Red Wine Polyphenols Inhibit Vascular Smooth Muscle Cell Migration Through Two Distinct Signaling Pathways

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Background—Red wine polyphenols (RWPs) have been shown to have an antiatherogenic activity mainly through antioxidative effects on LDL oxidation. Although vascular smooth muscle cell (SMC) migration is critical to atherosclerosis formation, the effect of RWPs on SMC migration has not been elucidated. In this study, we investigated whether RWPs could affect the migration of cultured SMCs stimulated by growth factors.

Methods and Results—RWP concentration dependently inhibited platelet-derived growth factor (PDGF)-BB–induced and serum-induced SMC migration in wounding assay and Boyden chamber assay. However, these inhibitory effects of RWPs were not seen in serum-stimulated vascular endothelial cell migration in either assay. Moreover, specific inhibitors of phosphatidylinositol-3'-kinase (PI3K) and p38 mitogen-activated protein kinase (p38 MAPK), but not of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), reduced PDGF-BB–induced SMC migration. To elucidate the signaling mechanism underlying the RWP effects, we investigated the effects of RWPs on the activity of PI3K and the phosphorylation of MAPK pathways in PDGF-BB–stimulated SMCs. RWPs inhibited the PI3K activity and p38MAPK phosphorylation, but not ERK1/2 phosphorylation, in a concentration-dependent manner. Moreover, the phosphorylation of MKK3/6, an upstream kinase of p38 MAPK, was also inhibited by RWP treatment in a concentration-dependent manner, suggesting that the inhibitory effect of RWPs on the p38MAPK pathway works upstream of MKK3/6. The concentration-effect relationship of RWPs necessary for the inhibition of PI3K and p38MAPK pathways was similar to that of cell migration assays.

Conclusions—RWPs inhibit the SMC migration through the inhibition of 2 distinct signaling pathways and thus exert antiatherogenic actions. (Circulation. 2002;105:2404-2410.)

Key Words: muscle, smooth | endothelium | atherosclerosis

Lower rates of mortality from coronary heart disease have been observed in the French population despite its having a high-cholesterol diet. Red wine has been assumed to account for the phenomenon, which is known as the “French paradox.”1 Recently, it has been demonstrated that red wine polyphenols (RWPs) have various antiatherogenic effects, such as antioxidation of LDL-cholesterol,2-3 inhibition of adhesion molecule expression in cytokine-stimulated vascular endothelial cells (ECs),4 inhibition of platelet aggregation,5 induction of NO release, and vasorelaxation.6

It has been shown that development of atherosclerosis is characterized by EC dysfunction, proliferation and migration of vascular smooth muscle cells (SMCs), and increased extracellular matrix deposition.7 SMC migration is believed to play a major role in the pathogenesis of many vascular diseases, including progressive intimal thickening after coronary intervention.8 It has been previously reported that activation of the phosphatidylinositol 3'-kinase (PI3K) pathway is a rapid response to platelet-derived growth factor (PDGF), one of the most potent chemoattractants for SMCs, and is implicated in cellular motility, such as actin reorganization and membrane ruffling, in several cell types, including SMCs.9,10 Moreover, it has been demonstrated recently that activation of mitogen-activated protein kinase (MAPK) pathways also plays a role in cell migration. Specifically, it has been reported that p38MAPK, one of the MAPK pathways, may mediate the migratory action of tracheal SMCs in response to PDGF.11

We have recently shown that RWPs inhibit SMC proliferation and that the downregulation of a cell-cycle regulator, cyclin A, is at least in part responsible for this growth inhibition.12 However, the effect of RWPs on SMC migration is not known. Thus, in the present study, we investigated whether RWPs could affect PDGF-BB–induced SMC migration and activation of intracellular signaling pathways that are known to mediate cell migration.
Methods

Preparation of Red Wine Polyphenolic Compound
RWPs were extracted from pulverized red wine (7600 mL in total; Suntory Co) by adsorption chromatography using a Diaion HP-20 column as described. The total RWP fraction (24 g total) was obtained by eluting the column with 100% ethanol. This compound was dissolved in 50% ethanol using ultrasonication. The effects of RWPs on our experiments were compared with those of ethanol at a final concentration of 0.5% as vehicle.

Cell Culture
Rat aortic SMCs (RASMCS) were isolated from Sprague-Dawley rats, and human aortic SMCs (HASMCs) were purchased (BioWhit-taker, Inc.) in cell migration assays, migration of SMCs was compared with that of serum-stimulated ECs, bovine carotid ECs (BCECs), or human umbilical vascular endothelial cells (HUVECs). All cells were subcultured as described.

Cell Migration Assay
Two migration assays were performed. For the monolayer-wounding cell migration assay (wounding assay), SMCs that had been grown to confluence in 6-well culture plates were subjected to wounding as previously described. The cells were preincubated with serum-free medium for 24 hours and with RWPs at final concentrations of 1 to 100 μg/mL for 10 hours. Cells layers were scraped with a sterile single-edged razor blade and reincubated with RWPs at each concentration and then stimulated with PDGF-BB (10 ng/mL; human recombinant, Sigma) or 10% serum. After 30 hours of incubation at 37°C, the cells were fixed and stained with Giemsa stain. The fixed cells were microscoped, and the number of cells migrated across the regions of the wound edge was counted. In addition, to investigate the effect of RWPs on EC migration, the migration of 10% serum-stimulated BCECs or HUVECs was examined.

In the second assay, migration of SMCs or ECs briefly exposed to chemotoxant and RWPs was assayed using a 96-well modified Boyden chamber housing a collagen-precultured polycarbonate filter with 8.0-μm pores, as previously described. Lower chambers were filled with each chemotaxant, PDGF-BB (0.1 to 20 ng/mL) or 10% serum, and RWPs (1 ~ 100 μg/mL) in serum-free medium. Under this condition, the duration of RWP treatment was the same as that of the chemotoxant. An equal number of cells (5 × 10^4 cells/mL) in 225 μL was loaded into the upper chamber. After incubation for 6 hours, migrating cells on the lower surface of the filter were determined by counting 4 high-power (×200) fields of constant area per well. Moreover, the effect of RWPs on cell migration induced by PDGF-BB in both the upper and lower chambers, which represents chemokinetics, was also investigated. To examine whether the signaling pathway of PI3K or MAPK plays a role in SMC migration, the cells were pretreated with specific inhibitors, wortmannin (PI3K inhibitor; Sigma), SB203580 (p38MAPK inhibitor; Sigma), or PD98059 (MEK1 inhibitor; New England BioLabs) for 30 minutes, and the inhibitors were added again in the upper and lower chambers using the Boyden chamber assay.

PI3K Activity
The PI3K activity was assayed by the method described with slight modifications. After serum-starved SMCs were pretreated with RWPs for 10 hours, the cells were stimulated by PDGF-BB for 10 minutes, and then cell lysates were incubated with antiphosphotyrosine antibody (PY-20) for 2 hours and complexed with protein A-Sepharose (Amersham Pharmacia Biotech AB) for 1 hour at 4°C. The immunoprecipitate was washed and resuspended in 50 μL PI3K buffer (10 mMol/L Tris, pH 7.5, 100 mMol/L NaCl, 1 mMol/L EDTA, and 100 μMol/L Na3VO4), and 10 μg of phosphatidylinositol was added. The reaction was started by the addition of [γ-32P]ATP, and the samples were incubated at 30°C for 15 minutes. The reaction was stopped by the addition of 20 μL of 8N HCl and 160 μL chloroform methanol (1:1). The lower phase containing phospholipids was recovered and spotted on silica gel thin-layer chromatography plates (Gel-60, Merck), impregnated with 1% (wt/vol) potassium oxalate and allowed to dry before sample application, and developed in a mixture of chloroform, methanol, 28% NH4OH, and water (120:94:4:23.2 mL). The radioactivity on the dried plate was visualized and quantified by a Phospho-imaging analyzer.

MAPK Phosphorylation
After serum-starved HASMCs were pretreated with RWPs for 10 hours, the cells were stimulated by PDGF-BB for 10 minutes. Total protein extracts (20 μg/lane) were separated by 12% SDS-polyacryl- amide gel electrophoresis and transferred to nitrocellulose filter. To assess the activation of 3 distinct MAPK pathways, ERK1/2, SAPK-1/JNK, and p38MAPK, the total and phosphorylated proteins were detected with each specific MAPK antibody (New England BioLabs). Moreover, to examine the activation upstream of the p38MAPK pathway, antiphosphorylated MKK3/6 antibody was used. Bound proteins were visualized with enhanced chemiluminescence reagents (Amersham).

Cell Proliferation and DNA Synthesis
Serum-starved subconfluent HASMCs plated in 24-well plates (2 cm2/well) were pretreated with RWPs for 10 hours. For cell prolif-eration assay, the cells were stimulated with PDGF-BB (10 ng/mL) for 60 hours, and then cell number was measured. For DNA synthesis assay, the cells were stimulated with PDGF-BB (10 ng/mL) for 22 hours and then were labeled with [methyl-3H]-thymidine (Du-Port NEN) at 1 μCi/mL for 3 hours. After the cells had been labeled, incorporated [3H]-thymidine in the cells was extracted in 0.2N NaOH and measured.

Statistical Analysis
When statistically significant, effects were found by ANOVA analysis (Newman-Keuls test). A value of P<0.05 was considered significant. All data in the text and figures are expressed as mean±SEM.

Results
PDGF-BB as well as serum-stimulated migration of RASMCS in the wounding assay and pretreatment with RWPs showed potent inhibition of migration (Figure 1, top). The results of quantitative assessment in this assay demonstrated that RWPs inhibited PDGF-BB–induced or serum-induced migration of RASMCS in a concentration-dependent manner (Figure 1, bottom). In contrast, RWPs did not affect the migration of BCECs stimulated with 10% serum. RWPs also inhibited the migration of HASMCs stimulated with serum in a similar concentration-dependent manner but did not inhibit migration of serum-stimulated HUVECs (data not shown).

Cell migration can be generally characterized by the following 3 forms: (1) random motion, which occurs in the absence of any stimulus; (2) chemokinesis, which is a random motion that is influenced by a stimulus; and (3) chemotaxis, which is directed motion toward a gradient of a stimulus. Growth factor–induced migratory action consists of chemokinetics and chemotaxis. Because the incubation time in the wounding assay was 30 hours, newly divided cells may result in the increase in migrated cells. We additionally performed a Boyden chamber assay to examine the effect of RWPs on cell migration for 6 hours. As shown in Figure 2A, stimulation of RASMCS with PDGF-BB in the lower chamber showed a concentration-dependent induction of cell migration, which presents the chemotactic effect, and RWPs
exhibited potent inhibitory effects on the PDGF-BB–induced chemotaxis of RASMCs (58%, 15%, and 4% of the control with 10 ng/mL PDGF-BB at RWP concentrations of 10, 30, and 100 μg/mL, respectively). These findings suggest that RWP exerted antimigratory effects on SMCs even with a short exposure. Moreover, RWP concentration-dependently inhibited PDGF-BB–induced chemotaxis of HASMCs (Figure 2B). This result suggests that RWP exerted an inhibitory effect on SMC chemotaxis regardless of the species. In contrast, RWP did not inhibit the chemotaxis of BCECs stimulated with 10% serum, even at the highest concentration (100 μg/mL) of RWP (Figure 2C).

To elucidate whether each intracellular signaling pathway, PI3K or MAPK, mediates PDGF-BB–stimulated SMC migration, we investigated the effect of a specific inhibitor of each pathway. Treatment of RASMCs with wortmannin or SB203580 significantly decreased the migration (chemotaxis) in a concentration-dependent manner (Figure 3A). In contrast, PD98059 did not show any significant effects on the SMC migration. Moreover, simultaneous treatment with wortmannin and SB203580 showed an additional inhibition of SMC migration in our experimental conditions (Figure 3B). These findings suggest that the pathways of both PI3K and p38MAPK, but not of ERK1/2, are positively implicated in PDGF-BB–induced SMC migration.

Potent SMC chemotaxis induced by PDGF-BB in the lower chamber was inhibited by RWP (30 μg/mL) in the lower chamber, and this inhibition was enhanced by addition of RWP in the upper chamber. RWP also inhibited the random motion (chemokinesis) induced by PDGF-BB in both the upper and lower chambers (Figure 3C).

The effect of RWP on the lipid kinase activity of PI3K in PDGF-BB–stimulated VSMCs was examined (Figure 4A). Stimulation of RASMCs or HASMCs with PDGF-BB significantly increased PI3K activity, and RWP pretreatment inhibited PI3K activity in a concentration-dependent manner. The effects of RWP on the phosphorylation of each MAPK pathway, ERK1/2, SAPK-1/JNK, and p38MAPK, were examined (Figure 4B). RWP pretreatment inhibited the p38MAPK activation to 28%, 5%, and 3% of the control at concentrations of 10, 30, and 100 μg/mL, respectively. Surprisingly, the phosphorylation of other pathways, ERK1/2 and SAPK1/JNK, was not inhibited by RWP except at the highest concentration (100 μg/mL). These results suggest that the inhibitory effect of RWP on the activation of MAPK pathways could be specific for the p38MAPK pathway. To
clarify the mechanism of the inhibition of p38MAPK phosphorylation by RWPs, we examined the effect of RWPs on the phosphorylation of MKK3/6, an upstream kinase of p38MAPK. The phosphorylation of MKK3/6 was also inhibited by RWPs in a concentration-dependent manner, suggesting that the inhibitory effect of RWPs on the p38MAPK pathway works upstream of MKK3/6. It is noteworthy that the concentrations of RWPs necessary for the inhibition of PI3K and p38MAPK pathways were almost the same as those for the inhibition of SMC migration.

Activation of each signaling in PDGF-BB–stimulated SMCs was concentration-dependently inhibited by pretreatment with each inhibitor, whereas phosphorylation of ERK1/2 and p38MAPK was slightly inhibited by wortmannin only at the maximum concentration (Figure 5). In our experiments, each inhibitor did not interfere with other pathways.

We have previously reported that RWPs inhibited the serum-stimulated SMC proliferation.12 It is well known that the ERK1/2 pathway is important in growth factor–induced proliferation.17 However, PDGF-BB–induced activation of ERK1/2 in SMCs was not significantly inhibited by RWPs, as shown in Figure 4B. To investigate whether RWPs could inhibit PDGF-BB–stimulated SMC proliferation, the effect of RWPs on cell proliferation and DNA synthesis in SMCs was examined. RWPs significantly inhibited the cell number and [3H]-thymidine uptake of HASMCs stimulated by PDGF-BB in a concentration-dependent manner (Figure 6). These data suggest that the antiproliferative effect of RWPs on SMCs might be independent of the classic MAPK pathway ERK1/2.

**Discussion**

In the present study, we demonstrated that RWPs potently inhibited PDGF-BB and serum-stimulated SMC migration but not serum-stimulated EC migration. Moreover, RWPs significantly inhibited the activities of 2 signaling pathways, PI3K and p38MAPK, but not other MAPK pathways. The concentrations of RWPs necessary for the inhibition of these pathways were similar to those for the inhibition of SMC migration. Despite many studies investigating the mechanisms of intimal development using injured vessels of an animal model, there is little evidence that this model is valid either for the spontaneous intimal formation.
Several laboratories have previously reported that PI3K is indispensable for cell migration induced not only by PDGF-BB but also by other growth factors in several cell types. One study showed that replacement of 2 tyrosine residues within the PI3K-binding sites of the PDGF β-receptor causes a loss of chemotactic response to PDGF-BB; however, another study demonstrated that PI3K inhibitors did not inhibit chemotaxis in SMCs and Swiss 3T3 cells. In the present study, the inhibition of PI3K by wortmannin partially reduced PDGF-BB–induced SMC migration. The underlying mechanism of the discrepant observations about the functional role of PI3K in PDGF-induced SMC migration remains to be elucidated. It also has been demonstrated that cytoskeletal organization changes dynamically when cells increase their motility, and PI3K activity associates with an increase of cell motility. Small G proteins downstream of the PI3K pathway, Rac and Rho, are known to be involved in PDGF-stimulated cellular responses, such as Rac-mediated lamellipodia and Rho-mediated stress fiber and focal adhesion formation. The potent inhibitory effect of RWPs on SMC migration might be associated with, at least in part, the downregulation of these signaling pathways downstream of PI3K.

It has been reported that Akt, which is downstream of PI3K, is a major mediator for growth factors in cell survival. Under our experimental conditions, phosphorylation of Akt might not be important for cell survival, because RWPs at the highest concentration (100 μg/mL) significantly downregulated the Akt phosphorylation; however, RWPs did not induce apoptosis in PDGF-BB–stimulated or serum-stimulated SMCs (data not shown).

Recently, several reports have shown that p38MAPK activation is important for the cytoskeletal formation, including actin reorganization, and phosphorylation of heat shock protein 27 (hsp27), which is downstream of p38MAPK and has been shown to play a role in membrane ruffling. In the present study, we showed that treatment of PDGF-BB–stimulated SMCs with RWPs resulted in inhibition of p38MAPK phosphorylation but did not affect other MAPK pathways. We also demonstrated that p38MAPK inhibition by SB203580 showed a potent inhibition on SMC migration, but ERK1/2 inhibition by PD98059 did not show the significant inhibition, in good agreement with previous studies. These results suggest that RWPs would exert the antimigratory effect on SMCs via the inhibition of cytoskeletal formation in part.

Matsumoto et al have reported that expression of dominant-negative Ras inhibited PDGF-BB–induced migration in ECs expressing wild-type PDGF β-receptor. They demonstrated that dominant-negative Ras inhibited PDGF-BB–induced activation of p38MAPK as well as that of ERK1/2 and inhibition of p38MAPK by SB203580 significantly decreased EC migration, but inhibition of ERK1/2 by PD98059 did not. In the present study, because RWPs (3 to 30 μg/mL) did not inhibit PDGF-BB–induced ERK1/2 phosphorylation, it is very likely that RWPs did not interfere with the pathway from the PDGF receptor, via Ras, to ERK1/2. These findings suggest that the site of RWP action may be between Ras and p38MAPK. The phosphorylation of MKK3/6, upstream of

Figure 4. A, Effects of RWPs on PI3K activity in SMCs. After serum-starved RASMCs or HASMCs were pretreated with RWPs for 10 hours, the cells were stimulated with PDGF-BB (10 ng/mL) for 10 minutes. Immunoprecipitated cell lysates by anti-phosphotyrosine antibody (PY20) were assayed for PI3K activity. Radiolabeled phosphatidylinositol 3′ phosphate (PI[3]P), the enzymatic product of PI3K, was separated by thin-layer chromatography. Results of measured radioactivity are shown in the bottom panel. One representative result of 3 separate experiments is shown. B, Effects of RWPs on MAPK phosphorylation in SMCs. After pretreatment of serum-starved HASMCs with RWPs for 10 hours, the cells were stimulated with PDGF-BB (10 ng/mL) for 10 minutes. Western immunoblotting was performed using specific antibodies for each MAPK pathway, either in total or in phosphorylated form (p-). Moreover, effects of RWPs on MKK3/6 phosphorylation (p-MKK3/6) were determined using a specific antibody.
p38MAPK, was also inhibited by RWPs, suggesting that RWPs work upstream of p38MAPK. With regard to the inhibitory effect of RWPs on the p38MAPK pathway, additional investigation will be necessary to identify the exact point where RWPs work.

In our previous study, RWPs downregulated expression of cyclin A gene, one of the cell-cycle regulators, through downregulation of transcription factors.12 In the present study, RWPs strongly inhibited the proliferation and DNA synthesis of PDGF-BB–stimulated SMCs; however, the ERK1/2 phosphorylation, which is known to relate to cell growth,17 was not inhibited by RWPs (up to 30 μg/mL). These results suggest that the inhibitory effects of RWPs on cell-cycle progression in SMCs do not depend on the ERK1/2 pathway. On the other hand, it has been recently reported that p13K is associated with cell-cycle regulation, indicating that p13K inhibitors induce G1 arrest of several cells through upregulation of p27Kip1, one of the cyclin-dependent kinase inhibitors.28,29 Moreover, a key role of p27Kip1 in SMC migration using SMCs from p27Kip1−/− mice has been demonstrated recently.30 To clarify the mechanism of the antiproliferative and antimigratory effects of RWPs on SMCs, we investigated the effect of RWPs on the p27Kip1 expression in SMCs. Serum stimulation suppressed the induction of p27Kip1 in serum-starved SMCs, and treatment of serum-stimulated SMCs with PI3K inhibitor, wortmannin or LY294002, significantly induced the p27Kip1 expression. It is of interest that RWPs (30 μg/mL) also upregulated the p27Kip1 expression in serum-stimulated RASMCs (data not shown). This result suggests that RWPs suppress cell-cycle progression not only through the downregulation of cyclin A expression but also through the upregulation of p27Kip1 and that RWPs may inhibit intracellular signaling cascades activated by stimulators of cell proliferation and migration at an upstream site.

The concentration of RWPs in blood after red wine intake has not been determined, because RWPs consist of polymers as well as monomers, and for now it is impossible to measure the concentration of polymers in blood. Several researchers have shown that the absorption efficiency is ~1% to 5% for monomers.31 If the absorption efficiency of polymers is similar to this value, the calculated final concentration of RWPs in blood could be 1 to 10 μg/mL after intake of 30 to 300 mL red wine. Furthermore, if this concentration of RWPs in blood is maintained over the long term by the regular consumption of red wine, it might be possible that RWPs would exert antimigratory effects on SMCs in vivo.

When detachment of endothelial monolayer is induced by several injuries, EC migration is mainly responsible for the wound repair of damaged vessels. We previously showed that RWPs had no inhibitory effect on proliferation, DNA synthesis, and cyclin A gene expression in serum-stimulated ECs.12 In the present study, RWPs did not inhibit serum-induced EC migration in either migration assay. Although RWPs had no significant effects on proliferation and migration of ECs, the mechanism of these phenomena is unclear. However, we found that RWPs inhibited expression of vascular cellular adhesion molecule-1 in tumor necrosis factor-α–stimulated HUVECs in a concentration-dependent manner (data not shown). Moreover, it has been recently reported that gallates, a component of RWPs, inhibited cytokine-induced adhesion molecule expression in ECs through a reduction of nuclear factor-κB activity.4 These beneficial effects of RWPs on ECs suggest that RWPs may play a role not only in the maintenance of vascular structural integrity but also in the functional restoration of damaged ECs. In the future, the effects of RWPs on EC function, including membrane sensitivity and intracellular signaling, need to be elucidated.

In summary, RWPs have potent inhibitory effects on SMC migration and proliferation in the context of atherogenesis. Our findings on the intracellular signaling pathways modulating the proliferation and migration of SMCs may provide a new insight into the mechanism of the French paradox and
may enable us to innovate a therapeutic approach for the prevention of atherosclerotic progression.

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