Temporal PTEN inactivation causes proliferation of saphenous vein smooth muscle cells of human CABG conduits

Amit K. Mitra, Guanghong Jia, Deepak M. Gangahar, Devendra K. Agrawal

Abstract

Internal mammary artery (IMA) coronary artery bypass grafts (CABG) are remarkably resistant to intimal hyperplasia (IH) as compared to saphenous vein (SV) grafts following aorto-coronary anastomosis. The reason behind this puzzling difference still remains an enigma. In this study, we examined the effects of IGF-1 stimulation on the PI3K-AKT/PKB pathway mediating proliferation of smooth muscle cells (SMCs) of IMA and SV origin and the specific contribution of phosphatase and tensin homologue (PTEN) in regulating the IGF-1-PI3K-AKT/PKB axis under these conditions. Mitogenic activation with IGF-1, time-dependently stimulated the phosphorylation of PI3K and AKT/PKB in the SV SMCs to a much greater extent than the IMA. Conversely, PTEN was found to be significantly more active in IMA SMCs. Transient overexpression of PTEN in SMCs of SV and IMA inhibited AKT/PKB activity and upstream of AKT/PKB, caused a reduction of IGF-1 receptors. Downstream, PTEN overexpression in SV SMCs induced the transactivation of tumour suppressor protein p53 by down-regulating the expression of its inhibitor MDM2. However, PTEN overexpression had no significant effect on MDM2 and p53 expression in IMA SMCs. PTEN overexpression in SV SMCs induced the transactivation of tumour suppressor protein p53 by down-regulating the expression of its inhibitor MDM2. However, PTEN overexpression had no significant effect on MDM2 and p53 expression in IMA SMCs. PTEN overexpression inhibited IGF-1-induced SMC proliferation in both SV and IMA. PTEN suppression, induced by siRNA transfection of IMA SMCs diminished the negative regulation of PI3K-PKB signalling leading to greater proliferative response induced by IGF-1 stimulation. Thus, we show for the first time that early inactivation of PTEN in SV SMCs leads to temporally increased activity of the pro-hyperplasia PI3K-AKT/PKB pathway leading to IH-induced vein graft occlusion. Therefore, modulation of the PI3K-AKT/PKB pathway via PTEN might be a novel and effective strategy in combating SV graft failure following CABG.

Keywords: CABG • internal mammary artery • intimal hyperplasia • IGF-1 • PTEN • restenosis • saphenous vein • vein-graft disease

Introduction

Coronary artery disease leading to myocardial infarction and ischaemia is an important cause of morbidity and mortality worldwide. One of the treatment modes consists of coronary artery bypass grafts (CABG) using either arterial or venous conduits, including saphenous vein (SV) and internal mammary artery (IMA). However, the long-term outcome of the procedure is severely limited due to closure of the graft vessel due to intimal hyperplasia (IH). Interestingly, the SV is singularly more prone to IH-induced occlusion leading to vein graft failure, while the IMA is almost totally resistant to restenosis. Almost 20% of all CABG procedures using SV lead to vein graft failure within 1 year and about 40% at 2 years [1, 2]. The reason for this still remains an enigma.

Smooth muscle cells (SMCs) within the medial layer of blood vessels play a critical role in the pathogenesis of fibroproliferative vasculopathies such as atherosclerosis, restenosis and bypass vein graft failure [3, 4]. Under physiological conditions, the SMCs are maintained in a quiescent state. However, following injury,
these cells undergo a phenotypic modulation into a proliferative state and migrate and multiply within the intimal region of the vessel causing luminal occlusion. The entire process involves injury to the endothelium, inflammation, SMC proliferation and de novo synthesis of extracellular matrix. The synthesis of extracellular matrix component leads to vessel remodelling and these results in narrowing of the lumen.

Several growth factors including IGF-1, angiotensin II and platelet-derived growth factor lead to matrix production [5, 6]. An important goal is therefore to identify the molecular pathways and their regulators involved in SMC proliferation and using this information to develop novel therapeutic targets. Transplanting a venous conduit from the iliac artery to the arterial circulation exposes it to shear stress which leads to endothelial injury [7] which is accompanied by an accumulation of inflammatory cells and the release of growth factors and cytokines [8, 9]. The role of various growth factors including insulin-like growth factor (IGF)-1 have been reported [10, 11]. Activation of IGF-1 receptors trigger signalling pathways involving PI3K and AKT/PKB [12, 13] and are up-regulated in SMC proliferation [13]. The IGF-1-PI3K-AKT/PKB axis mediates the balance between survival, apoptosis and proliferation. Thus, modulation of this axis could be a viable option in inhibiting SMC hyperplasia. A possible regulatory molecule could be the tumour suppressor protein, phosphatase and tensin homologue (PTEN) [14].

PTEN is an inositol 3-phosphatase and an endogenous inhibitor of PI3K [15]. It regulates cell growth and apoptosis [16, 17]. PTEN has been shown to be a dual protein and lipid phosphatase and can hydrolyse 3’-phosphoinositol products to prevent downstream activation of AKT/PKB which is an effector molecule of PI3K [18]. Adenoviral-mediated overexpression of PTEN blocked the mitogenic effects of platelet-derived growth factor (PDGF). Overexpression of PTEN causes G1 cell cycle arrest [19] and apoptosis of SMCs by down-regulating the PI3K-AKT/PKB pathway [20]. The majority of studies relating to PTEN and SMC hyperplasia have been conducted in animal models with limited information in SV SMCs [21]. We, for the first time, have investigated and compared the activity of PTEN in IGF-1-stimulated human SMCs of SV and IMA origin and demonstrate the early inactivation of PTEN in SV SMCs to induce IH secondary to SMC proliferation.

Methods

Human tissue collection

The protocol for this study was approved by the Institutional Review Board of Creighton University. All samples were collected anonymously. The excess SV and IMA bypass conduits left over following CABG surgery were obtained from 38 patients (age 46–78 years with a median of 59.5 year) undergoing CABG procedures. Matched samples of both SV and IMA were obtained from the same patient. Specimens were collected with minimal delay in the University of Wisconsin (UW) solution and immediately transported to the laboratory. UW solution is regularly used for organ transport for transplant purposes and retains the viability of tissues for at least 24 hrs [22]. Strict aseptic technique was followed for subsequent processing of tissues.

Smooth muscle cell isolation and culture

SMCs from the tissue samples were isolated by a method previously reported by our laboratory for carotid plaque SMC culture [23] with minor modifications. Briefly, the SVs and IMA were dissected free of all fat and connective tissue using a dissecting microscope. The adventitia was stripped away and the endothelial cells were removed by blunt dissection. The specimens were minced and subjected to enzymatic digestion with elastase (2%) and collagenase (1%) (SIGMA, St. Louis, MO, USA) in Dulbecco’s Modified Eagle’s Medium (DMEM). The cellular digests were filtered through 100 μm sterile cell strainer and then centrifuged at 1000 rpm for 10 min. The cell pellet was washed twice in DMEM with 10% foetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml). The final cell pellet was re-suspended in pre-warmed smooth muscle cell medium (SMCM; ScienCell Research Laboratories, San Diego, CA, USA) supplemented with 10% heat inactivated FBS and incubated at 37°C with 5% CO2. Subcultured straws were used between the 3rd and 7th passage in order to maintain as close to normal phenotype as possible. Purity of the isolated SMCs was confirmed by their characteristic ‘hill-valley’ growth pattern and positive immunostaining with smooth muscle α-actin (Dako, Carpentry, CA, USA) and caldesmon (Biogenex, San Ramon, CA, USA). For most experiments, cells at 80–90% confluence were incubated in serum-free medium for 24–48 hrs in order to render them quiescent. Mitogenic stimulation was achieved using recombinant human IGF-1 (Peprotech, Rocky Hill, NJ, USA).

Immunoblotting

SMCs after various treatments were harvested and lysed in ice-cold RIPA buffer (Sigma, St. Louis, MO, USA) and sonicated to disrupt the cell membrane. The lysates were centrifuged at 13,000 rpm for 10 min. at 4°C and supernatant collected. The protein concentration was estimated using BCA Protein Assay (BioRad Laboratories Inc., Hercules, CA, USA). Proteins were resolved using SDS-PAGE and transferred onto Trans-Blot transfer medium (BioRad Laboratories, Hercules, CA, USA). Membranes were probed with the following primary antibodies: anti-PTEN monoclonal antibody, anti-phospho-PTEN (Ser380), anti-phospho-AKT/PKB (Ser473) (Cell Signaling Tech., Danvers, MA, USA), IGF-1Rα, MDM2, PCNA, p53 and GAPDH (Santa Cruz Biotech., Santa Cruz, CA, USA). Membranes were incubated with species appropriate Horseradish peroxidase (HRP)-conjugated secondary antibody and developed using SuperSignal West Dura Stable Peroxide Buffer. Bands were visualized and densitometry analysis was performed using UVP Bioimaging system (UVP, Minneapolis, MN, USA).

Cellular activation of signalling ELISA (CASE)

CASE (SuperArray Bioscience Corporation, MD, USA) is an assay method to detect protein kinase cascade activated by an extracellular stimulus in whole cells by monitoring the phosphorylation status of the protein. The entire assay occurs directly in cell culture wells and thus eliminates the need for cell lysates, Western blotting or the need for radioactive nucleotides for in vitro assays. The method is as per the manufacturer’s protocol. Briefly, SV and IMA SMCs were cultured in 96-well plates. For each treatment point, two wells of cells at the same density and treated in identical fashion were used. Each assay included a blank control (with no cells), null controls (cells incubated with the secondary antibody but no primary antibody) and experimental controls (cells not subjected to the experimental conditions). Assays were done on approximately 80% confluent cells. Cells
were treated with IGF-1 (100 ng/ml) over a time course of 0–24 hrs and then fixed in the wells using a formaldehyde-based fixing reagent. The wells were washed three times with washing buffer followed by the addition of quenching buffer for 20 min. at room temperature. The wells were further washed twice and incubated overnight at 4°C with phospho-protein or pan-protein-specific antibody. The wells were washed three times and incubated with secondary antibody for 1 hr at room temperature further washed and treated with developing solution for the formation of end-point blue coloration which was terminated using the stop solution provided. The absorbance was read on a multi-well plate reader at 450 nm. The data was normalized to the relative cell number.

**Gene silencing**

siRNA transfection was used to inhibit the expression of PTEN gene in IMA SMCs using siRNA duplex against the PTEN gene. The siRNA duplex sequence was 5’-GAAUAUCAGUACU ACUUAACA-3’ (sense) and 3’- UUCCUUAUAUAGAUCGAAAGAAUUGU-5’ (antisense). Using 6-well plates, cells (2 × 10^5) were seeded in 2 ml of antibiotic-free SMGM supplemented with 10% FBS 24 hrs followed by transfection with 0.25–1 μg of the siRNA duplex and incubated at 37°C for 5–7 hrs using lipofectAMINE 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). Degree of the gene silencing was determined by Western blotting and protein down-regulation was confirmed by demonstrating 70–80% reduction in signal intensity as compared with control non-transfected cells.

**Proliferation assay**

The 5-bromodeoxyuridine (BrdU) incorporation assay was carried out by using a cell proliferation ELISA BrdU kit (Roche Applied Science, Germany). The cells were labelled with 10 μM of BrdU solution and incubated for 24 hrs at 37°C. The cells were dried and fixed, and the cellular DNA was denatured with FixDenat solution for 30 min. at room temperature. A mouse anti-BrdU monoclonal antibody conjugated with peroxidase was added to each well and the plates were incubated again at room temperature for 2 hrs, tetramethylbenzidine was added and the cells were incubated for 30 min. at room temperature. Finally, the absorbance of the samples was measured by a microplate reader at 370 nm.

**Results**

**Involved of PI3K in IGF-1-mediated activation**

Mitogenic stimulation with IGF-1 (100 ng/ml) caused progressive increase in total PI3K expression over the course of 24 hrs (Fig. 1A), which was significantly greater in SV SMCs than in IMA SMCs (Fig. 1B). We also observed a progressive increase in the phosphorylated form of PI3K which persisted at 24 hrs in SV SMCs (Fig. 1A), but not in IMA SMCs (Fig. 1B). These results indicate that PI3K, which is an early mediator in the IGF-PI3K-AKT/PKB axis, was quantitatively increased and exhibited greater temporal activation in SMCs of SV.

**Effect of IGF-1 on PTEN expression and activation**

PTEN was expressed in both SV and IMA SMCs after stimulation with IGF-1 (100 ng/ml) over a time course of 0–24 hrs. However, there was a greater increase in total PTEN expression in the IMA as compared to the SV SMCs (Fig. 2). Conversely, the phosphorylated PTEN (Ser-380) increased rapidly and continued to do so even at 24 hrs in SV SMCs, in contrast to IMA SMCs, and showed a marked decrease at 24 hrs (Fig. 2). Since the activity as well as the stability of PTEN is dependent on the state of phosphorylation, our results indicate that reduced phosphorylation of PTEN maintains the active state of the molecule in IMA SMCs.

**PTEN expression decreases the phosphorylation of AKT/PKB in SV SMCs**

To characterize the role of PTEN in SMC proliferation, we transiently overexpressed PTEN in SV SMCs. As presented above, we observed distinctly greater degree of activation of AKT/PKB in SMCs of SV. Numerous clones showing increased PTEN expression were
Fig. 1 CASE assay of PI3K phosphorylation. Saphenous vein (SV) and internal mammary artery (IMA) smooth muscle cells (SMCs) were stimulated with insulin-like growth factor (IGF)-1 (100 ng/ml). (A) Total and phosphorylated fractions of PI3K in SV SMCs (B) Total and phosphorylated fractions of PI3K in IMA SMCs. (**P < 0.001; *P < 0.05; n = 5)

Fig. 2 Comparative expression of total and phosphorylated phosphatase and tensin homologue (PTEN). SV and IMA SMCs were stimulated with IGF-1 (100 ng/ml). (A) Western blot and densitometry quantification of total PTEN levels. The graphs represent densitometry analysis of the bands. GAPDH was used to show equal loading (**P < 0.001; *P < 0.05; n = 5). (B) PTEN phosphorylation (Ser-380) was determined by Western blotting and densitometry quantification. The graphs represent densitometric analysis of the bands from five individual experiments. (**P < 0.001; *P < 0.05)
screened by the level of expression of PTEN and confirmed by Western blotting at 48 hrs (Fig. 3A). Cytotoxicity following transfection protocol was assessed by trypan blue dye-positive cell count and was less than 10%. Following transfection, cells were stimulated with IGF-1 (100 ng/ml) over a time course of 4–24 hrs. There was an increased phosphorylation (Ser-473) of AKT/PKB up to 4 hrs, which then decreased over the period of 24 hrs as compared to non-transfected cells (Fig. 3B).

PTEN decreases cell surface IGF-1R expression

We analysed the effect of overexpression of PTEN on the molecules upstream of PI3K-AKT/PKB in SV SMCs. Following stimulation with IGF-1 (100 ng/ml), the overall levels of IGF-1R (β-subunits) increased up to 2 hrs followed by significant decrease at 4 and 12 hrs (Fig. 4A). This was in contrast to the SV SMCs transfected with the empty vector, which showed significant increase in the IGF-1R following IGF-1 treatment and this was similar to our previous results of normal (non-transfected) SMCs stimulated with IGF-1. Inhibition of the PI3K pathway using LY294002 caused a down-regulation of IGF-1R which was highly significant at 12 hrs (Fig. 4A). In IMASMCs, IGF-1 (100 ng/ml) alone significantly increased IGF-1R expression from 2 to 4 hrs followed by decrease at 12 hrs (Fig. 4A). The effect of IGF-1 to increase IGF-1R in IMASMCs was blocked by PTEN overexpression at 4 and 12 hrs (Fig. 4B).

PTEN promotes p53 activity and regulates cellular levels of MDM2

We examined possible downstream targets of AKT/PKB and the effect of PTEN expression on the activity of MDM2, a known effector of AKT/PKB action and negatively regulates p53, which is a negative regulator of proliferation. SV and IMASMCs stimulated with IGF-1 showed significantly increased MDM2 levels in SV SMCs at 12 and 24 hrs (Fig. 5A). In IMASMCs, the increase was less marked and was not temporally significant. This could be attributed to the higher levels of PTEN activation demonstrated in IMASMCs. It was surprising to see the sustained MDM2 expression in both groups of cells, since MDM2 normally has a half life of less than 1 hr. This could be due to prolonged IGF-1 stimulation. Cells after similar treatment were analysed for p53 function. Western blot assay showed a marked increase in p53 activity in the IMASMCs as compared to the SV (Fig. 5B). We anticipated that expression of PTEN would up-regulate p53 activity and have an inverse effect on MDM2 levels. To test this, SMCs of SV and IMASMCs overexpressing PTEN were stimulated with IGF-1 and analysed for MDM2 and p53 levels. PTEN-overexpression significantly reduced IGF-1-induced MDM2 levels and significantly increased IGF-1-induced p53 levels in SV SMCs (Fig. 6A and B). However, PTEN overexpression had no significant effect on MDM2 and p53 expression in IMASMCs (Fig. 6B). Interestingly, the MDM2 and p53 levels in PTEN-overexpressed SV SMCs were similar to those in PTEN-overexpressed IMASMCs. Taken together, these results show that conditions, which favour the down-regulation of MDM2, such as increased PTEN activity, inhibit the proliferative effects of PI3K-AKT/PKB axis via up-regulation of p53 in SV SMCs.

PTEN underexpression causes increased AKT/PKB activity

To demonstrate the effect of underexpression of PTEN, we tested the influence of PTEN siRNA transfection in IMASMCs. As shown earlier, PTEN activity was far greater in IMASMCs as compared to the SV. Therefore, it follows that silencing the PTEN gene could produce the opposite effects to that seen in normal IMASMCs. Immunoblotting IMASMCs transfected with PTEN siRNA confirmed silencing of PTEN expression. Cell viability was found to be >90%. We then examined the effect of PTEN silencing on the
phosphorylation activity of AKT/PKB. Transfected cells were stimulated with IGF-1 over a period of 4–24 hrs. Non-transfected cells similarly treated were used as control. Western blot analysis confirmed an increased level of p-AKT/PKB (Ser-473) at up to 24 hrs followed by a decrease, which however, did not reach the basal level and was statistically significant (Fig. 7). The reason for this late stage decrease is not known. Non-transfected cells did not show the extent of AKT/PKB activation as compared to transfected SMCs. However, the pattern of increase was similar to that seen in CASE assay of normally expressing PTEN IMA SMCs.

Fig. 4 Expression of IGF-1 receptors in PTEN overexpressed SMCs of SV and IMA. IGF-1R expression was assayed by Western blotting in response to PTEN overexpression in SMCs of SV and IMA, after mitogenic stimulation with IGF-1 (100 ng/ml). (A) Expression of IGF-1 receptors in PTEN-overexpressed SV SMC. (B) Expression of IGF-1 receptors in PTEN-overexpressed IMA SMCs. Protein lysates were subjected to immunoblotting with an antibody against the β-subunit of the IGF-1R. The graphs represent densitometry analysis of the bands from five individual experiments. (**P < 0.001; *P < 0.05) To determine whether the PI3K/AKT/PKB pathway mediates the downregulation of IGF-1R, the transfected SV SMCs were incubated with LY294002 (25 μM) for 1 hr prior to IGF stimulation. An empty vector control was used in all the experiments.
Effect of PTEN gene silencing on cell proliferation of SV and IMA SMCs

Proliferative properties of SMCs from PTEN siRNA transfected and non-transfected cells were analysed following IGF-1 stimulation. PTEN overexpression inhibited the SMC proliferation in both SV and IMA (Fig. 8A). Meanwhile, Using Western blot analysis of PCNA (a known marker of cell proliferation), PTEN siRNA-transfected IMA SMCs showed markedly greater proliferation as compared to non-transfected cells (Fig. 8B). This demonstrates the impact of dysregulation of PTEN function on the proliferative properties of SMCs.
Discussion

SMC proliferation leading to IH is the primary pathology associated with vein graft failure [24]. PI3K and AKT/PKB are key molecules which mediate the propagation of mitogenic signals to the translation of cell cycle proteins [25, 26]. Activation of PI3K-AKT/PKB pathway has been shown to promote proliferation in a variety of cell types [27–30] and there are reports that have shown increased DNA synthesis following mitogenic stimuli to be mediated via the PI3K-AKT/PKB pathway [31, 32]. AKT/PKB is directly involved in cell cycle regulation through its downstream target GSK-3β, by preventing the phosphorylation and degradation of cyclin D1 [33] and also by negatively regulating the cyclin-dependent kinase inhibitors p21 and p27 [34, 35]. It also plays an important role in cell growth by directly phosphorylating the mammalian target of rapamycin or mTor and also inactivating tuberin (TSC2) which is an mTor inhibitor [36, 37]. Thus, it is apparent that the IGF-1-PI3K-AKT/PKB axis is of paramount importance in the proliferation of SMCs and subsequent IH formation and graft failure.

In this study, we compared the effects of IGF-1 stimulation on PI3K and AKT/PKB activity. IGF-1 selectively promoted both PI3K expression and phosphorylation in both SV and IMA SMCs. However, the effect was far greater in the SV as compared to IMA. A similar effect was observed in the expression and phosphorylation of AKT/PKB following similar mitogenic stimuli. This suggests that there is a temporally increased IGF-1-induced activation of the PI3K-AKT/PKB pathway in the SV SMCs, which could result in a greater activation of downstream effector molecules responsible for cell cycle activation and thus contribute to the greater proliferative rates of SV SMCs.

There are reports on the histological differences between SV and IMA conduits as well as the differences in their biochemical composition [38]. This may be involved, at least in part, in the different PTEN expression and activity in SV and IMA. Also, kinase(s) that regulates PTEN might be differentially expressed and activated in SV and IMA. Obviously, further studies are warranted to elucidate and dissect cellular and molecular mechanisms underlying different expression of PTEN in SV and IMA.

Cancer cells are perhaps the best model of unregulated cell proliferation and PTEN has been found to be mutated or dysfunctional in a wide spectrum of human cancers. The function of PTEN is lost by mutation, deletion or promoter methylation [39] and recently a mutation of the PI3KCA gene encoding the p110 subunit of PI3K has been detected in several human tumours. Unchecked PI3K/AKT/PKB activity as a result of PTEN dysfunction, have been observed in breast, ovarian, pancreatic and some other form of malignancies. However, there appears to be an AKT/PKB independent pathway associated with PTEN deletion. Activation of PI3K/AKT/PKB pathway secondary to PTEN inactivation, resulted in an up-regulation of p53, possibly as a brake mechanism against the unchecked pro-hyperplasia action of PI3K pathway and up-regulation of p53 genes leads to growth suppression [40]. There appears an extensive volume of literature citing the association between PI3K, AKT/PKB, PTEN, p53, MDM2, cyclins, cdk's, cki's and other proteins involved in the cell cycle machinery in numerous cancer conditions [41–44]. It is, therefore, quite plausible that such an association also exists in IH since both cancer cells and...
IH SMCs appear to function as autonomous units independent of normal regulatory mechanisms.

PTEN regulates the functioning of PI3K through hydrolysis of PI(3,4)P2 and PIP3 and would be expected to inhibit the processes leading to SMC proliferation. Thus, dysregulation of PTEN function could be a possible cause for the observed increased proliferative properties of SMCs from venous CABG conduits. In normal SMCs, PTEN function contributes to loss of AKT/PKB signalling and a quiescent phenotype. However, following mitogenic stimuli, there is PTEN inactivation and constitutive AKT/PKB signalling and a phenotypic modulation to the proliferative phenotype [45]. The PTEN tail is important for phosphorylation activity and maintaining PTEN stability [46]. Recent studies have shown that phosphorylation causes decreased activity and greater stability, while the non-phosphorylated form of PTEN is potentially more active but less stable [47].

Vascular injury results in PTEN inactivation and increased PI3K-AKT/PKB signalling. In this study, we tested the effects of overexpressing and silencing PTEN in SV and IMA SMCs focusing on the regulatory activity of PTEN. The potential ability of SMCs to replicate under mitogenic stimuli could occur due to loss or inactivation of negative growth regulators. We have demonstrated the decreased activity of PTEN leading to increased activity of the PI3K-AKT/PKB activity to be a potential contributory factor in SV SMC proliferation. Overexpression of PTEN caused a reduction in cell surface IGF-1 receptors via the PI3K-AKT/PKB pathway, which could possibly result in the sustained mitogenic effect of IGF-1 in SV SMCs and not in IMA. We have demonstrated

Fig. 8 Effect of PTEN gene silencing on the proliferation of SMCs in SV and IMA. siRNA transfected SMCs were incubated with IGF-1 (100 ng/ml) for 24 and 48 hrs. (A) BrDU incorporation of SV and IMA SMCs after PTEN overexpression and activation with IGF-1 for 24 hrs. (B) Proliferation was assessed by detection of PCNA by Western blotting and the results are shown as densitometric quantification. Proliferation of SMCs is considerably increased in PTEN-silenced IGF-1-stimulated IMA SMCs as compared to the non-transfected controls. (**P < 0.001, *P < 0.05; n = 5)
that gene silencing of PTEN resulted in the abolition of the inhibitory response to mitogen-mediated growth response in SMCs of IMA. Furthermore, we have shown distinct correlation between PTEN and the downstream effect of AKT/PKB substrate PTEN. PTEN is linked to the PI3K pathway.

References

1. Fitzgibbon GM, Kafka HP, Leach AJ, Keon WJ, Hooper GD, Burton JR. Coronary bypass graft fate and patient outcome: angiographic follow-up of 5,065 grafts related to survival and reoperation in 1,388 patients during 25 years. J Am Coll Cardiol. 1996; 28: 616–26.

2. Velth PJ, Gupta SK, Ascer E, White-Flores S, Samson RH, Scher LA, Towne JB, Bernhard VM, Bonier P, Flinn WR. Six-year prospective multicenter randomized comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infringuinal arterial reconstructions. J Vasc Surg. 1986; 3: 104–14.

3. Lusis AJ. Atherosclerosis. Nature. 2000; 407: 233–41.

4. Ross R. Atherosclerosis is an inflammatory disease. Am Heart J. 1999; 138: S419–20.

5. Bailey WL, LaFleur DW, Forrester JS, Fagin JA, and Sharifi BG. Stimulation of rat vascular smooth muscle cell glycosaminoglycan production by angiotensin II. Atherosclerosis. 1994; 111: 55–64.

6. Sharifi BG, LaFleur DW, Pirola CJ, Forrester JS, Fagin JA. Angiotensin II regulates tenasin gene expression in vascular smooth muscle cells. J Biol Chem. 1992; 267: 23910–15.

7. Kennedy JH, Lever MJ, Addis BJ, Paneth M. Changes in vein interstitium following distension for aortocoronary bypass. J Cardiovasc Surg. 1989; 30: 992–5.

8. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med. 2002; 8: 1249–56.

9. Mitra AK, DeCore MG, Agraval DK. Cells, cytokines and cellular immunity in the pathogenesis of fibroproliferative vasculopathies. Can J Physiol Pharmacol. 2005; 83: 701–15.

10. Bayes-Genis A, Conover CA, Schwartz RS. The insulin-like growth factor axis: A review of atherosclerosis and restenosis. Circ Res. 2000; 86: 125–30.

11. Miao RQ, Murakami H, Song Q, Chao L, Chao J. Kallistatin stimulates vascular smooth muscle cell proliferation and migration in vitro and neointima formation in balloon-injured rat artery. Circ Res. 2000; 86: 418–24.

12. Bornfeldt KE, Raines EW, Nakano T, Graves LM, Krebs EG, Ross R. Insulin-like growth factor-I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. J Clin Invest. 1994; 93: 1266–74.

13. Stewart CE, Rotwein P. Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. Physiol Rev. 1996; 76: 1005–26.

14. Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA. 1999; 96: 4240–5.

15. Huang J, Niu XL, Pippen AM, Annex BH, Kontos CD. Adenovirus-mediated intraarterial delivery of PTEN inhibits neointimal hyperplasia. Arterioscler Thromb Vasc Biol. 2005; 25: 354–8.

16. Chung TW, Lee YC, Ko JH, Kim CH. Hepatitis B Virus X protein modulates the expression of PTEN by inhibiting the function of p53, a transcriptional activator in liver cells. Cancer Res. 2003; 63: 3453–8.

17. Lu Y, Lin YZ, LaPushin R, Cuevas B, Fang X, Yu SX, Davies MA, Khan H, Furui T, Mao M, Zinner R, Hung MC, Steck P, Siminovitch K, Mills GB. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene. 1999; 18: 7034–45.

18. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem. 1998; 273: 13375–8.

19. Moon SK, Kim HM, Kim CH. PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kappaB and AP-1 in vascular smooth muscle cells. Arch Biochem Biophys. 2004; 421: 267–76.

20. Li J, Simpson L, Takahashi M, Miliareis C, Myers MP, Tonks N, Parsons R. The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res. 1998; 58: 5667–72.

21. Hata JA, Petrofski JA, Schroder JN, Williams ML, Timberlake SH, Pippen A, Corwin MT, Solan AK, Jakoi A, Gehrig TR, Kontos CD, Milano CA. Modulation of phosphatidylinositol 3-kinase signaling...
reduces intimal hyperplasia in aortocoronary saphenous vein grafts. J Thorac Cardiovasc Surg. 2005; 129: 1405–13.

22. Abebe W, Cavallari N, Agrawal DK, Rowley J, Thorpe PE, Hunter WJ, Edwards JD. Functional and morphological assessment of rat aorta stored in University of Wisconsin and Eurocollins solutions. Transplantation. 1993; 56: 808–16.

23. Dhume AS, Agrawal DK. Inability of vascular smooth muscle cells to proceed beyond S phase of cell cycle, and increased apoptosis in symptomatic carotid artery disease. J Vasc Surg. 2003; 38: 155–61.

24. Akowuah EF, Sheridan PJ, Cooper GJ, Newman C. Preventing saphenous vein graft failure: does gene therapy have a role? Ann Thorac Surg. 2003; 76: 959–66.

25. Rameh LE, Cantley LC. The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem. 1999; 274: 8347–50.

26. Thomas G, Hall MN. TOR signalling and control of cell growth. Curr. Opin Cell Biol. 1997; 9: 782–7.

27. Andjelkovic M, Jakubowicz T, Cron P, Thomas G, Hall MN. TOR signalling and control of cell growth. Curr. Opin Cell Biol. 1997; 9: 782–7.

28. Nave BT, Owens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J. 1999; 344: 427–31.

29. Franke TF, Yang Si, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tischler PN. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell. 1995; 81: 727–36.

30. Peruzzi F, Prisco M, Dew M, Salomoni P, Grassilli E, Romano G, Calabretta B, Baserga R. Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. Mol Cell Biol. 1999; 19: 7203–15.

31. Higaki M, Shimokado K. Phosphatidylinositol 3-kinase is required for growth factor-induced amino acid uptake by vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 1999; 19: 2127–32.

32. Imai Y, Clemons DR. Roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways in stimulation of vascular smooth muscle cell migration and deoxyribonucleic acid synthesis by insulin-like growth factor-I. Endocrinology. 1999; 140: 4228–35.

33. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 1998; 12: 3499–511.

34. Gesbert F, Sellers WR, Signoretti S, Loda M, Griffin JD. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27kip1 through the phosphatidylinositol 3-Kinase/AKT pathway. J Biol Chem. 2000; 275: 39223–30.

35. Zhao BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol. 2001; 3: 245–52.

36. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/Akt pathway. Mol Cell. 2002; 10: 151–62.

37. Nave BT, Owens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J. 1999; 344: 427–31.

38. Canham PB, Finlay HM, Boughner DR. Contrasting structure of the saphenous vein and internal mammary artery used as coronary bypass vessels. Cardiovasc Res. 1997; 34: 557–67.

39. Blanco-Aparicio C, Renner O, Leal JF, Carnero A. PTEN, more than the AKT pathway into a protein complex. J Biol Chem. 2000; 275: 39223–30.

40. Lee C, Kim JS, Waldman T. Activated PI3K signaling as an endogenous inducer of p53 in human cancer. Cell Cycle. 2007; 6: 394–6.

41. Mehrian-Shai R, Chen CD, Shi T, Horvath S, Nelson SF, Reichardt JF, Sawyer CL. Insulin growth factor-binding protein 2 is a candidate biomarker for PTEN status and PI3K/Akt pathway activation in glioblastoma and prostate cancer. Proc Natl Acad Sci USA. 2007; 104: 5563–8.

42. Priuli M, Calastrelli A, Bruno P, Amalia A, Paradiso A, Canti G, Nicolini A. Preferential chemosensitivity of PTEN-mutated prostate cells by silencing the Akt kinase. Prostate. 2007; 67: 782–9.

43. Wang Y, Hou P, Yu H, Wang W, Ji M, Zhao S, Yan S, Sun X, Liu D, Shi B, Zhu G, Condouris S, Xing M. High Prevalence and Mutual Exclusivity of Genetic Alterations in the PI3K/AKT Pathway in Thyroid Tumors. J Clin Endocrinol Metab. 2007; 92: 2387–90.

44. Zhang M, Fang X, Liu H, Wang S, Yang D. Blockade of AKT activation in prostate cancer cells with a small molecule inhibitor, 9-chloro-2-methyllellipticinium acetate (CMEP). Biochem Pharmacol. 2007; 73: 15–24.

45. Mourani PM, Garli P, Wenzlau JM, Carpenter TC, Stembank KR, Weiser-Evans MC. Unique, highly proliferative growth phenotype expressed by embryonic and neointimal smooth muscle cells is driven by constitutive Akt, mTOR, and p70S6K signaling and is actively repressed by PTEN. Circulation. 2004; 109: 1299–306.

46. Guzeloglu-Kayisli O, Kayisli UA, Al-Rejail R, Zheng W, Luleci G, Arici A. Regulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression by estradiol and proges- terone in human endometrium. J Clin Endocrinol Metab. 2003; 88: 5017–26.

47. Vazquez F, Grossman SR, Takahashi Y, Rokas MW, Nakamura N, Sellers WR. Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J Biol Chem. 2001; 276: 48627–30.

48. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997; 387: 296–9.