TNF-α–Induced Migration of Vascular Smooth Muscle Cells Is MAPK Dependent

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Abstract—Migration of vascular smooth muscle cells (VSMC) is a key event in neointimal formation and atherosclerosis that may be linked to the accumulation of inflammatory cells and release of chemotactic cytokines. Tumor necrosis factor-α (TNF-α) induces chemotaxis of inflammatory cells and fibroblasts, but little is known about chemotactic signaling by TNF-α in VSMC. The aim of this study was to investigate the role of TNF-α in VSMC migration and to elucidate the chemotactic signaling pathways mediating this action. TNF-α (50 to 400 U/mL) induced migration of cultured rat aortic VSMC in a dose-dependent manner. Because activation of the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (MAPK) is known to be required in platelet-derived growth factor–directed and angiotensin II–directed migration of these cells, we used the MAPK-inhibitor PD98059 to determine if chemotactic signaling by TNF-α involves the MAPK pathway as well. We found that TNF-α–directed migration was substantially inhibited by PD98059. TNF-α (100 U/mL) transiently activated MAPK with a maximal induction 10 minutes after stimulation that returned to baseline levels by 2 hours after treatment. Only a single peak of increased MAPK activity was seen. PD98059 also blocked TNF-α–stimulated MAPK activation in a concentration-dependent manner, which is consistent with its inhibition of TNF-α–directed migration. To identify which TNF-α receptor is involved in TNF-α–induced MAPK activation, antibodies against the p55 TNF-α receptor-1 (TNF-R1) and the p75 TNF-α receptor-2 (TNF-R2) were used. VSMC express both receptors, but TNF-α–induced MAPK activation was inhibited only by the TNF-R1 antibody. The TNF-R2 antibody had no effect. Thiazolidinediones are known to inhibit TNF-α signaling in adipose tissue and attenuate platelet-derived growth factor–directed and angiotensin II–directed migration in VSMC. We therefore investigated the effects of the thiazolidinediones troglitazone (TRO) and rosiglitazone (RSG) on TNF-α–induced migration. Both TRO and RSG inhibited migration, but neither attenuated TNF-α–induced MAPK activation, indicating that their antimigration activity was exerted downstream of MAPK. These experiments provide the first evidence that early activation of MAPK is a crucial event in TNF-α–mediated signal transduction leading to VSMC migration. Moreover, inhibition of TNF-α–directed migration by the insulin sensitizers TRO and RSG underscores their potential as vasculoprotective agents.

Key Words: signal transduction ■ muscle, smooth ■ atherosclerosis ■ MAPK ■ cytokine ■ migration

Vascular smooth muscle cell (VSMC) migration is a critical event in lesion development and progression in restenosis and atherosclerosis.1 Migration of VSMC is a complex response that occurs during several pathological processes that involve accumulation of inflammatory cells and the release of chemotactic cytokines.2 Cytokines such as tumor necrosis factor-α (TNF-α) are pluripotent mediators of inflammation3 and have been implicated in the chemotactic response of inflammatory cells4 and fibroblasts.5 Little is known, however, about the chemoattractant effects of TNF-α in the vasculature. In addition to its role as an immune modulator, TNF-α may also play an important role in atherogenesis and restenosis.6 TNF-α has been found to be expressed in VSMC after balloon injury7 and in restenotic lesions8 but not in the normal vasculature. Moreover, the presence of TNF-α has been demonstrated in intimal VSMC9 and in plaques of atherosclerotic arteries10 as well as in models of transplantation-associated atherosclerosis.11 In an animal model of coronary-graft atherosclerosis, blockade of TNF-α with a soluble TNF-α receptor12 inhibited acute coronary neointimal formation. Thus, cells of the arterial wall can both produce and respond to this cytokine in vivo. Thus TNF-α is a highly important cytokine in the injured vasculature, where it may function to regulate the expression of growth factors (platelet-derived growth factor [PDGF], vascular endothelial cell growth factor [VEGF], fibroblast...
TNF-α—Directed Migration Is MAPK Dependent

growth factor [FGF]), adhesion molecules, cytokines, and extracellular matrix degrading metalloproteinases as well as directly affect VSMC growth and migration. To better define the role of TNF-α in the injured vasculature, we investigated its effect on VSMC migration and identified the signaling pathways mediating this process.

Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM), glutamine, antibiotics, HEPES, DMSO, and monoclonal antibody against smooth muscle α-actin were obtained from Sigma (St Louis, Mo); rat recombinant TNF-α was from R&D systems (Minneapolis, Minn); and Hybond enhanced chemiluminescence nitrocellulose membrane, horseradish peroxidase–linked anti-rabbit antibody, and enhanced chemiluminescence Western blotting detection reagents were from Amersham Life Sciences (Arlington Heights, Ill). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, Calif).

Culture plastic ware and cell culture inserts (8 well format; Falcon) and transwell chambers were obtained from Costar. Cell fixation and staining was performed with the Quik-Diff stain set from DAE. Sprague-Dawley rats were from Charles River, Mass. The MAPK-ERK Kinase (MEK) inhibitor PD98059 and phosphospecific and total-extracellular signal-regulated kinase (ERK)1/ERK2 mitogen-activated protein kinase (MAPK) rabbit antibodies were purchased from New England BioLabs (Beverly, Mass). Troglitazone (TRO) was kindly provided by Parke Davis; rosiglitazone (RSG, formerly BRL 49653) was a generous gift from Smith Kline Beecham. Antibodies against TNF-α receptor-1 and receptor-2 were obtained from Santa Cruz Biotechnologies (Santa Cruz, Calif). The bromodeoxyuridine (BrDU) detection kit was purchased from Boehringer Mannheim.

Cell Culture

Rat aortic smooth muscle cells from thoracic aortas of 2- to 3-month-old Sprague-Dawley rats were prepared and cultured as described previously, and the procedures followed were in accordance with institutional guidelines. A monoclonal antibody against smooth muscle α-actin was used to assess the purity of the smooth muscle cell cultures. Flow cytometry of anti–smooth muscle α-actin antibody–stained cells revealed a purity of 95±3%. For all experiments early passaged (5 or less) VSMCs were used, and each individual experiment represented in the n value was performed with an independent preparation of VSMC.

Migration

VSMC migration was examined in transwell cell culture chambers with gelatin-coated polycarbonate membranes as described previously. In this assay, movement of VSMC through the coated membrane toward chemoattractants measures both invasion and chemotaxis. Cells were pretreated with PD98059 (10 or 30 μmol/L), TRO (1 to 20 μmol/L), RSG (0.1 to 10 μmol/L), or vehicle (0.4% FBS/DMEM) for 30 minutes at 37°C. Cell attachment to the gelatin-coated membrane was not affected by any of the inhibitors (data not shown). Inhibitors were added both to the upper and the lower compartments and were present throughout the duration of the experiment. Migration was induced by addition of TNF-α (50 to 400 U/mL) to the lower compartment. After a 4-hour migration period, cells that had undergone migration toward the lower membrane surface were counted per HPF. To determine the effect of TNF-α on DNA-synthesis, incorporation of the thymidine analogue BrDU was measured with the BrdU labeling and detection kit II from Boehringer Mannheim. VSMC were incubated either with TNF-α (100 U/mL) in serum-free medium or were kept in serum-free medium as control. Cell nuclei incorporating BrDU appeared brown and were counted in 4 to 6 different HPFs per well and related to total cell number.

Statistics

ANOVA or paired or unpaired t test was performed for statistical analysis as appropriate. A probability value <0.05 was considered to be statistically significant. Data are expressed as mean±SEM.

Results

TNF-α Stimulates VSMC Migration

To investigate the directed migration responses of VSMC toward TNF-α, experiments were performed with a transwell migration chamber assay. Using increasing concentrations of TNF-α (50 to 400 U/mL), we observed a dose-dependent increase in directed migration of cultured VSMC (Figure 1). The cytokine induced a modest response at 50 U/mL with 7.2±1.2 cells/HPF, representing a 1.7±0.3-fold stimulation.
Cells were incubated with the MEK inhibitor PD98059 for 30 minutes before TNF-α (100 U/mL) was added. MAPK inhibition with PD98059 at 10 μmol/L significantly reduced migratory response and abolished TNF-α-induced migration at 30 μmol/L. VSMC migration is shown as x-fold induction over control. Experiments were repeated 3 times and done in duplicate. Data are expressed as mean±SEM. *P<0.05 vs TNF-α alone.

compared with control (4.2±0.3 cells/HPF). Significantly augmented migration occurred at 100 U/mL and 200 U/mL TNF-α, leading to a 2.74±0.13-fold and 3.4±0.1-fold stimulation, respectively (both P<0.05 compared with control). At the highest concentration tested, there was no further increase in migration toward TNF-α (400 U/mL) (3.38±0.14-fold over control, P<0.05).

**TNF-α-Induced Migration Is MAPK-Dependent**

Although an induction of the ERK1/ERK2 MAPK pathway by TNF-α in VSMC had not yet been demonstrated, MAPK activation is known to be an important step in PDGF-directed and angiotensin II–directed migration. In addition, TNF-α has been shown to induce MAPK activation in several other cell types including fibroblasts, for which the cytokine is chemotactic. We therefore tested the specific MEK inhibitor PD98059 to examine a possible involvement of the MEK/MAPK pathway in TNF-α–induced migration. The data in Figure 2 demonstrate that treatment of VSMC with the MAPK pathway inhibitor PD98059 at 10 μmol/L and 30 μmol/L significantly inhibited their response toward TNF-α (100 U/mL) (66±7.2% and 100±6%, respectively; P<0.05). At the concentrations used, PD98059 did not cause any cytotoxic effects. There was no evidence of cell detachment or loss of plasma membrane integrity, as evidenced by the uptake of trypan blue.

**TNF-α Transiently Activates MAPK**

To corroborate our findings that the pharmacological inhibitor PD98059 of the MAPK pathway inhibited TNF-α–induced chemotactic signaling in VSMC, we examined the effect of TNF-α on MAPK activation. VSMC were made quiescent by serum starvation, and after stimulation with TNF-α the activation and phosphorylation of MAPK was assessed by immunoblotting with a phosphospecific ERK1/ERK2 MAPK antibody. In parallel experiments, the amount of total ERK1/ERK2 MAPK was determined in the same cell extract with the use of an antibody that recognizes all ERK1/ERK2 MAPKs independent of their phosphorylation state. Quiescent cells in the control groups exhibited low MAPK activity, as evidenced by the faint bands detected with the phosphospecific antibody (Figure 3). The residual MAPK activity in the controls probably is due to the fact that a small percentage of serum (0.4%) was present, which is known to stimulate MAPK. Stimulation with TNF-α for 10 minutes induced MAPK activation in a dose-dependent manner, resulting in a 11.5±2.3-fold increase in phosphorylated, activated MAPK compared with untreated control at a concentration of 100 U/mL (P<0.05). At higher concentrations of TNF-α, no further increase in MAPK activation was observed, indicating that TNF-α–inducible MAPK activity had plateaued (Figure 3). Interestingly, TNF-α at 200 U/mL induced more VSMC migration than was observed in cells stimulated with 100 U/mL TNF-α. Increased VSMC migration at concentrations of TNF-α higher than required to induce maximal MAPK activation therefore may be mediated through pathways other than MAPK.

Since different growth factors and peptides activate MAPK either transiently (5 to 10 minutes peak) or induce an additional second sustained peak in MAPK activity (5 to 10 minutes initial peak, followed by second peak 1 to 2 hours later), we performed a time-course study of MAPK induction by TNF-α. As shown in Figure 4, TNF-α (100 U/mL) induced a rapid and transient activation of MAPK that...
returns to baseline values within 60 minutes. No second peak or sustained late phase in TNF-α–stimulated MAPK activity was detected. TNF-α did not affect the amount of total MAPK protein during the investigated time course (Figure 4 lane II).

Treatment with PD98059 (1 to 30 μmol/L) blocked TNF-α–stimulated MAPK activation in a concentration-dependent manner (Figure 5). TNF-α–induced MAPK activation was inhibited by 37±5% and 74±8% at 1 μmol/L and 10 μmol/L PD98059, respectively. At 30 μmol/L, PD98059 completely abolished the effect of TNF-α on MAPK activation (P<0.05).

Figure 4. MAPK activation by TNF-α is monophasic and transient. Serum-starved VSMC were treated with TNF-α (100 U/mL) for 10 to 180 minutes, and protein samples were immunoblotted with I, a phosphospecific ERK1/ERK2 MAPK antibody, or II, an antibody against total ERK1/ERK2 MAPK. Western blots shown are representative of 4 experiments with different cell preparations. Co indicates control.

Figure 5. PD98059 dose-dependently inhibits TNF-α–induced MAPK activation. Cells were incubated with MEK-inhibitor PD98059 (1 to 30 μmol/L) for 30 minutes before TNF-α (100 U/mL) was added. After stimulation with TNF-α for 10 minutes, cell lysates were immunoblotted for I, activated, phosphorylated ERK1/ERK2 MAPK, or II, total ERK1/ERK2 MAPK (top, representative immunoblots). Results of densitometric analysis of 3 different experiments are shown in the bottom panel. MAPK activity was measured by densitometry and is expressed in arbitrary units. Data are shown as mean±SEM. *P<0.05 vs TNF-α alone. Co indicates control.

Figure 6. TNF-α activates MAPK in VSMC through the p55 TNF-α receptor-1. Quiescent rat aortic VSMC were pretreated for 30 minutes with increasing concentrations of p55 TNF-α receptor-1 antibody (1:1000–1:100) or p75 TNF-α receptor-2 antibody (1:1000–1:100) followed by addition of TNF-α (100 U/mL). After coincubation with TNF-α and receptor antibodies for 10 minutes, cells were harvested and protein samples were immunoblotted with I, a phosphospecific ERK1/ERK2 MAPK antibody, or II, an antibody against total ERK1/ERK2 MAPK. Western blots shown are representatives of 4 experiments with different cell preparations. Co indicates control.

Figure 7. Both TNF-α receptors are present in VSMC. Control cell extracts were separated on 7.5% PAGE-SDS gels, followed by Western blot analysis with antibodies against p55 TNF-α receptor-1 or p75 TNF-α receptor-2. As shown, distinct bands of the right sizes were detected for both TNF-α receptors. Experiment was repeated with a different set of protein samples, revealing the same results.
II–mediated migration of VSMC.19,20 Because of those findings, we examined the effects of the TZDs TRO and rosiglitazone RSG on TNF-α–induced migration. The data in Figure 8 show that migration of VSMC toward the cytokine was significantly inhibited by treatment with TRO 10 μmol/L and 20 μmol/L by 68±5.8% and 99±7.2%, respectively (both P<0.05 vs TNF-α 100 μmol/L alone). An even more potent effect was observed for RSG that completely abolished migration toward TNF-α at 10 μmol/L (P<0.05) (Figure 8).

At all concentrations of TRO (1 to 20 μmol/L) or RSG (0.1 to 10 μmol/L) used in migration assays, we observed no cytotoxic effects evidenced by the lack of cell detachment or the absence of a significant number of cells staining positively for trypan blue.

**TZDs Act Downstream of TNF-α–Induced MAPK Activation**

To determine whether TRO and RSG inhibited migration by targeting the MAPK pathway, we investigated their effect on TNF-α–induced MAPK activation. VSMC were made quiescent by 24-hour serum starvation, then pretreated with TRO or RSG for 30 minutes, followed by stimulation with TNF-α (100 U/mL) for 10 minutes. Neither TRO nor RSG attenuated the MAPK activation in response to TNF-α, indicating that the TZDs inhibit VSMC migration downstream of MAPK activation, a signaling step required for TNF-α–directed migration (Figure 9).

**TNF-α Is Not Mitogenic for VSMC**

Because VSMC accumulation in restenosis and atherosclerosis results from a combination of cell growth and migration, it was of interest to determine whether TNF-α also functions as a mitogen for VSMC. Using the thymidine analogue BrdU, we examined the effect of TNF-α on VSMC proliferation. Treatment with TNF-α (100 U/mL) for 24 hours did not result in a significant increase in DNA-synthesis compared with control, as determined by BrdU-incorporation (TNF-α: 9.8±0.9%; control: 8.4±0.7%).

**Discussion**

TNF-α is a cytokine that is released by inflammatory cells at sites of vascular injury and is expressed in the arterial wall under pathological conditions, in which it is associated with lesion formation.3,6–11 Migration of VSMC from the tunica media to the intima is one of the major pathological vascular responses that leads to the development and progression of intimal thickening.1 Previous studies demonstrated that TNF-α is a potent migration factor for different cell types, such as fibroblasts and inflammatory cells.4,5

The present investigation demonstrates that (1) TNF-α is a potent migration factor but not a growth factor for VSMC; (2) TNF-α stimulates only an early activation (10 minutes) of MAPK but not a second, delayed peak in activity (2 to 6 hours), which may explain why this cytokine is a potent migration factor but a weak mitogen; (3) TNF-α–induced MAPK activation is mediated by the p55 TNF-α receptor and not the p75 TNF-α receptor; and (4) TZDs inhibit TNF-α–stimulated VSMC migration.

The mechanisms and intracellular signaling pathways leading to VSMC migration are not completely understood. Among the cytosolic events in response to migration factors, recent studies have recognized the ERK1/ERK2 MAPKs as key signaling steps for this process in VSMC.18,19,26 However, migration of VSMC has also been linked to other signaling molecules, such as increased phosphatidylinositol turnover leading to the activation of phospholipase C27 as well as increased intracellular calcium and activation of the calcium/calmodulin-dependent kinase II.28–31 It has been shown that PDGF-induced chemotactic signaling involves activation of the calcium/calmodulin kinase II, which in turn leads to phosphorylation and activation of myosin light chain kinase.32 Yet, another recent study revealed that myosin light chain kinase, which phosphorylates and reorganizes cytoskeletal components that facilitate cell movement,33 is also a substrate for MAPK.34,35 The importance of the MAPK-pathway in VSMC migration is supported by findings showing that inhibition of MAPK with antisense oligodeoxynucle-
TNF-α–Directed Migration Is MAPK Dependent

To further elucidate the signaling steps involved with TNF-α–induced MAPK activation, we examined the role of the TNF-α receptors. In other cell types, 2 main TNF-α receptors have been described: the p55 TNF-α receptor-1 (TNF-α-R1), and the p75 TNF-α receptor-2 (TNF-α-R2). Although many cell types express both receptors, the majority of TNF-α–induced signaling events are mediated by TNF-α-R1. In VSMC, TNF-α induces signaling through the TNF-α-R1 that leads to MAPK activation, which is required for cell migration.

In addition to its role in directed migration toward TNF-α and other chemokines, MAPK is also involved in mitogenic signaling by growth factors in VSMC. VSMC proliferation and migration both importantly contribute to the accumulation of these cells in vascular lesions. A potential candidate for a therapeutic approach for preventing lesion formation in the arterial wall could be the novel insulin-sensitizing class of TZDs. In the present study, we were able to demonstrate that 2 TZDs, TRO and RSG, inhibited TNF-α–directed migration of VSMC. Furthermore, we previously showed that TRO inhibits PDGF-directed and angiotensin II–directed migration, and that migratory responses toward both chemoattractants are MAPK dependent. The precise mechanism by which TZDs inhibit MAPK-dependent migration pathways remains to be elucidated, although our results suggest an effect downstream of MAPK, since neither TRO nor RSG affected TNF-α–stimulated MAPK activation.

Because of the enormous clinical relevance of VSMC accumulation in the development and progression of atherosclerosis and restenosis, strategies targeting the MAPK pathway as a common signaling step in VSMC migration and proliferation may provide new therapeutic approaches for the treatment and prevention of these vascular diseases.

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