Inhibition of Vascular Smooth Muscle Cell Proliferation and Neointimal Formation in Injured Arteries by a Novel, Oral Mitogen-Activated Protein Kinase/Extracellular Signal–Regulated Kinase Inhibitor

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Background—Mitogen-activated protein kinases (MAPKs) are rapidly induced after arterial injury in different animal models. However, their precise role in vascular smooth muscle cell (VSMC) proliferation and neointimal formation in vivo remains to be determined.

Methods and Results—We investigated the properties of a novel, selective inhibitor of the upstream kinase, MAPK/extracellular signal–regulated kinase, that is orally active (PD0185625). In vitro, PD0185625 was shown to abrogate p44/p42 MAPK activation in VSMCs after serum stimulation. This was associated with a dose-dependent inhibition of VSMC proliferation. In vivo, PD0185625 was administered orally to rats (200 mg · kg\(^{-1}\) · d\(^{-1}\)) beginning 2 days before balloon injury of the left carotid artery and for 2 weeks thereafter. Treatment with PD0185625 led to nearly complete inhibition of p44/p42 MAPK activation after balloon injury. This resulted in a significant decrease in VSMC proliferation (BrdU incorporation) at day 7 after injury. Moreover, neointimal formation was significantly reduced in PD0185625-treated animals at 14 and 28 days after arterial injury. We found that PD0185625 did not increase the rate of apoptotic cell death but prevented cell cycle progression and induced a G1 block.

Conclusions—PD0185625 reduced neointimal formation after arterial injury. The mechanism involved inhibition of VSMC proliferation via a G1 block of the cell cycle. Orally active selective MAPK inhibitors could represent a novel therapeutic approach for vascular diseases. (Circulation. 2004;110:3367-3371.)

Key Words: atherosclerosis ■ restenosis ■ signal transduction

Proliferation and migration of vascular smooth muscle cells (VSMCs) play important roles in the development of atherosclerotic diseases.\(^1\) However, the precise signaling pathways involved in that physiopathology are not completely understood. Mitogen-activated protein kinases (MAPKs) are a family of protein serine/threonine kinases regulating cell proliferation, motility, survival, and differentiation.\(^2\) The first member of this family to be characterized was p44/p42 MAPK, which is activated after phosphorylation by the dual-specificity kinase MAPK/extracellular signal–regulated kinase (MEK1). Although p44/p42 MAPK has been reported to be induced after arterial injury in different animal models,\(^3\) its specific role in VSMC proliferation and neointimal formation in vivo still remains to be determined.

In the present study, we investigated the role of p44/p42 MAPK in response to arterial injury by using a novel, selective MEK inhibitor that is orally active (PD0185625). Our results indicate that PD0185625 can successfully abrogate p44/p42 MAPK activation in vivo, which results in reduced VSMC proliferation and neointimal formation after arterial injury. We also demonstrate that PD0185625 does not increase the rate of apoptotic cell death but acts via a G1 block of the cell cycle.

Methods

Animal Experiments

Male Sprague-Dawley rats (400 g; Charles River Inc, Saint-Constant, Québec, Canada) were used for all experiments. The left carotid artery was denuded of its endothelium by intraluminal passage of a 2F embolectomy balloon catheter introduced through the external carotid artery. PD0185625 (Pfizer) was administered orally to rats beginning 2 days before balloon injury and for 2 weeks thereafter. The choice of doses used in our experiments was based on preliminary experiments with an ex vivo assay to assess the inhibition of MAPK activation in tumors excised from mice after treatment with PD0185625 (Dr Judith Sebolt-Leopold, personal communication, 2003). Rats were humanely killed by intravenous barbiturate overdose 30 minutes after balloon injury (time of maximal p44/p42 MAPK activation\(^4\) to 7) for analysis of...
p44/p42 MAPK and at days 14 or 28 for intima-media ratio measurements. A subgroup of animals received an intraperitoneal infusion of BrdU (100 mg/kg) 24 hours before euthanization at day 7 for analysis of cellular proliferation. Detection of DNA synthesis (BrdU incorporation) and apoptosis (terminal dUTP nick end-labeling [TUNEL]) in vivo was performed as previously described. All protocols were approved by the Comité Institutionnel de Protection des Animaux (CIPA) of Centre Hospitalier de l’Université de Montréal (CHUM).

Cell Culture
Rat VSMCs were isolated from the thoracic aorta by the explant technique. For all experiments, cells were serum-starved for 48 hours before serum stimulation. VSMC proliferation was assessed with the ELISA 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Promega). Cell cycle was assessed by flow cytometry (Becton-Dickinson) with propidium iodide (PI) staining. Apoptotic cells were determined by binding of annexin V along with PI exclusion.

Western Blotting
Phosphospecific p44/p42 MAPK Western blots were performed as previously described.

Statistical Analysis
All results are expressed as mean±SEM. Student’s t test was used for comparisons between 2 means. A value of P<0.05 was interpreted to denote statistical significance.

Results

Effect of PD0185625 on p44/p42 MAPK Activation and VSMC Proliferation In Vitro
Treatment with PD0185625 dose-dependently inhibited VSMC proliferation after serum stimulation in vitro (Figure 1A). At a dose of 50 μmol/L, PD0185625 maximally inhibited VSMC proliferation and completely abrogated serum-induced p44/p42 MAPK activation (Figure 1B). Fluorescence-activated cell sorting analyses revealed that treatment of VSMCs with PD0185625 prevented cell cycle progression and induced a G1 block (Figure 1C). However, treatment with PD0185625 was not associated with an increased rate of apoptosis in VSMCs (Figure 1D).

Inhibition of p44/p42 MAPK Activation by PD0185625 After Arterial Injury
An important increase in p44/p42 MAPK activity was documented 30 minutes after balloon angioplasty in rat carotid arteries (Figure 2A). Animals treated orally with PD0185625 showed a dose-dependent reduction in p44/p42 MAPK activation, with nearly complete inhibition obtained at a dose of 200 mg·kg\(^{-1}\)·d\(^{-1}\) (Figure 2A).

Effect of p44/p42 MAPK Inhibition In Vivo on Cellular Proliferation and Cellular Apoptosis After Arterial Injury
Cellular proliferation and apoptosis were evaluated at day 7 after arterial injury by BrdU incorporation and TUNEL staining, respectively. Treatment with PD0185625 (200 mg·kg\(^{-1}\)·d\(^{-1}\)) led to a significant reduction in the percentage of BrdU-positive cells in the neointima (19±3% vs 47±5%, P<0.005) and in the media (3.3±0.5% vs 7.5±0.9%, P<0.05; Figure 2B, top). However, PD0185625 therapy did not increase the rate of apoptotic cell death in injured arteries (Figure 2B, bottom).

Effect of p44/p42 MAPK Inhibition In Vivo on Neointimal Formation
As shown on Figure 2C, neointimal formation was significantly reduced in PD0185625-treated animals by day 14 after arterial injury (intima-media ratio, 0.65±0.05 vs 0.93±0.07, P<0.005). The inhibiting effect of PD0185625 on neointimal formation was even more pronounced by day 28 after arterial injury (intima-media ratio, 0.69±0.07 vs 1.33±0.11, P<0.005).

Discussion
p44/p42 MAPK activity is rapidly induced after arterial injury in different animal models, and it has been suggested that this might trigger a series of molecular events leading to neointimal formation. However, defining the specific role of p44/p42 MAPK activation in neointimal formation has previously been complicated by the lack of a specific and potent pharmacologic inhibitor that can be used in vivo. Previous studies that used local inhibition of p44/p42 MAPK to prevent neointimal formation have reported variable results. For instance, gene transfer of a dominant-negative mutant of p44/p42 MAPK has been shown to prevent VSMC proliferation and neointimal thickening in balloon-injured arteries. On the other hand, local administration of a selective inhibitor of MEK1 (PD98059) reduced medial cell replication after arterial injury but did not inhibit intimal cell replication. These apparently conflicting results might be at least partly due to variations in the efficiency of p44/p42 MAPK inhibition in vivo. Moreover, such studies are performed with invasive surgical procedures and lengthy delivery times that are not applicable for clinical use. The current study provides the first comprehensive analysis of the role of p44/p42 MAPK activation in response to arterial injury by using a potent, systemic inhibitor (PD0185625) that is orally active.

We first demonstrated that PD0185625 can completely abrogate p44/p42 MAPK activation in serum-stimulated VSMCs and that this led to an important reduction in cellular proliferation (Figure 1A and 1B). We also showed that PD0185625 did not induce apoptosis but had a cytostatic effect on VSMCs, preventing cell cycle progression via a G1 block (Figure 1C and 1D). Interestingly, the 2 agents that are currently used as coatings to prevent in-stent restenosis in patients (rapamycin and paclitaxel) are also cytostatic as opposed to cytotoxic. In recent clinical trials, drug-eluting stents have been shown to reduce restenosis and target-vessel revascularization. However, these devices have potential limitations. First, the agents used cannot be given over a long time, considering the limited amount of drug that can be placed on the stent. Second, this strategy necessitates deployment of a stent, which might not be possible or desirable in certain situations (complex anatomy, diffuse disease, very small vessels, etc). Finally, one major limitation of drug-eluting stents is their cost, which is currently 2 to 3 times more
Figure 1. PD0185625 inhibits p44/p42 MAPK activation and VSMC proliferation in vitro. A, Quantification of cellular proliferation was performed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay in presence of different concentrations of PD0185625, n=7 per group. *P<0.005 vs control. B, Cellular extracts were prepared at different times after serum stimulation in VSMCs treated or not with 50 μmol/L PD0185625. p44/p42 MAPK activation was evaluated by Western blotting and phosphospecific antibody. Similar results were obtained in 4 different experiments. C, Cell cycle analysis was performed by flow cytometry after 24 hours of treatment with PD0185625. Percentages of cells in each phase of cell cycle are indicated in table below graph. Similar results were obtained in 3 different experiments. D, Representative flow cytometric dot plots of annexin V–fluorescein isothiocyanate (FITC)/PI dual-color flow cytometry after 24 hours of treatment with 50 μmol/L PD0185625 or mitomycin C (used as positive control for apoptosis). Bottom right region represents apoptotic cells. Similar results were obtained in 3 different experiments. All other abbreviations are as defined in text.
than conventional stents. Therefore, the use of oral agents might provide a less expensive and in certain situations, a more effective means of dealing with restenosis or progressive atherosclerosis.

Although promising results were shown in preclinical animal experiments, previous studies of systemic therapy to prevent restenosis in humans have been largely disappointing. Negative results have been attributed to a low antiproliferative effect of the agents used and/or inadequate local drug concentrations. In the present study, we showed that the activation of p44/p42 MAPK in injured arteries was almost completely abolished in animals treated orally with PD0185625, which indicates that this drug can reach adequate local concentrations (Figure 2A). Moreover, the pharmacologic effect of PD0185625 was confirmed by demonstrating a significant reduction in cellular proliferation and neointimal formation after arterial injury in treated animals (Figure 2B and 2C). Interestingly, the reduction of neointimal formation in animals treated with PD0185625 for 14 days was even more pronounced at 28 days versus 14 days after angioplasty (Figure 2C). This suggests that a short-course treatment during the active phase of VSMC proliferation after arterial injury might be sufficient to inhibit neointimal formation and prevent restenosis.

In summary, the present study highlights the important role of p44/p42 MAPK for neointimal formation in response to arterial injury. We have shown for the first time that an oral inhibitor of the upstream kinase MEK has a cytostatic effect on VSMCs and can inhibit neointimal formation in vivo. These results could provide important insights for the development of novel therapeutic strategies against progressive atherosclerotic diseases.

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