J. Cell. Mol. Med. Vol 13, No 1, 2009 pp. 177-187

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

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Received: September 19, 2007; Accepted: March 10, 2008

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 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,

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doi:10.1111/j.1582-4934.2008.00311.x

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 segment into 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 G_1 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 transplantation 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% CO_2. Subcultured strains 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, Carpenteria, 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 BioRad 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 (Pierce Biotech, Rockford, IL, USA). 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 were normalized to the relative cell number.

Transient cell transfection

The SV SMCs were transfected using pORF9-hPTEN (an expression vector containing the human PTEN open-reading frame) (InvivoGen, CA, USA). The hPTEN gene consists of an intronless ORF from the ATG to the stop codon. The ORF size was 1212 bp and cloning fragment size was 1246 bp. The protocol for growth of pORF transformed bacteria and the selection of bacterial clones were as per the manufacturer's protocol. Briefly, the lyophilized *E. coli* was re-suspended with 1 ml of LB medium and bacteria from this suspension were streaked onto ampicillin LB agar plates. The plates are incubated at 37°C overnight. Isolated single colony of the bacteria was grown in LB medium overnight at 37°C. The pORF plasmid DNA was extracted using the QUIAGEN plasmid Midi kit (Quiagen, CA, USA) and the yield determined spectrophotometrically. For each transfection, SV SMCs (2×10^6) were plated in 6-well plates and grown in SMCM containing 10% FBS, until cell density reached 70% confluence. The expression plasmid DNA (15 μg in 800 μl of optiMEM medium) was mixed with 15 μl lipofectAMINE (GibcoBRL, Grand Island, NY, USA) and incubated at room temperature for 30 min. The SMCs were washed with serum-free medium and the lipofectAMINE/DNA mixture was added and incubated at 37°C for 5 hrs and for a further 48 hrs in the presence of growth medium containing 20% FBS. As a control, SV SMCs were also transfected with an empty vector (pORF9-MCS, Invivogen, CA, USA). In each experiment, the transfection efficiency was assessed using Western blotting of transfected and identically treated non-transfected cells. The transfection efficiency ranged from 70–78% and the viability, as assessed by trypan blue dye exclusion, ranged between 88 and 96%.

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′-GAAUAUCUAGUACUU ACUUUAACA-3′ (sense) and 3′-UUCCUUAUAGAUCAUGAAUGAAAUUGU-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.

Statistical analysis

All data are represented as mean ± SEM. Data were analysed using one way ANOVA with Tukey's post test for analysis of intra and intersample groups. For the study, $P < 0.05$ was considered significant.

Results

Involvement 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

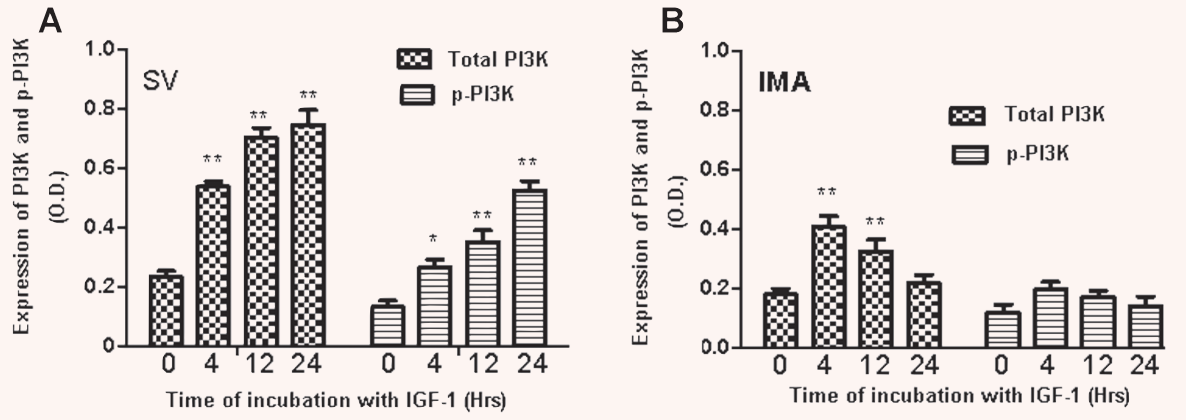

Fig. 1 CASE assay of PI3K phosphorylation. Saphenous vein (SV) and internal mammary artery (IMA) smooth muscle cells (SMCs) were stimulated with insulin-like growth factor (IGF)-1 (100 ng/ml). (**A**) Total and phosphorylated fractions of PI3K in SV SMCs (**B**) Total and phosphorylated fractions of PI3K in IMA SMCs. (** $P < 0.001$; * $P < 0.05$; $n = 5$)

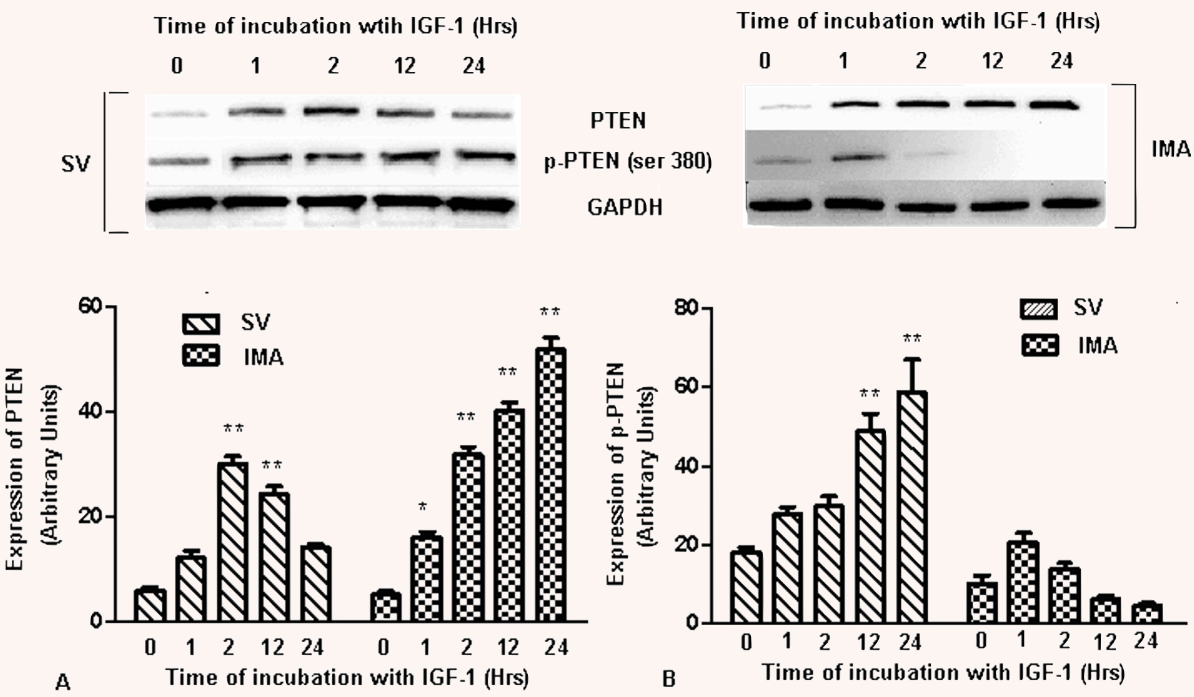

Fig. 2 Comparative expression of total and phosphorylated phosphatase and tensin homologue (PTEN). SV and IMA SMCs were stimulated with IGF-1 (100 ng/ml). (**A**) Western blot and densitometry quantification of total PTEN levels. The graphs represent densitometry analysis of the bands. GAPDH was used 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. (** $P < 0.001$; * $P < 0.05$)

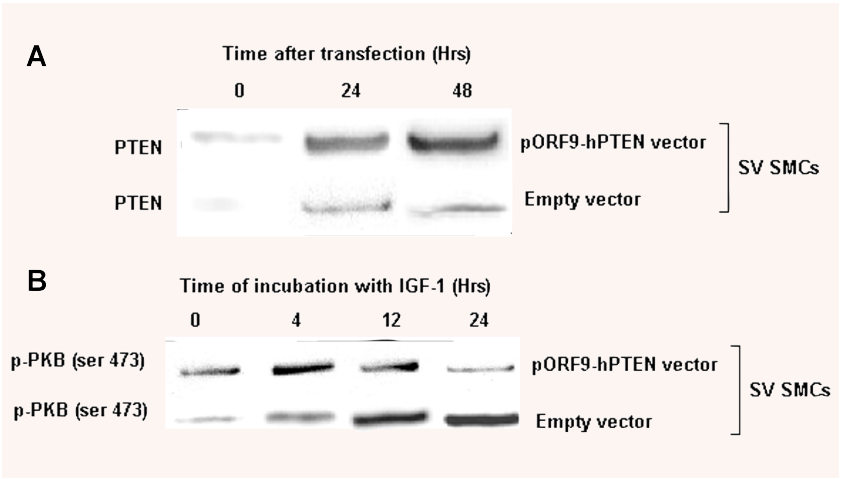

Fig. 3 Effect of PTEN overexpression on the activation of AKT/PKB. (**A**) SV SMCs were transiently overexpressed with PTEN using pORF9-hPTEN vector ($n = 5$). A negative control using empty (pORF9-MCS) vector was used. (**B**) The transfected cells were stimulated with IGF-1 (100 ng/ml) over a time course. The activation of AKT/PKB was determined by Western blotting using antibody against AKT/PKB (ser 473). 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 IMA SMCs, IGF-1 (100 ng/ml) alone significantly induced 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 IMA SMCs 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 IMA SMCs stimulated with IGF-1 showed significantly increased MDM2 levels in SV SMCs at 12 and 24 hrs (Fig. 5A). In IMA SMCs, the increase was less marked and was not temporally significant. This could be attributed to the higher levels of PTEN activation demonstrated in IMA SMCs. 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 IMA SMCs 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 IMA 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 IMA SMCs (Fig. 6B). Interestingly, the MDM2 and p53 levels in PTEN-everexpressed SV SMCs were similar to those in PTEN-everexpressed IMA SMCs. 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 IMA SMCs. As shown earlier, PTEN activity was far greater in IMA SMCs as compared to the SV. Therefore, it follows that silencing the PTEN gene could produce the opposite effects to that seen in normal IMA SMCs. Immunoblotting IMA SMCs 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

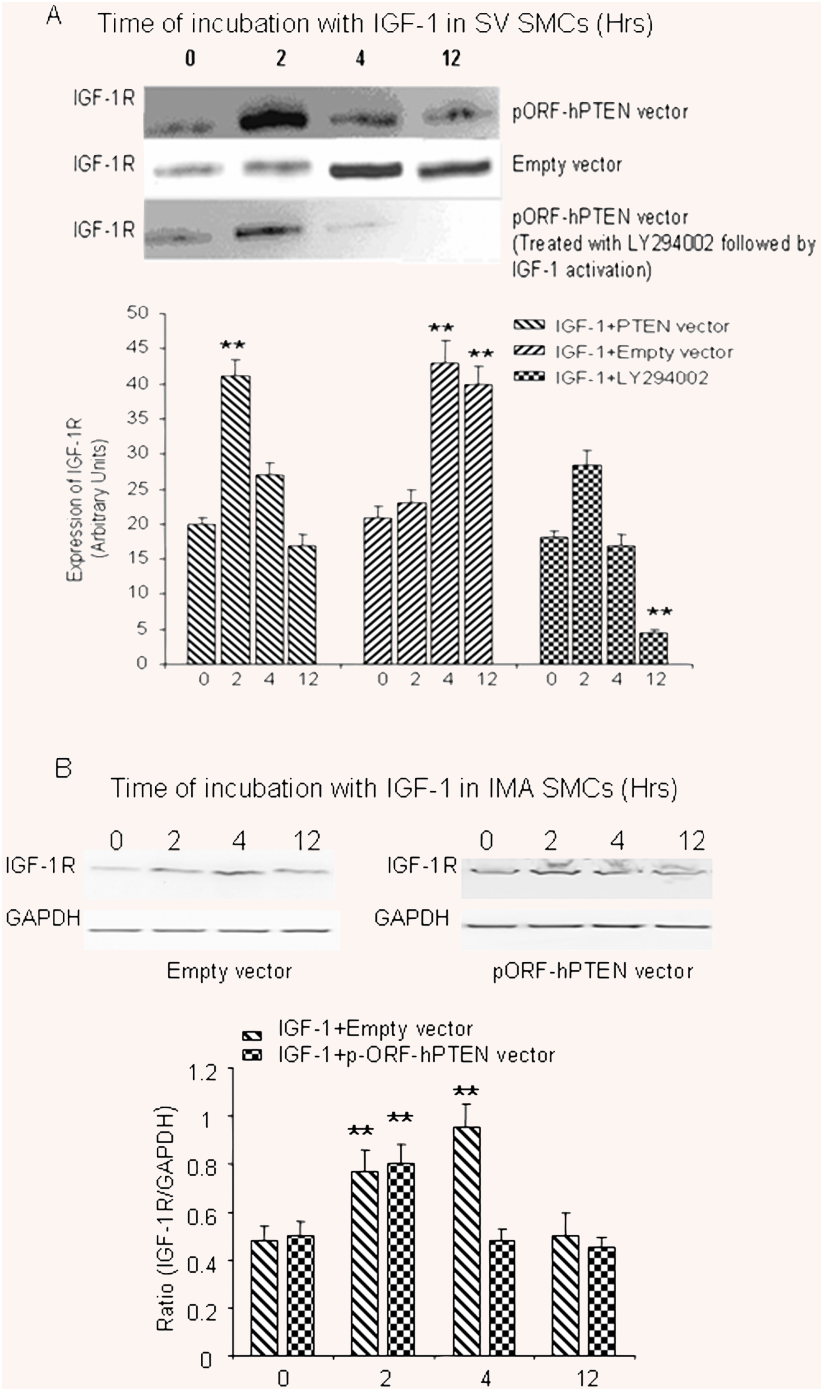

Fig. 4 Expression of IGF-1 receptors in PTEN overexpressed SMCs of SV and IMA. IGF-1R expression was assayed by Western blotting in response to PTEN overexpression in SMCs of SV and IMA, after mitogenic stimulation with IGF-1 (100 ng/ml). (**A**) Expression of IGF-1 receptors in PTEN-overexpressed SV 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 down-regulation 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.

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.

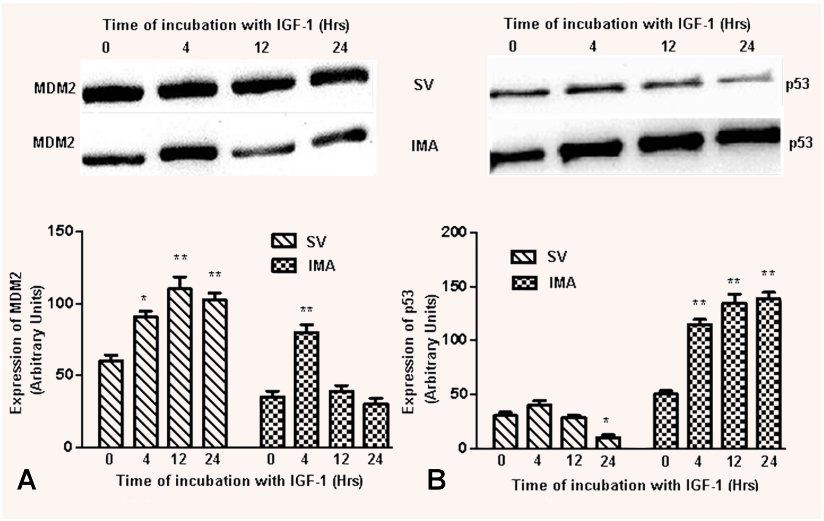

Fig. 5 Comparative expression of p53 and MDM2 in SV SMCs following IGF-1 activation. SMCs were simulated with IGF-1 (100 ng/ml) over a time course. p53 and MDM2 were detected by Western blotting and presented as densitometric quantification of the bands from five individual experiments. (** $P < 0.001$; * $P < 0.05$)

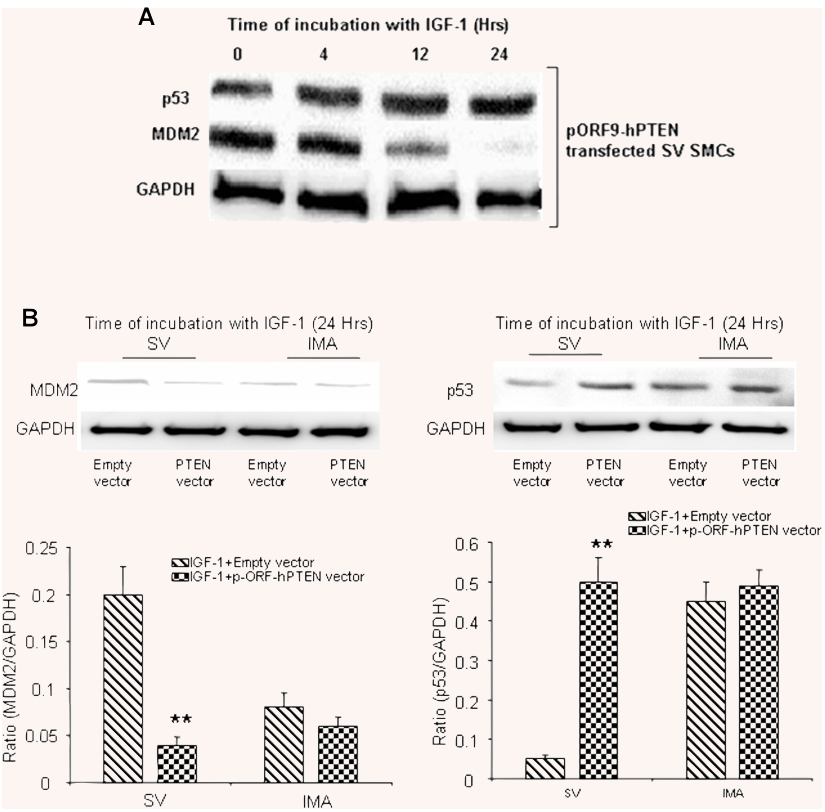

Fig. 6 Effect of PTEN overexpression on the regulation of MDM2 and p53 co-activation in SMCs of SV and IMA. (**A**) SV SMCs were transfected with pORF9-hPTEN. Transfected SMCs were stimulated with IGF-1 (100 ng/ml) over a time course of 0–24 hrs. (**B**) Ex-pression of MDM2 and p53 in SV and IMA SMCs following transfection with empty vector or PTEN vector and activation with IGF-1 (100 ng/ml) for 24 hrs. MDM2 and p53 levels were detected by Western blotting using appropriate primary antibodies. GAPDH was used as a loading control. ($n = 5$; ** $P < 0.001$)

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.

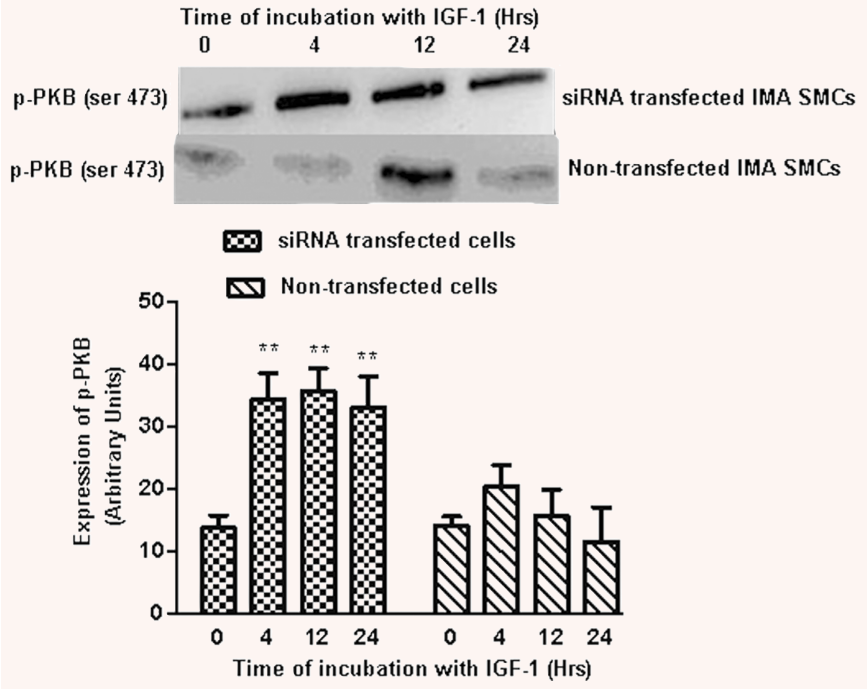

Fig. 7 To examine the effect of PTEN gene silencing on AKT/PKB activation. In order to observe the effects of PTEN gene silencing, IMA SMCs were transfected with a duplex siRNA. siRNA transfected IMA SMCs were stimulated with IGF-1 (100 ng/ml) over a period of 4–24 hrs. p-AKT/PKB protein levels were determined by anti-AKT/PKB (Ser-473) Western blotting and the data presented as densitometric quantification from five individual experiments. (** $P < 0.001$)

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 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

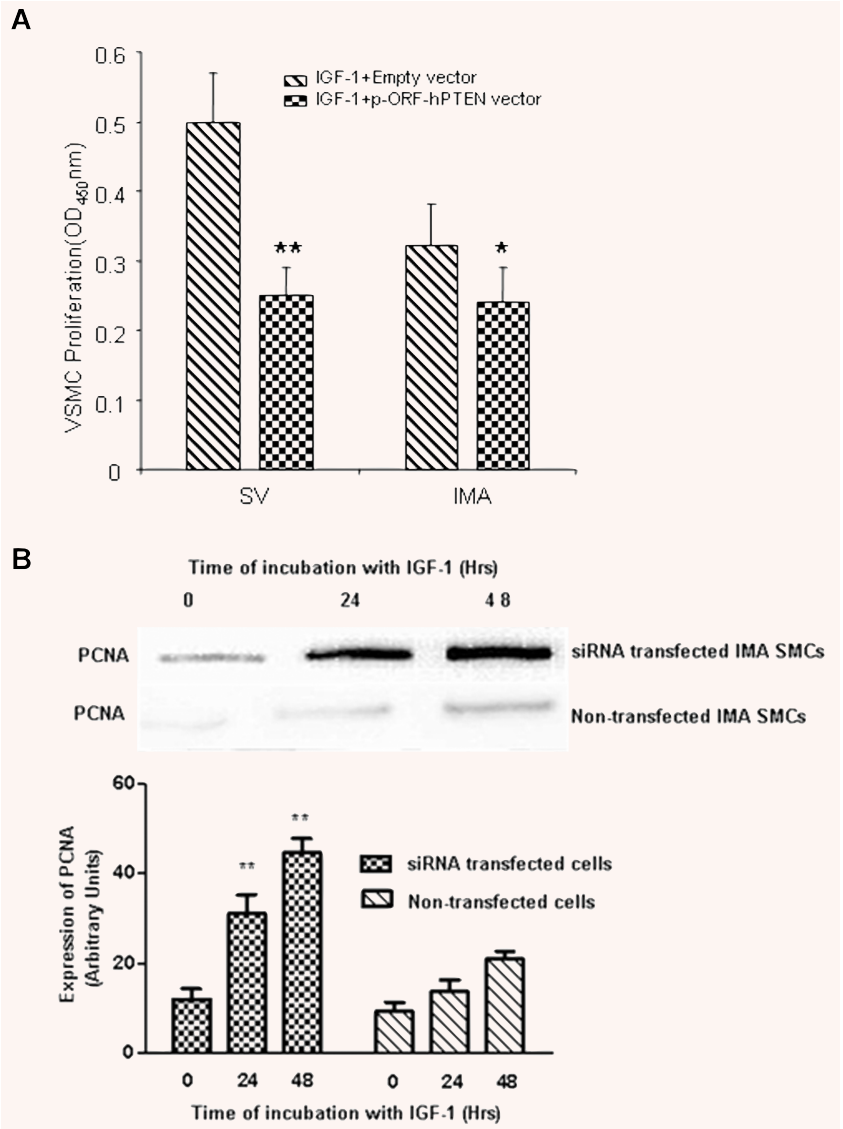

Fig. 8 Effect of PTEN gene silencing on the proliferation of SMCs in SV and IMA. siRNA transfected SMCs were incubated with IGF-1 (100 ng/ml) for 24 and 48 hrs. (**A**) BrDU incorporation of SV and IMA SMCs after PTEN overexpression and activation with IGF-1 for 24 hrs. (**B**) Proliferation was assessed 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. ($**P < 0.001$, $*P < 0.05$; $n = 5$)

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

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 MDM2 and its relationship to p53 activity. p53 is up-regulated during cell stress and induces cells cycle arrest. MDM2 targets p53 and induces its degradation [48]. Treatment with PI3K inhibitors abrogates the PI3K signalling and diminishes the cellular contents of MDM2 [49], thus indicating that the functioning of MDM2/p53 is linked to the PI3K pathway.

Vein graft failure is a common clinical problem and negates the beneficial effects of CABG procedures. Numerous growth factors and cytokines cause the propagation of pro-hyperplasia signalling pathways culminating in cell cycle progression leading to SMC proliferation. New strategies leading to prolonged vein graft lifespan are needed. It is therefore important to have a better understanding of the process of IH and the key molecules involved. The findings from this study has clearly demonstrated the key role of PTEN in regulating IGF-1/PI3K/AKT/PKB signalling, one of the possible proliferation-inducing pathways. Further studies would result in developing pharmaceutical or possibly gene-based therapies modulating the activity of PTEN in order to regulate the PI3K-AKT/PKB pro-hyperplasia pathway.

Acknowledgements

This study was supported by National Institutes of Health grants R01HL070885 and R01HL07349 (to D.K.A.), and Creighton University LB692 Nebraska Tobacco Settlement Grant (to D.K.A.). We gratefully acknowledge the contributions of the Nebraska Heart Hospital, Lincoln, NE for providing the tissue samples for our study.

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