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Indole-3-carbinol blocks platelet-derived growth factor-stimulated vascular smooth muscle cell function and reduces neointima formation *in vivo*

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Abstract

The purpose of this study was to determine the effect and associated cell signaling mechanisms of indole-3-carbinol (I3C) on platelet-derived growth factor (PDGF)-BB-induced proliferation and migration of cultured vascular smooth muscle cells (VSMCs) and neointima formation in a carotid injury model. Our data demonstrated that I3C inhibited PDGF-BB-induced proliferation of VSMCs in a dose-dependent manner without causing cell cytotoxicity, as assessed by 5-bromo-2′-deoxyuridine incorporation and WST-1 assays. Further studies revealed that the antiproliferative effect of I3C was caused by the arrest of cells in both the G0/G1 and S phases. Moreover, I3C treatment inhibited migration of VSMCs and partly reversed the expression of smooth-muscle-specific contractile markers. We also demonstrated that I3C-induced growth inhibition was associated with an inhibition of the expression of cyclin D1 and cyclin-dependent kinase 4/6, as well as an increase in $p27^{Kip1}$ levels in PDGF-stimulated VSMCs. These beneficial effects of I3C on VSMCs appeared to be at least partly mediated by the inhibition of Akt and the subsequent activation of glycogen synthase kinase (GSK) 3β. Furthermore, using a mouse carotid artery injury model, we found that treatment with 150 mg/kg I3C resulted in a significant reduction of the neointima/media ratio and cells positive for proliferating cell nuclear antigen. These results demonstrate that I3C can suppress the proliferation and migration of VSMCs and neointima hyperplasia after vascular injury via inhibition of the Akt/GSK3β pathway and suggest that this might be feasible as part of a therapeutic strategy for vascular proliferative diseases.

Keywords: Indole-3-carbinol; Neointima; Vascular smooth muscle cell; Platelet-derived growth factor

1. Introduction

Vascular smooth muscle cells (VSMCs) are the principal cell type involved in the pathogenesis of atherosclerosis, restenosis after percutaneous coronary intervention and late failure of vein grafting [1]. Physiologically, VSMCs are quiescent and primarily responsible for the contraction and relaxation that regulate blood pressure and vascular tone. However, during neointima development after vascular injury, growth and prothrombotic factors released from platelets and leucocytes trigger VSMCs to migrate from the media to the intima, where they proliferate and undergo phenotypic changes [2]. Given the prominent role of VSMCs, inhibition of pathological VSMC proliferation and migration is a critical step in the prevention and treatment of neointima development. Therefore, identifying pharmacological interventions with combined antiproliferative and antimigration properties is a promising approach to improving existing therapeutic strategies.

Phytochemicals from plants have recently received increasing attention. Epidemiological studies show that increased consumption of whole grains, vegetables and fruits is associated with a lower risk of cancer and cardiovascular disease death [3,4]. These studies suggest that dietary phytochemicals are the principal sources and building blocks of potent therapeutic molecules. These natural products include flavonoids, isoflavones and indole-3-carbinol (I3C). I3C is produced by cruciferous vegetables such as cauliflower, broccoli and Brussels sprouts. The major component of cruciferous vegetables is a 3-indolylmethyl glucosinolate which is readily hydrolyzed to give I3C (Fig. 1A) [5]. Owing to the vinyl hemiaminal moiety of the indole ring, in acidic conditions, I3C undergoes a series of condensation reactions to give a broad spectrum of products, including the biologically-active dimer 3,3′-diindolylmethane. I3C has been demonstrated to have inhibitory effects on the growth of a variety of cancer cells, including those of the prostate, breast, colon, lung and endometrium [6]. Several lines of evidence connect these inhibitory effects to the pleiotropic effects of I3C on multiple signaling targets related to cell cycle control, apoptosis, signal transduction, oncogenesis, hormonal homeostasis

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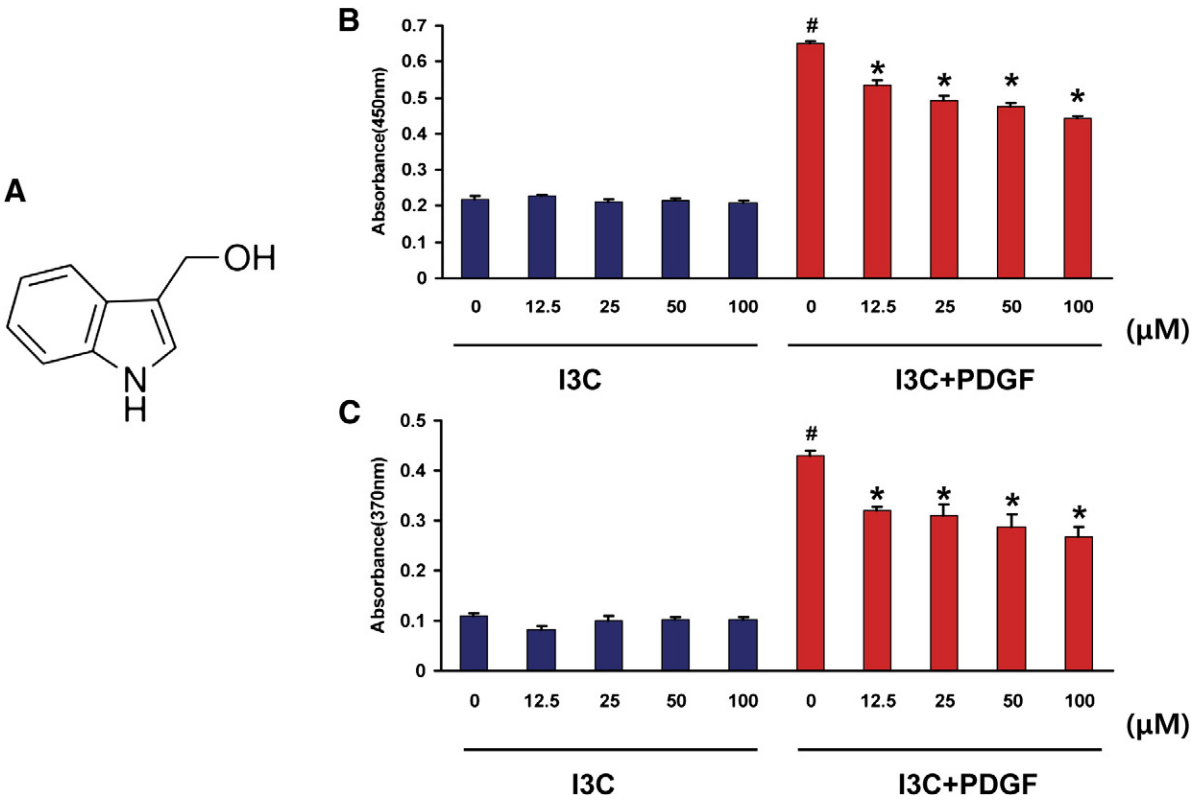

Fig. 1. The dose-dependent inhibitory effects of I3C on the proliferation of VSMCs and DNA synthesis stimulated by PDGF-BB. VSMCs were incubated with increasing concentrations of I3C (12.5 to 100 μM) for 48 h in the absence or presence of PDGF. (A) Chemical structure of I3C. (B) Cell viability was examined using the WST test. Data were expressed as the mean OD450±S.E.M. (#$P<.01$ versus the control group; *$P<.01$ versus PDGF alone; $n=6$). (C) BrdU incorporation was determined using an ELISA-based assay. DNA synthesis was expressed as mean OD370±S.E.M. (#$P<.01$ versus the control group; *$P<.01$ versus PDGF alone; $n=6$).

and transcriptional regulation [6,7]. *In vitro* studies have indicated that I3C is a potent inhibitor of cyclin-dependent kinases (CDKs) 2, 4 and 6, and this inhibition is accompanied by the up-regulation of $p21^{Waf1}$ and $p27^{Kip1}$, the down-regulation of retinoblastoma protein phosphorylation and subsequent G0/G1 cell cycle arrest [7]. The signaling pathway responsible for the inhibitory effect of I3C on cell proliferation has been extensively investigated in cancer cell culture models. Howells et al. [8] demonstrated that the antiproliferative effects of I3C appeared to be mediated by inactivation of Akt and its downstream effector, the nuclear transcription factor NF-κB [9]. Moreover, I3C has been shown to inhibit the mitogen-activated protein kinase (MAPK) pathway [10] and constitutively active signal transducer and activator of transcription 3 [11] in pancreatic carcinoma cells. In addition to these antiproliferative effects, there is compelling evidence that I3C inhibits the migration and invasion of breast cancer cells by activating the function of invasion suppressor molecules, including E-cadherin, catenins and BRCA1, and by decreasing expression of the chemokine receptor CXCR4 and the matrix metalloproteinase (MMP)-9 [12,13]. I3C has also been reported to have anti-inflammatory effects through the inhibition of inflammatory factor production [14].

Although the antiproliferative and antimigration effects of I3C on cancer cells are well documented, the direct role and mechanism of I3C in cardiovascular disease remain unknown. In the present study, we examined the potential therapeutic effects of I3C as a novel treatment for vascular remodeling after injury. We analyzed the effects of I3C on VSMC proliferation and migration, as well as its impact on neointima formation following vascular injury *in vivo*. To examine the mechanisms underlying its activity, we investigated the signaling pathways that are possibly influenced by I3C.

2. Materials and methods

2.1. Materials

I3C was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human platelet-derived growth factor-BB (PDGF-BB) was obtained from Prospec (Rehovot, Israel). The antibodies used to recognize the total and phosphorylated forms of extracellular-signal-regulated kinase 1/2 (ERK1/2), JNK1/2, p38, AKT and glycogen synthase kinase (GSK) 3β were ordered from Cell Signaling Technology (Danvers, MA, USA). Anti-smooth muscle alpha-actin (SM α-actin) and SM22α were purchased from Abcam (Cambridge, MA, USA). Antibodies against CDk4, CDk6, cyclin D1, $p27^{Kip1}$, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and proliferating cell nuclear antigen (PCNA) were purchased from Cell Signaling. Complete protease inhibitor, PhosSTOP, Cell Proliferation Reagent WST-1 and Cell Proliferation ELISA, BrdU (colorimetric), kit were obtained from Roche Diagnostics (Mannheim, Germany). All other reagents were from Sigma-Aldrich except where specified. For the *in vitro* study, I3C was dissolved in dimethyl sulfoxide (DMSO) medium. DMSO alone (without I3C) served as a control and did not show any effect on cell viability, cell proliferation or related molecular mechanisms (data not shown).

2.2. Cell culture

Primary VSMCs were enzymatically isolated from the thoracic aortas of Sprague–Dawley rats and grown in Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal bovine serum. The purity of the VSMCs was characterized based on morphology and positive staining of SM α-actin. The cells used in this study were between passages 5 and 12. The VSMCs were grown to 60% to 80% confluence and serum-starved for 24 h. Quiescent cells were pretreated with various concentrations of I3C for 1 h prior to stimulation with PDGF-BB (20 ng/ml).

2.3. Measurement of cell proliferation and DNA synthesis

Cell proliferation was determined by a nonradioactive colorimetric WST-1 assay according to the manufacturer's instructions. VSMCs (5×10^3/well) were grown to 60% confluence, and their growth was arrested in 96-well microplates. After preincubation with various concentrations of I3C for 1 h, the cells were treated with PDGF-BB (20 ng/ml) for 48 h in the presence or absence of I3C and loaded with WST-1 for the final 2 h. The color

intensity was measured at 450 nm. DNA synthesis was assessed in VSMCs by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation using a cell proliferation enzyme-linked immunosorbent assay (ELISA) kit. The cells were seeded and stimulated as for the WST-1 assay. BrdU was added for the final 2 h of treatment.

2.4. Cell cycle progression assays

Cell cycle progression was determined using propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS). Briefly, cells at 70% confluence were synchronized for 24 h, preincubated with I3C (100 μM) for 1 h and subsequently treated with PDGF-BB (20 ng/ml) for 24 h. The cells were then trypsinized and fixed with 70% ethanol overnight. The fixed cells were collected by centrifugation, washed once in phosphate-buffered saline, incubated with 1 ml PI staining buffer (20 μg/ml PI and 50 μg/ml RNase A) and analyzed using FACS. Cell cycle distributions were analyzed using Multicycle AV software (Phoenix Flow Systems, San Diego, CA, USA).

2.5. Migration assays

Migration assays were performed using the Transwell system (a 6.5-mm polycarbonate membrane with 8-μm pores; Corning, NY, USA). Cells (50 μl, 5×10^4) were seeded into the upper chamber and allowed to attach for 30 min. The monolayers were then treated for 1 h by adding 50 μl of I3C solution (2×) to the upper chamber and 600 μl of the I3C solution (1×) to the lower chamber. PDGF-BB was added to the bottom chamber as the chemoattractant. Cells were allowed to migrate through the membrane to the lower surface for 6 h. Cells on the upper surface of the membrane that had not migrated were scraped off using Q-tips, and cells that had migrated to the lower surface were fixed and stained with 0.1% crystal violet/20% methanol and counted. Migrated cell numbers were calculated as the number of cells migrated per high-power field (200×).

2.6. Western blotting

VSMCs were cultured in a 6-cm dish, grown to 70% to 80% confluence and starved in serum-free medium for 24 h. The cells were then treated with I3C (100 μM) for 2 h before exposure to 20 ng/ml PDGF-BB for the indicated time. The cells were lysed in RIPA (RadioImmunoPrecipitation Assay) buffer containing protease and phosphatase cocktails. Protein extracts (20 μg) were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to Immobilon-FL transfer membranes (Millipore) and probed with various antibodies. After incubation with a secondary IRDye, 800CW-conjugated antibody, signals were visualized using an Odyssey Imaging System. Specific protein expression levels were normalized to GAPDH for total protein or to total proteins for phosphorylated protein.

2.7. Endovascular carotid artery guidewire injury

All animal experimentation protocols were performed under institutional guidelines for animal welfare in accordance with the Animal Care and Use Committee of Renmin Hospital, Wuhan University. Ten-week-old male c57BL/6 mice underwent endothelial denudation injuries by the insertion of a guidewire (0.38 mm in diameter, No. C-SF-15-15, Cook, Bloomington, IN, USA) as previously described [15]. Briefly, using aseptic techniques, the left carotid artery was exposed, and the distal bifurcation of the carotid artery was encircled proximally and ligated distally with 8-0 silk sutures. A guidewire was introduced into the arterial lumen through a transverse arteriotomy created between the sutures, and the guidewire was then advanced toward the aortic arch and withdrawn five times. The guidewire was then removed, the proximal 8-0 suture was ligated, and the incision was closed with 5-0 sterile surgical gut. Sham surgery without injury was performed on the right side. After surgery, the mice were fed either normal rodent chow or normal rodent chow containing 0.09% I3C (w/w). This diet corresponded to 150 mg/kg I3C under the assumption that a 30-g mouse consumes 5 g of chow. The mice were maintained on these diets for 28 days before euthanasia. The right and left carotid arteries were isolated, fixed in buffered formalin phosphate and processed for morphometric and immunohistochemical analyses.

2.8. Evaluation of neointima formation

For morphometric analysis, paraffin-embedded sections (5 μm thick) were cut at equally spaced intervals in the middle of the injured and control common carotid artery segments and stained with hematoxylin and eosin to demarcate cell types. Fifteen sections from each carotid artery were reviewed and scored under blind conditions. The intimal (I) and medial (M) areas were measured using Image Proplus6.0 software, and the I/M ratios were calculated.

2.9. Proliferating cell nuclear antigen immunohistochemistry

Immunostaining for PCNA was performed as previously described [16]. An anti-PCNA monoclonal antibody complemented by a biotinylated anti-mouse secondary antibody was applied to perfusion-fixed, paraffin-embedded tissues. Slides were treated with an avidin–biotin block, exposed to diaminobenzidine followed by hematoxylin and analyzed using light microscopy. The data are presented as the number of PCNA-positive cells within the neointima.

2.10. Statistical analysis

The results are expressed as means±S.E.M. Statistical analysis was performed by a one-way analysis of variance followed by Dunnett's multiple-comparison tests. A difference with a P value<.05 was considered significant.

3. Results

3.1. I3C inhibits PDGF-BB-stimulated VSMCs proliferation

The abnormal proliferation of VSMCs contributes to vascular lesion formation. I3C is a proven antitumor compound that leads to growth suppression of cancer cells; however, whether I3C suppresses the growth of VSMCs is unknown. To evaluate the effect of I3C on cell proliferation, we used a WST-1 cell proliferation assay. Stimulation with PDGF-BB for 48 h increased the proliferation of VSMCs approximately threefold compared with nontreated control cells (Fig. 1B). After pretreatment with I3C (12.5 to 100 μM), these PDGF-BB-induced cell numbers decreased significantly in a concentration-dependent manner; the greatest suppression of proliferation was observed with 100 μM I3C. Cells treated with increasing concentrations of I3C alone (12.5 to 100 μM) for 48 h showed no significant difference in viability compared with untreated cells, suggesting that I3C was not cytotoxic at the concentrations tested (Fig. 1B). Therefore, we chose to use 100 μM I3C in the remainder of the study. We further investigated the inhibitory effects of I3C on DNA synthesis using a BrdU incorporation assay. Fig. 1C illustrated that treatment with PDGF-BB increased DNA synthesis in VSMCs, whereas I3C significantly inhibited DNA synthesis in a dose-dependent manner.

3.2. I3C induces G1 and S arrests in VSMCs

Flow cytometric analysis was used to determine whether the antiproliferative effect of I3C was due to arrest at a specific point of the cell cycle. As shown in Fig. 2, flow cytometric analysis of the DNA content indicated that PDGF-BB treatment alone significantly increased the percentage of cells in S phase while decreasing the G0/G1 populations. I3C at a concentration of 100 μM significantly increased the fraction of G0/G1-phase cells and reduced the fraction of G2/M-phase cells but did not change the fraction of S-phase cells. These data indicated that I3C blocked S-phase cell cycle progression in addition to G1 arrest (Fig. 2A and B).

3.3. I3C inhibits cyclin, CDK expression and $p27^{Kip1}$ degradation in PDGF-BB-exposed VSMCs

Phase-specific cyclin–CDK complexes regulate progression through the cell cycle to confer specificity and order to this process [17]. To characterize the mechanism responsible for I3C-induced cell cycle arrest, the effects of I3C on cell cycle events such as the expression of cyclins and CDKs were analyzed using Western blotting. As shown in Fig. 3, PDGF-BB (20 ng/ml) induced significant increases in the expression of cyclin D1, CDK4 and CDK6, whereas treatment with I3C (100 μM) suppressed the up-regulation of these molecules (Fig. 2C).

$p27^{Kip1}$, a CDK inhibitor, binds to and inactivates a wide spectrum of cyclin–CDK complexes, leading to cell growth arrest at the G1 and G1/S boundary. We also examined the effects of I3C on the expression of $p27^{Kip1}$. $p27^{Kip1}$ was constitutively expressed in serum-starved quiescent VSMCs and down-regulated by PDGF-BB. In contrast, $p27^{Kip1}$ down-regulation was partly reversed by pretreatment with I3C (Fig. 2C).

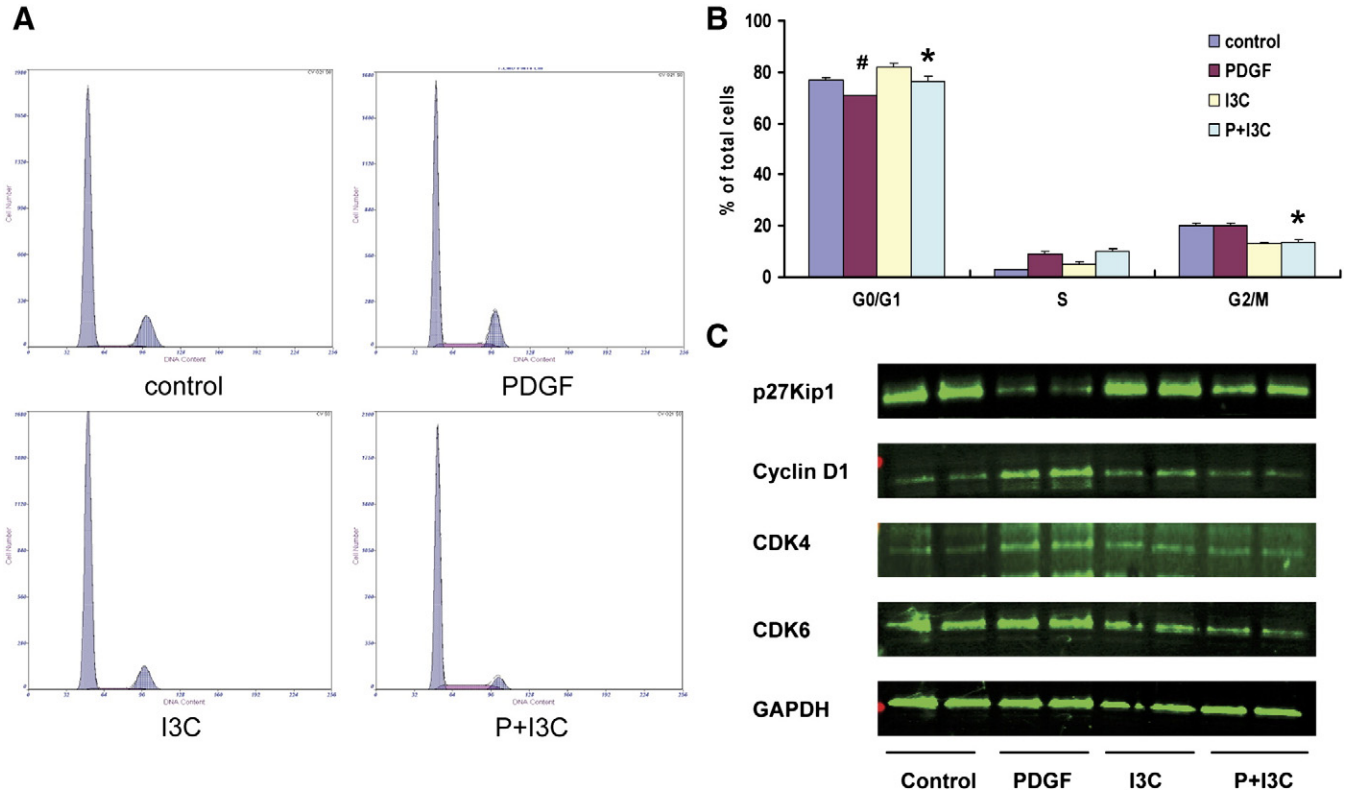

Fig. 2. I3C modulates cell cycle progression in VSMCs. VSMCs were grown with I3C (100 μM) in the absence or presence of PDGF-BB (20 ng/ml) for 24 h, and the cell cycle distribution was evaluated using flow cytometric analysis. (A) Representative cell cycle profiles are shown. (B) Quantification of VSMCs in the G0/G1, S and G2/M phases, as determined based on flow cytometric evaluation (#P<.01 versus the control group; *P<.01 versus PDGF alone; n=3). (C) Expression of cell cycle proteins was determined by Western blotting. GAPDH served as a loading control.

3.4. I3C prevents the migration of PDGF-BB-stimulated VSMCs

The migration of smooth muscle cells from the media to the intima also plays a critical role in vascular lesion formation. We next examined whether I3C plays a role in the regulation of VSMC migration. As indicated in Fig. 3A, treatment with PDGF-BB (20 ng/ml) caused an almost twofold increase in the migration of VSMCs; however, pretreatment with I3C (100 μM) significantly reduced PDGF-BB-induced migration. The number of migrated cells was significantly decreased by using I3C (Fig. 3B). These results suggest that I3C is a potent inhibitor of VSMC migration.

3.5. Effect of I3C on the phenotypic modulation of VSMCs

In response to vascular injury or growth factor signaling, VSMCs can switch between differentiated and dedifferentiated phenotypes.

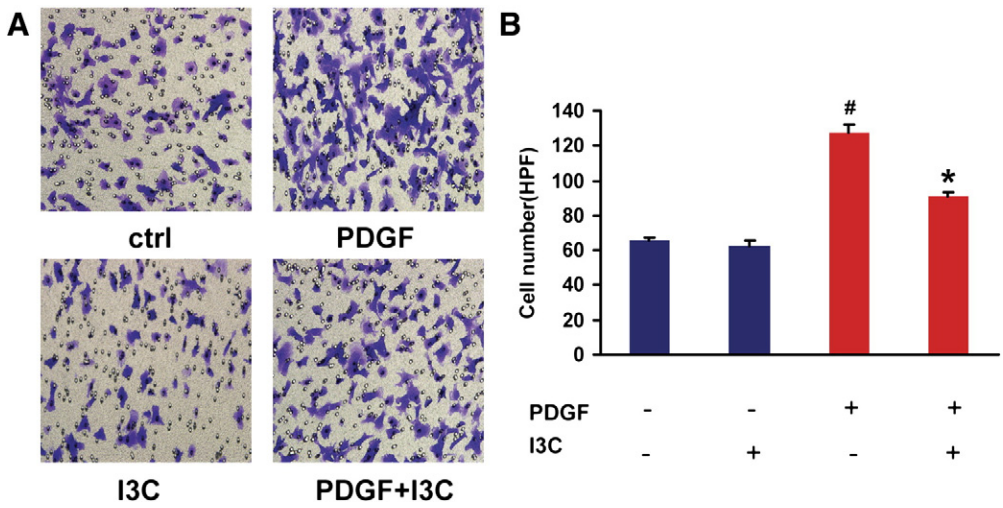

Fig. 3. I3C inhibits PDGF-BB-induced cell migration. (A) VSMCs were cultured in a cell migration filter insert and stimulated with PDGF-BB for 6 h with or without treatment with I3C (100 μM). (B) Cellular migration was determined by counting the cells that migrated through the pores. The results are expressed as means±S.E.M. (#P<.01 versus the control group; *P<.01 versus PDGF alone).

Dedifferentiated phenotypes are characterized by abnormal proliferation, migration, increased matrix secretion and decreased expression of a variety of SMC-specific contractile genes, including SM α-actin and SM22α [18]. To further evaluate the phenotypic modulation of VSMCs by I3C, we measured the expression of SM α-actin and SM22α by Western blot analysis. After pretreatment with I3C (100 μM) for 2 h, quiescent VSMCs were stimulated with PDGF-BB (20 ng/ml) for 48 h in the presence or absence of I3C. As indicated in Fig. 4, PDGF-BB significantly reduced the protein levels of SM α-actin and SM22α. Moreover, pretreatment with I3C partially blocked the repressive effect of PDGF, suggesting that I3C plays a role in maintaining the quiescent (differentiated) state of VSMCs.

3.6. Molecular mechanisms involved in the inhibition of proliferation and migration of VSMCs by I3C

We then analyzed the molecular mechanisms of phenotypic modulation by I3C in VSMCs. Previous studies have demonstrated that the phosphatidylinositol 3-kinase (PI3K)/Akt/GSK3β and MAPK signal transduction pathways are critically involved in VSMC proliferation and migration. Therefore, we first evaluated the effect of I3C on the PDGF-BB-induced activation of the Akt/GSK3β pathway. The cells were treated with PDGF-BB, and the phosphorylation of Akt and GSK3β was measured. Our data showed that PDGF-BB induced rapid and sustained activation of Akt and GSK3β without affecting the total levels of these molecules (Fig. 5). We then examined the effects of I3C on the kinetics of PDGF-induced Akt/GSK3β activation. The cells were pretreated with 100 μM I3C for 2 h and then treated with PDGF-BB for time indicated in the figure. As shown in Fig. 5, PDGF-BB-induced Akt/GSK3β activation was significantly impaired by I3C. The observed inhibitory effects of I3C on PDGF-BB-induced Akt /GSK3β activation were not due to the decreases in total protein levels.

We then examined the effects of I3C on the PDGF-BB-induced activation of MAPK pathways by treating VSMCs with PDGF-BB for time indicated in the figure. Significant activation of ERK1/2, p38 and JNK1/2 was first observed 5 min after PDGF treatment, and this activation was sustained for 15 min, as assessed by comparison with controls via Western blotting. However, I3C did not exhibit any inhibitory effect on the phosphorylation of ERK1/2, p38 and JNK1/2 (Fig. 5).

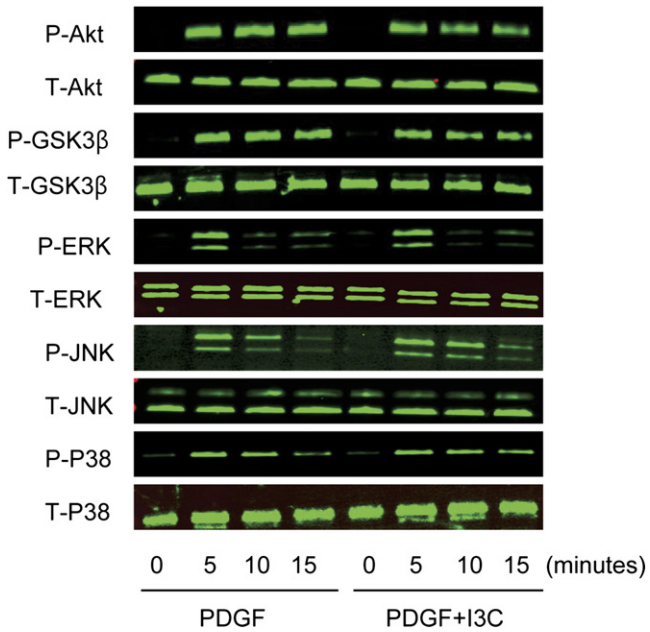

Fig. 5. Treatment with I3C inhibits the PDGF-stimulated phosphorylation of Akt and GSK-3β but has no effect on ERK1/2, p38 or JNK. Cells were treated with or without (control) I3C for 2 h, followed by PDGF (20 ng/ml) for the indicated times. Protein levels of phospho-Akt, Akt, phospho-GSK-3β, GSK-3β, phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK and JNK were determined based on Western blot analysis. One representative photo was obtained from three experiments.

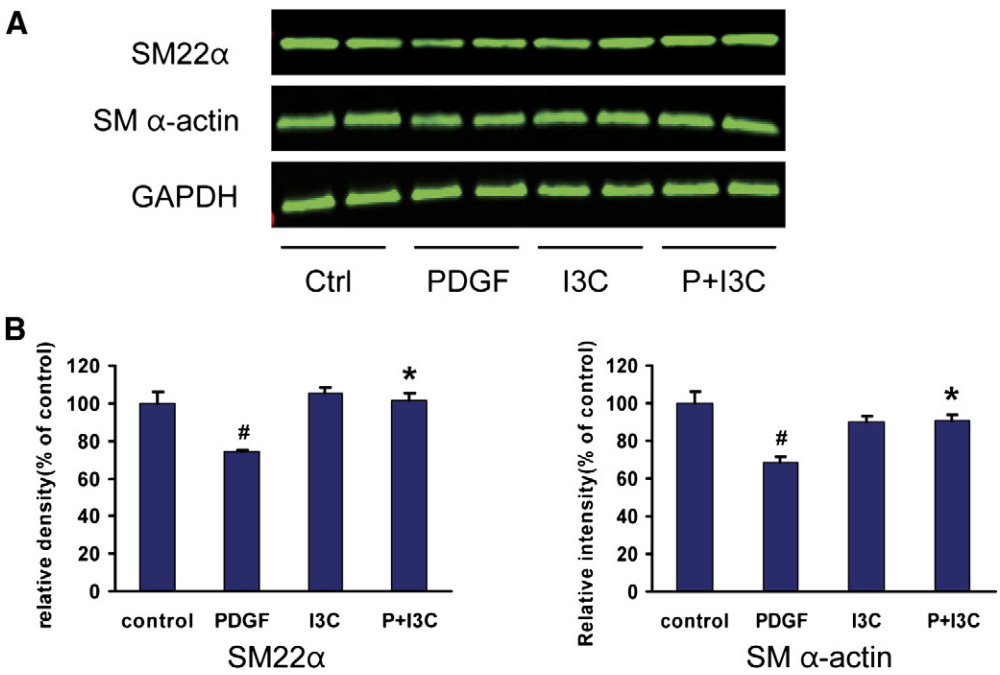

Fig. 4. Effects of I3C on the regulation of smooth muscle gene expression. (A) VSMCs were preincubated for 2 h with I3C and then stimulated with PDGF (20 ng/ml) for 48 h. The protein levels of SM α-actin and SM22α were determined using Western blot analysis and quantified using densitometry. (B) Bar graphs showing quantification of the Western blots. The results are expressed as percentages of the control (#P<.05 versus the control group; *P<.05 versus PDGF alone).

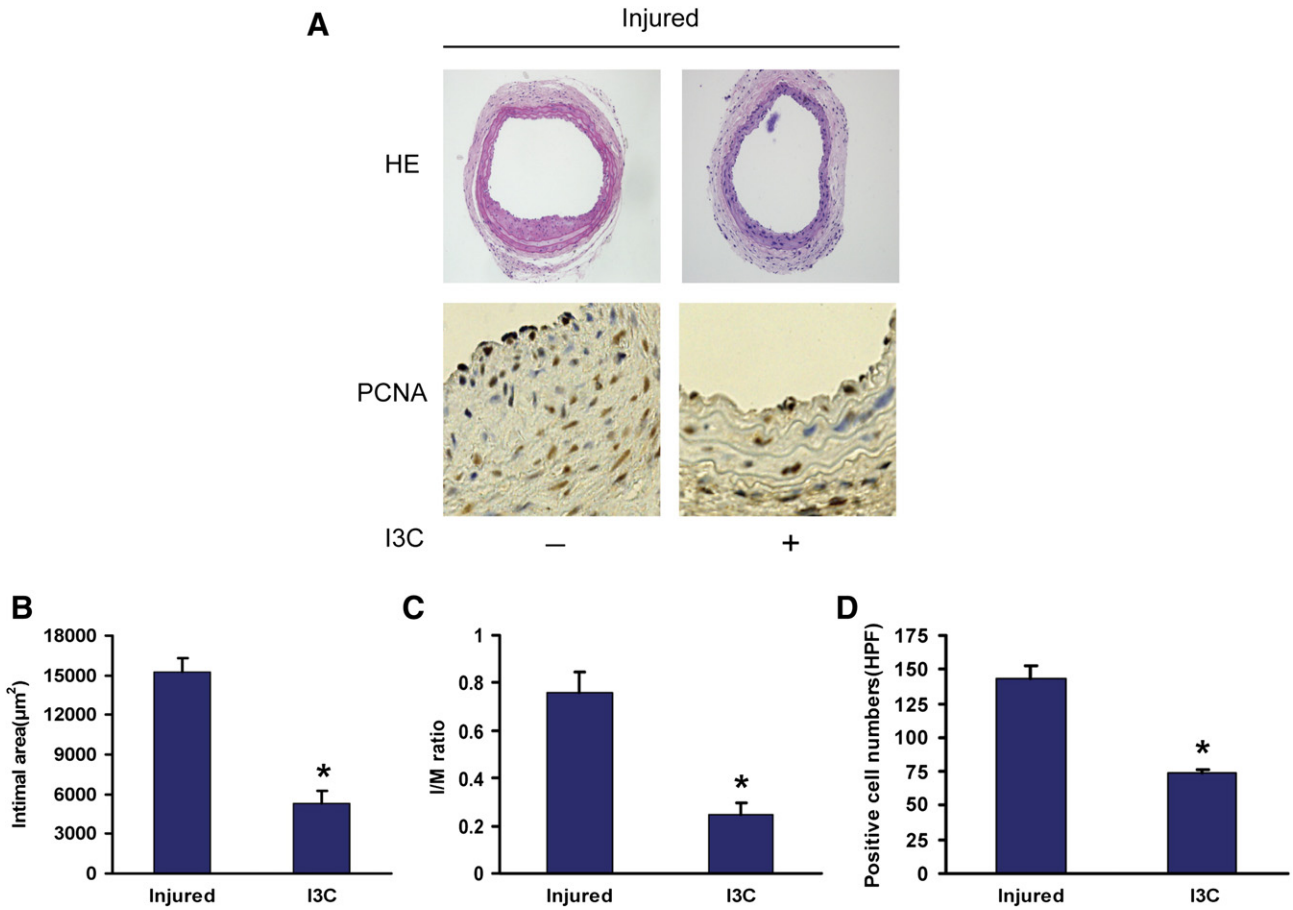

Fig. 6. I3C prevents neointimal formation and cell proliferation induced by guidewire injury. (A) Representative section of the injured carotid artery of a mouse from either the untreated control group or the I3C-treated group ($n=6$, *$P<.01$ versus the injured control). (B–D) Quantification of the I/M ratio, intimal area and PCNA-positive cells of mouse carotid arteries from either the control group or the I3C-treated group ($n=9$; *$P<.01$ versus the injured control).

3.7. Effects of I3C on neointima formation and cell proliferation in vivo

To evaluate the effect of I3C on neointima formation, mouse carotid arteries were harvested 28 days after injury and subjected to morphometric analysis. Representative sections from control and I3C-treated injured carotid arteries are shown in Fig. 6A. The formation of the neointima was significantly increased in guidewire-injured carotid arteries. In contrast, the I/M ratios and neointima areas were significantly reduced in I3C-treated carotid arteries compared to injured controls (intimal area: 5343±932.11 μm^2 versus 15,243.53± 1052.22 μm^2, respectively; I/M ratios: 0.25±0.05 versus 0.76±0.09, respectively). To assess VSMC growth, arterial sections were stained with an anti-PCNA antibody (Fig. 6A). Treatment with I3C significantly diminished the injury-induced increase in PCNA-positive staining in the neointima of carotid arteries; the number of PCNA-positive cells in the guidewire-injured carotid artery was 50% that of the injured control (Fig. 6D).

4. Discussion

In this study, we demonstrated that I3C inhibited PDGF-induced vascular smooth muscle cell proliferation and migration, as well as phenotypic changes, *in vitro* without cytotoxic effects. The effect of I3C was also observed *in vivo*; oral administration of I3C effectively attenuated neointima hyperplasia in guidewire-injured mouse carotid arteries. We also showed that I3C-induced growth inhibition was associated with the inhibition of cyclin D1, CDK4 and CDK6 expression, as well as an increase in $p27^{Kip1}$ levels in PDGF-stimulated VSMCs. These beneficial effects of I3C on VSMCs were associated with inhibition of the Akt/GSK3β pathway. These findings suggest that I3C may protect against vascular remodeling after injury.

Multiple lines of evidence have shown that phenotypic modulation is an important phenomenon in VSMC activation. VSMCs retain remarkable plasticity and can undergo rather profound and reversible changes in phenotype in response to changes in local environmental signaling. These changes are normally induced by balloon injury and growth factor signaling [2]. In response to vascular injury, the phenotype of VSMCs switches from a quiescent, differentiated state to a dedifferentiated state characterized by dramatically increased cell proliferation, migration and synthetic capacity. At the same time, the expression of smooth muscle-specific contractile markers, such as SM α-actin and SM22α, is decreased [18]. This dedifferentiated phenotype contributes to the pathogenesis of many cardiovascular disorders, including atherosclerosis, restenosis after angioplasty or bypass, and hypertension [19–21]. It is well established that PDGF-BB is a key mediator of VSMC phenotypic switching [22]. In accordance with previous studies, we observed that PDGF-BB induced VSMC proliferation and migration while decreasing SM α-actin and SM22α expression. More importantly, our *in vitro* study demonstrated that treatment with I3C significantly inhibited PDGF-BB-induced cell proliferation and migration while reversing the expression of SM α-actin and SM22α. These results suggest that I3C may help to maintain VSMCs in a quiescent state.

In maturated vascular tissue, most of the VSMCs are quiescent. However, in response to the stimulation of growth factors produced

after vascular injury, activated VSMCs reenter the cell cycle to initiate abnormal proliferation. I3C has been demonstrated to cause cell cycle arrest in a variety of cancer cell lines [23–25]. For this reason, we hypothesized that I3C would block VSMC proliferation through cell cycle arrest. As expected, our results demonstrated that I3C treatment for 24 h led to a significant increase in the number of cells in the G0/G1 phase and a reduction in the number of cells in the G2/M phase, indicating that I3C inhibits cell cycle progression in both the G0/G1 phase and the S phase. Previous studies have revealed that I3C induces G1 cell cycle arrest in PC3 prostate cancer cells and breast cancer cells [8,10]; the differences between our study and those results may be due to differences in the cell type studied. The cell cycle is regulated by the synthesis, activation and degradation of molecules that modulate the proliferative phenotype [26]. D-type cyclins form active complexes with CDK4 and CDK6 to phosphorylate retinoblastoma protein, inducing the release of transcription factors and initiating cell cycle entry and G1/S-phase transition [17]. A growing body of evidence suggests that cyclin D1, CDK4 and CDK6 are up-regulated after PDGF-BB stimulation *in vitro* or vascular injury *in vivo* [27]. Because the cells in our experiments exhibited G1 phase arrest, we examined the effects of I3C on the expression of cyclin D1, CDK4 and CDK6. Our experiment indicated that I3C treatment of VSMCs resulted in significant down-modulation of cyclin D1, CDK4 and CDK6, although to differing extents. The activity of the cyclin/CDK complex is dependent upon the balance between cyclins and CDK inhibitors (CKIs). One of the CKIs, $p27^{Kip1}$, can bind to and inhibit a wide spectrum of cyclin/CDK complexes, such as cyclin D–CDK4/6 and cyclin E–CDK2, and arrest cell growth at the boundary between G1 and G1/S. In addition, $p27^{Kip1}$ also inhibits the cyclin A–CDK2 complex, which, owing to its importance in S-phase progression, may be responsible for the S-phase arrest by I3C. In healthy arteries, $p27^{Kip1}$ is constitutively expressed and negatively regulates the growth of VSMCs. However, in response to arterial injury, $p27^{Kip1}$ is down-regulated [28]. Chen and coworkers delivered adenovirus that encoded $p27^{Kip1}$, thereby reducing neointimal hyperplasia in rat carotid arteries when applied at the time of angioplasty [29]. Tanner and coworkers also found that adenovirus-mediated overexpression of $p27^{Kip1}$ reduced intimal VSMC proliferation and the I/M area ratio after vascular injury and gene transfer to pig arteries [30]. Therefore, pharmacological interventions that induce $p27^{Kip1}$ expression may also inhibit VSMC proliferation. In the present study, the selective up-regulation of $p27^{Kip1}$ expression by I3C was consistent with the inhibitory effects of I3C on the expression of several cell cycle proteins and cell proliferation. Taken together, these data suggest that the cell cycle arrest in VSMCs following I3C treatment is mainly due to the inhibitory action of $p27^{Kip1}$ on CDK complexes and the inhibition of the expression of CDK kinase components (including cyclin D1, CDK4 and CDK6).

Cyclin D1 and $p27^{Kip1}$ have been suggested as regulators of cell migration in recent studies [31,32]. To investigate whether the effect of I3C on cell cycle protein contributes to its inhibitory effect on cell migration, we measured the expression of cyclin D1/p27Kip1 after treatment with PDGF for 6 h in the presence or absence of I3C. The results demonstrated that the expression of cyclin D1/p27Kip1 was not significantly changed after 6-h incubation with I3C compared with treatment with PDGF alone (data not shown). This indicates that there may have other mechanisms involved. As we mentioned above, I3C inhibits the migration of breast cancer cells by decreasing expression of the chemokine receptor CXCR4 and the MMP-9; however, whether I3C has the same effects on VSMC needs further investigation.

Given that phenotype modulation of VSMCs contributes to the formation of advanced atherosclerotic lesions and in-stent restenosis, I3C may be beneficial regarding these vascular injuries. Indeed, we demonstrated that I3C attenuated the increase in PCNA-positive cells in the neointimal region and significantly reduced intimal hyperplasia following guidewire injury of the mouse carotid artery. These results bolstered our *in vitro* findings and provide direct evidence that I3C can protect against pathological vascular remodeling after vascular injury. Clinical studies have established that ingestion of 400 mg of I3C twice daily is well tolerated [33]. In the present study, mice were treated with I3C orally at a dosage of 150 mg/kg per day, which is equivalent to a dose of 12.2 mg/kg in an average adult human [34]. This dose is within the range that has been used in clinical trials. In our protocol, treatment with I3C was initiated after vascular injury and proved to be able to attenuate vascular remodeling. Furthermore, I3C inhibited the PDGF-induced proliferation and migration of VSMCs *in vitro* without cytotoxic effects, increasing the clinical relevance of our findings. Therefore, I3C may be a strong candidate for the treatment of vascular remodeling owing to its practicality and safety in potential therapeutic applications. To extend the benefits observed in mice to the clinical scenario, translational strategies will be the subject of further research.

To explore the molecular mechanism underlying the inhibition by I3C of the phenotypic modulation of VSMCs, we tested the roles of PI3K/Akt and MAPK signaling in this process. The PI3K/Akt signaling pathway is implicated in multiple cellular processes, including migration and proliferation events downstream of growth factors such as PDGF. Akt has various downstream targets, including GSK3β, and it phosphorylates GSK3β and decreases the catalytic activity of this molecule [35]. Given the critical role of Akt/GSK3β in the regulation of VSMC proliferation and migration, we examined the activation of this signaling pathway. Consistent with previous studies, PDGF induced a rapid and sustained activation of Akt/GSK3β. More importantly, pretreatment with I3C significantly attenuated the phosphorylation of Akt/GSK3β, which is consistent with the changes observed in the expression of cyclin D1 and $p27^{Kip1}$. Activation of GSK-3β has been found to regulate cyclin D1 stability and expression. By inducing cyclin D1 export from the nucleus to the cytoplasm for proteolysis, GSK-3β decreases the availability of cyclin D1 [17]. GSK-3β also has been found to inhibit the translocation of the transcription factor "nuclear factor of activated T cells" into the nucleus, and this process was recently found to induce cyclin D1 expression [31]. Furthermore, GSK-3β inhibition has been shown to decrease expression of the CDK inhibitor $p27^{Kip1}$ [36]. Moreover, Bianchi and coworkers found that GSK-3β phosphorylated a serine residue of FAK to negatively modulate FAK catalytic activity, which is important in cell migration [37].

Apart from regulating cell proliferation and migration, Akt is also involved in phenotype modulation. It has been reported that the activation of Akt by PDGF leads to serum response factor (SRF) redistribution to the cytosol and reduction of the DNA binding activity of SRF, which suppresses SM α-actin and SM22α expression [38]. Combining these previous results with our findings, we speculate that the Akt/GSK3β signaling pathway at least partly contributes to the inhibitory effect of I3C on the proliferation, migration and phenotype modulation of VSMCs and subsequent neointima development.

We also examined the effect of I3C on the activation of MAPK, another important player implicated in the VSMC proliferation and migration induced by PDGF-BB. Unlike its effect on the Akt pathway, I3C failed to attenuate the PDGF-stimulated phosphorylation of ERK, JNK and p38 MAPK. These results indicate that the PDGF-BB-mediated early signal activations, such as those of ERK1/2, JNK and p38, may be not involved in the inhibitory effect of I3C on the phenotypic modulation of VSMCs.

In addition to what we have investigated, there may be some other mechanisms through which I3C inhibits neointima formation. I3C has been reported to have anti-inflammatory effects through the inhibition of inflammatory factor production in macrophage [14]; moreover, I3C was found to have antioxidative effects by up-

regulating nuclear erythroid related factor 2-mediated signaling and antioxidative stress response [39]. However whether I3C has the same effects on VSMC needs further investigation.

In conclusion, our data indicate that I3C inhibits the phenotypic modulation of VSMCs induced by PDGF-BB and attenuates the neointimal formation response to vascular injury. This process is associated with an inhibition of cyclin D1 expression and an increase in $p27^{Kip1}$ accumulation via blockade of the Akt/GSK3β signaling cascade. The results of this study indicate the possibility that I3C is a potent agent for the prevention of vascular proliferative disease.

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