGF-β

RSMC/control media RSMC/TGF-β media

RSMC/control media RSMC/TGF-β media
Fig. 2

A. BM Migration

B. MSC Migration
Fig. 3

A. 

B. 

C. 

D. 

E. 

F.
Fig. 6

A: Control Media
B: TGF-β Media
C: TGF-β/AdSmad3 Media

D: 
<table>
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<tr>
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<td>β-Tubulin</td>
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E: 
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<td>Calponin</td>
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<td>β-Tubulin</td>
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</table>
Fig. 7

A. AdNull  B. AdSmad3

C. Quantification of MCP-1 staining

D. Uninjury  E. Injury

*
Fig. 8

A. MCP-1 (pg/ml)

B. MCP-1 (ng/ml)

C. MCP-1 (pg/ml)

D. MCP-1 (pg/ml)

E. BM Migration

F. MSC Migration

TGF-β | AdSmad3 | Ro-32-0432 (5 µM)
- | + | + + + + +
+ | + | + + + + +
+ | + | + + + + +

MCP-1
- | + | + + + + +
+ | + | + + + + +
+ | + | + + + + +

Rottlerin
0 | 0 | 0.5 | 1 | 5 | 10 (µM)

Control | AdPKCd

TGF-β | AdSmad3 | Rottlerin
- | + | +
+ | + | +
+ | + | +
Transforming growth factor-beta promotes recruitment of bone marrow cells and bone marrow-derived mesenchymal stem cells through stimulation of MCP-1 production in vascular smooth muscle cells
Fan Zhang, Shirling Tsai, Kaori Kato, Dai Yamanouchi, Chunjie Wang, Shahin Rafii, Bo Liu and K. Craig Kent

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