Thymidine Phosphorylase Gene Transfer Inhibits Vascular Smooth Muscle Cell Proliferation by Upregulating Heme Oxygenase-1 and p27KIP1

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Objective—Thymidine phosphorylase (TP) reportedly promotes endothelial cell migration and induces heme oxygenase (HO)-1 expression. However, its effect on vascular smooth muscle cells (VSMCs) is poorly understood. In this study, we examined the effect of TP on VSMCs in vitro and in vivo.

Methods and Results—Phagemid vector encoding human TP gene was transfected into rat VSMCs, and a clone overexpressing TP was selected (C2). C2 showed a slower migration and proliferation than VSMCs cloned with empty vector (pC) under basal, serum-stimulated, and hypoxic conditions. This decrease in proliferation correlated with TP-induced HO-1 expression and was reversed by inhibitors of either TP or HO activity. Furthermore, in C2, the cyclin-dependent kinase inhibitor (p27KIP1) was much more abundant than in pC, and the cell cycle was arrested at the G1 phase. TP or HO activity inhibitors decreased p27KIP1 expression in C2 to the level seen in pC. Adventitial TP gene delivery significantly reduced neointimal VSMC migration and neointima formation in balloon-injured rat carotid arteries.

Conclusions—TP overexpression upregulated HO-1 expression and consequently increased p27KIP1 in cultured VSMCs, and inhibited VSMC migration and proliferation in vitro and in vivo. TP represents a promising target for treating vascular obstructive disease. (Arterioscler Thromb Vasc Biol. 2005;25:1370-1375.)

Key Words: angiogenesis • gene therapy • thymidine phosphorylase • vascular obstructive disease • vascular smooth muscle cells

Dysregulated growth and motility of vascular smooth muscle cells (VSMCs) contribute to neointimal lesion development during the pathogenesis of vascular obstructive disease, including atherosclerosis, restenosis after percutaneous interventions, and bypass graft stenosis.1 Inhibition of this process is associated with reduced neointimal thickening in the setting of balloon angioplasty and chronic graft vessel disease.2 Thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor (PD-ECGF), has been reported to elicit a strong angiogenic response reflecting enhanced endothelial cell migration, but not proliferation.3,4 TP/PD-ECGF reportedly inhibits apoptosis and induced heme oxygenase-1 (HO-1) expression in cancer cells.5–8 In our previous study using the TP/PD-ECGF gene to alleviate myocardial ischemia in vivo, therapeutic effects involved the induction of angiogenesis including arteriogenesis, as well as decreased myocardial apoptosis, although downstream signals and molecular mechanisms underlying these effects remain obscure.9 In contrast, the effects of TP on VSMCs should not be neglected, because VSMCs are involved in neointima formation in the setting of vascular disease, arteriogenesis, and vessel maturation after angiogenesis.10,11

HO-1, also known as heat shock protein (HSP) 32, can be expressed in essentially any tissue on appropriate stimulation, and plays a physiological role in the vasculature because of its antiatherogenic properties.12 In particular, HO-1 is very sensitive to upregulation by a variety of stress mediators. HO-1 induction is strongly associated with protection against cellular injury and is considered a fundamental endogenous defense. The protective activity of HO-1 may involve degradation of the pro-oxidant heme substrate or possibly effects of the reaction products, carbon monoxide (CO), biliverdin, which is subsequently converted to bilirubin, or iron, which is bound by ferritin.12–16 CO has properties similar to those of nitric oxide and alters expression of inflammatory mediators, including tumor necrosis factor, IL-1, and IL-10.14,17 CO also can have a protective role against vascular injury, because it inhibits VSMC proliferation.18,19 Pretreatment with the HO-1 inducer, hemin, or adenovirus-mediated HO-1 gene transfer has been reported to inhibit neointimal formation induced by balloon injury via generation of CO.19,20

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Sengupta et al. have characterized the effect of TP/PD-EGF on endothelial cells. Although Somjen et al. reported that TP/PD-EGF inhibits DNA synthesis in cultured smooth muscle cells based on radiolabeled thymidine uptake and incorporation, previous reports have shown that the effects of TP/PD-EGF on cellular uptake of [methyl-3H]thymidine arise from its effect on the availability of thymidine in the extracellular culture medium, and the mechanism has not been elucidated. To expand our understanding of TP/PD-EGF effects on the vascular system, we presently focused on VSMCs. The human TP/PD-EGF gene was transfected into rat VSMCs in vitro and adventitia of balloon-injured rat carotid artery in vivo to study its effect on VSMC proliferation and migration as well as underlying mechanisms.

Materials and Methods

The phagemid expression vector (PBK-RSV; Stratagene, La Jolla, Calif), encoding the human TP gene or control PBK-RSV vector, was transfected into VSMCs; and expression of TP/PD-EGF by cells was detected by immunoblot analysis and a TP/PD-EGF activity assay. Thirteen clones that overexpressed TP/PD-EGF were selected. All of these VSMC clones overexpressing TP/PD-EGF showed a slower growth rate than empty vector-transfected control cells (pC). Clone 2 (C2) cells and the pC cells were used for the following experiments.

Proliferation of synchronized cells under serum stimulated conditions in the presence or absence of 200 μmol/L of an inhibitor of TP/PD-EGF (TPI; TAIHO Pharmaceutical, Tokyo, Japan), or 10 μmol/L tin-mesoporphyrin (SnMP, an inhibitor of HO activity; Porphyrin Products, Logan, Utah), or under hypoxic conditions (stimulated with CoCl2) was quantified with the MTT assay. In another experiment, serum-stimulated proliferation of wild-type VSMCs in the presence of cell lysate (C2, pC, or wild VSMC lysate) was quantified with the MTT assay. Apoptosis in the setting of hypoxia was detected using an apoptotic DNA ladder kit (Roche Diagnostics GmbH, Mannheim Germany). VSMC migration was analyzed by both QCM-FN, a quantitative cell migration assay kit (Chemicon international, Temecula, Calif), and a wound regeneration assay.

HO-1, HSP 25, p27KIP1, and cyclin D1 expression were analyzed by Western analysis. HO activity was detected as described by Turcanu et al. Cell cycle progression was analyzed using flow cytometry.

For in vivo experiments, 14 male Sprague-Dawley rats (290 to 310 grams; Sankyo Labo Service, Toyama, Japan) were anesthetized with pentobarbital (50 mg/kg), a 2-French balloon catheter was inserted into the aorta through the right external carotid artery, and angioplasty of the right common carotid artery was performed by an intraperitoneal overdose of pentobarbital 14 days later for around the common carotid artery for gene delivery. Rats were killed by an intraperitoneal overdose of pentobarbital 14 days later for cell lysis to CoCl2 at the indicated concentrations in the presence or absence of TPI for 24 hours, and proliferation was assessed with an MTT assay. Results represent the mean values for 6 separate experiments. *P<0.01, C2 vs pC; †P<0.01, C2 vs C2+TPI; ‡P<0.01, C2 vs pC and VSMC. C2 indicates VSMC transfected with the TP/PD-EGF gene, whereas pC indicates VSMC transfected with empty vector. ND indicates not detectable.

Results

Effect of TP/PD-EGF on VSMC Proliferation

TP/PD-EGF immunoblotting confirmed that C2 cells expressed more TP/PD-EGF than the pC cells, which, in fact, showed no detectable TP/PD-EGF expression. Corresponding to the results of Western analysis, TP/PD-EGF activity was markedly increased in the C2 cells, with no activity detected in the pC cells (Figure 1A).

Under serum-starved and serum-stimulated conditions (2.5%, 5%, or 10% fetal calf serum), C2 cells showed slower growth than pC cells (Figure 1B). Results are represented as fold change in the growth rate compared with control cells (0% fetal calf serum). Similar results were found for other tested clones (clone 7 and 13, data not shown). The addition of TPI did not influence the proliferation of pC cells at any concentration of fetal calf serum used but markedly increased the proliferation of C2 cells compared with pC cells under the same conditions. Furthermore, addition of cell lysate from wild VSMCs or pC cells did not influence the serum stimulated proliferation of wild VSMCs, but the C2 cell lysate significantly decreased the serum stimulated proliferation of wild VSMC at concentrations of 100, 50, and 25 μmol/L (Figure 1C).

To examine whether TP/PD-EGF can protect VSMCs from death during chemically induced hypoxia, we exposed the C2 and pC cells to CoCl2, which induces a form of hypoxia. CoCl2 dose-dependently decreased the cell growth rate in C2 and pC cells in the presence of 10% fetal calf serum. When the CoCl2 concentration exceeded 300 μmol/L, cytotoxicity became apparent based on the trypan blue exclusion assay, and no difference in viability was found between C2 and pC cells. However, C2 cells had a lower growth rate than pC cells at 150 to 300 μmol/L concentrations of CoCl2. This difference disappeared with the addition of TPI (Figure 1D). We used a DNA ladder assay to...
determine whether decreased C2 cell growth during chemically induced hypoxia was associated with apoptosis. No DNA fragments were detected at any concentration of CoCl₂ (data not shown).

**Effect of TP/PD-ECGF on VSMC Migration**

Because not only proliferation but also migration of VSMCs are major determinants of vascular lesion formation, we examined migration using the Boyden chamber assay and wound regeneration of cells after mechanical injury to a confluent monolayer. As shown in Figure 2A, migration of C2 was slower than pC based on the results of the Boyden chamber assay. Furthermore, a greater recovery of the wounded area induced by 10% fetal calf serum in the presence of 1 μg/mL actinomycin D, which was used to prevent the proliferation of cells, was observed in the pC cells, but not in C2 and C7 cells (Figure 2B).

**Effect of TP/PD-ECGF on HO-1 Expression**

Transfection of TP/PD-ECGF into VSMC markedly increased HO-1 expression under basal conditions. Serum stimulation dose-dependently increased the expression of HO-1 in both C2 and pC cells, although HO-1 expression remained higher in C2 than in pC cells (Figure 3A). When quiescent cells were exposed to 150 μmol/L CoCl₂, HO-1 expression increased significantly in C2 and pC cells compared with basal conditions, although the expression was higher in C2 than in pC cells (Figure 3B). Paralleling the increased expression of HO-1 observed by immunoblot analysis, HO activities were higher in C2 than in pC cells under serum-stimulated conditions (Figure 3C). Addition
of TPI or SnMP markedly decreased HO expression and activity in C2 cells to the level seen in pC cells (Figure 3B and 3C). Expression of HSP 25 in the 2 cell lines was constitutive and was not changed by serum or CoCl2 treatment (data not shown). Furthermore, addition of 2-deoxy-D-ribose (10^{-4} \text{ mol/L}) significantly reversed TPI inhibition of HO-1 expression under 10\% fetal calf serum-stimulated conditions (Figure 3D).

**HO Effect on VSMC Proliferation**

As shown in Figure 4, after serum deprivation for 48 hours, addition of 10\% fetal calf serum markedly stimulated proliferation in both cell lines. Adding SnMP to 10\% fetal calf serum media further increased proliferation of C2 cells to equal that in pC cells stimulated with 10\% fetal calf serum, although addition of 10\% fetal calf serum medium including SnMP also slightly increased proliferation of pC cells. A control experiment performed to determine the effect of SnMP on proliferation in the absence of serum showed that SnMP had no proliferative effect on the 2 cell lines under the concentrations that were used (data not shown).

**Effect of TP/PD-ECGF on the Cell Cycle and on Cell Cycle-Related Protein Expression**

To further examine the effect of TP/PD-ECGF on VSMC proliferation, cell cycle characteristics of the 2 cell lines were analyzed. As shown in Figure 5A, the percentage of C2 cells in the G1 phase was much greater than that of pC cells, whereas the percentage of C2 cells in the S and G2/M phase was significantly less than that of pC cells. Expression of p27^{KIP1} in C2 cells was greater than in pC cells, whereas no difference was seen between the 2 cell lines in the expression of cyclin D1, an early G1 marker. Neither TPI nor SnMP affected the expression of cyclin D1, but both significantly decreased the expression of p27^{KIP1} in C2 cells (Figure 5B). TPI and SnMP did not influence the expression of p27^{KIP1} in pC cells.

**Effect of TP/PD-ECGF on Neointimal Formation In Vivo**

X-gal staining revealed that chitosan hydrogel induced strong gene expression in the adventitia and vascular wall (Figure I, available online at http://atvb.ahajournals.org). As shown in Figure 6, neointimal formation was significantly reduced in TP/PD-ECGF–treated rats 14 days after arterial injury (intima/media ratio, 0.775±0.093 versus 1.795±0.124; \( P<0.001 \)). Immunohistochemical staining for smooth muscle cell \( \alpha \)-actin demonstrated that neointima is composed predominantly of VSMCs in the pCI/LacZ treated groups, but there were fewer VSMCs in the pClhTP group.

**Discussion**

This study demonstrated that TP/PD-ECGF gene transfer inhibits proliferation and migration of VSMC in vitro and in vivo, and the possible mechanism responsible for these effects is related to its enzyme activity and may act through increases in HO-1 and p27^{KIP1} expression.

TP/PD-ECGF has a chemotactic, but not a proliferative, effect on endothelial cells.\(^3\)\(^4\) Although TP/PD-ECGF has no mitogenic effect on endothelial cells, such as that of vascular endothelial growth factor, Sengupta et al reported that equimolar concentrations of TP/PD-ECGF and vascular endothelial growth factor induce similar monolayer recovery in wounded endothelium.\(^8\) Taking these observations together with our previous in vivo study into account,\(^9\) we believe that TP/PD-ECGF has a strong angiogenic effect. However, the effect of TP/PD-ECGF on other types of vascular cells, especially VSMCs, required further consideration.

In this study, we first demonstrated that after serum starvation for 48 hours, serum-stimulated proliferation of TP/PD-ECGF gene-transfected rat VSMCs (C2) was significantly decreased. Furthermore, adding the C2 lysate, which has higher TP activity, also decreased the proliferation of wild-type VSMC, in keeping
with the findings of Somjen et al. The degree of monolayer recovery of wounded C2 and C7 cells was lower than in pC cells, and these data are consistent with the results of Boyden chamber assay, suggesting that TP/PD-ECGF inhibits VSMC migration in vitro. Furthermore, in a balloon-injured rat carotid artery model, adventitial gene transfer of TP/PD-ECGF to injured vessels markedly decreased VSMC migration from the media to the intima, resulting in a reduction of intima formation in the TP/PD-ECGF–treated group. These effects of TP/PD-ECGF indicate an ability to decrease neointimal smooth muscle cell proliferation and migration, which are therapeutically important because of their role in reducing neointimal mass in occlusive vascular diseases and inhibiting further neointimal growth. Compared with the other angiogenic growth factors, such as vascular endothelial growth factor and fibroblast growth factor, which have been reported to stimulate, but not initiate, intimal thickening, our data suggest that TP/PD-ECGF is more suitable for gene therapy for occlusive diseases.

The second finding of this study was that TP/PD-ECGF decreased the growth rate of VSMC under CoCl2-induced hypoxia. This was unexpected, because we had suspected that TP/PD-ECGF would suppress VSMC apoptosis induced by hypoxia. However, we did not find evidence of DNA laddering in either cell line after exposure to 150 \( \mu \text{mol/L} \) to 1200 \( \mu \text{mol/L} \) of CoCl2 for 24 hours. This finding indicates that TP/PD-ECGF can induce other proteins, resulting in higher expression under hypoxic conditions and inhibition of proliferation of VSMCs.

In this study, we examined the possibility that the antiproliferative properties of TP/PD-ECGF on VSMCs are mediated by its enzyme-linked induction of HO-1 and p27\(^{kip1}\), as opposed to other small HSP, such as HSP 25, or cell cycle-related proteins such as cyclin D1. We found that transfection of the TP/PD-ECGF gene into rat VSMCs induced higher levels of HO-1 expression under basal, serum-stimulated, and CoCl2-induced hypoxic conditions. Inhibition of TP/PD-ECGF activity decreased HO-1 expression and activity in C2 cells to the level seen in pC cells under CoCl2-stimulated or serum-stimulated conditions, and 2-deoxy D-ribose, a product of thymidine metabolism by TP/PD-ECGF, significantly reversed the mediated TPI reduction in HO-1 expression under serum-stimulated conditions, suggesting that TP/PD-ECGF influences the signaling pathway of HO-1 induction, which is consistent with reported findings in cancer cells. This finding could account for the low C2 cell growth rate in the presence of CoCl2, because C2 cells showed a higher level of HO-1 expression under this condition, whereas HO-1 actions, including generation of its products, such as CO, inhibit VSMC proliferation. Visner et al. and Volti et al. reported that HO-1 induction inhibits VSMC proliferation, in agreement with our findings, but the responsible mechanisms have not been elucidated. In this study, we demonstrated that SnMP, an inhibitor of HO activity, increased C2 cell proliferation, similar to the effect of TPI, whereas addition of TPI or SnMP to the media significantly decreased p27\(^{kip1}\) expression in C2 cells. As described, CO has properties similar to those of nitric oxide, and Sato et al have reported that endothelial nitric oxide synthase gene transfer to VSMCs inhibits cell proliferation via upregulation of p27 and p21, resulting in a delay in cell cycle progression, but not via apoptosis. Igawa et al. reported that TP/PD-ECGF transfected into the head and neck squamous cell carcinoma cell line, IMC-3, showed a low level of p27\(^{kip1}\) expression, suggesting that TP/PD-ECGF regulates the p27\(^{kip1}\) pathway, and the regulation of p27\(^{kip1}\) expression may be cell-dependent. We therefore hypothesize that the antiproliferative effects of TP/PD-ECGF on VSMCs are mediated by HO-1 induced p27\(^{kip1}\) expression. With respect to related proteins, p27\(^{kip1}\) is a member of the family of cell cycle regulators called cyclin-dependent kinase inhibitors. These bind to “cyclin–CDK” complexes to cause cell cycle arrest in the G1 phase. Often known as a universal cyclin-dependent kinase inhibitors, p27\(^{kip1}\) interacts with all subtypes of “cyclin–CDK” complexes to inhibit cell cycle progression. In vitro, p27\(^{kip1}\) acts as an important regulator of aortic VSMC proliferation. In vivo, p27\(^{kip1}\) limits vascular remodeling in the systemic circulation after balloon injury. Considering our data (Figure 5) that the rate of passage through G1 was decreased in C2 cells, we further hypothesize that TP/PD-ECGF inhibits proliferation of VSMCs by increasing the expression of HO-1 and, in turn, that of p27\(^{kip1}\).

In summary, our results provide direct evidence that TP/PD-ECGF overexpression inhibits VSMC migration and proliferation in vitro and in vivo. These effects are mediated by the
induction of HO-1 and the resulting cell cycle effects involve increases in p27KIP1. In addition to the direct chemotactic effect of TP/PD-ECGF on endothelial cells, TP/PD-ECGF–derived HO-1 expression may promote endothelial cell proliferation and protect endothelial cells from injury. Thus, TP/PD-ECGF may prove important in the therapy of ischemic or vascular obstructive diseases in a number of different ways. We conclude that treatment of ischemic disease with TP/PD-ECGF warrants further preclinical investigation.

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