Original Article

Gambogic Acid Induces G0/G1 Cell Cycle Arrest and Cell Migration Inhibition Via Suppressing PDGF Receptor β Tyrosine Phosphorylation and Rac1 Activity in Rat Aortic Smooth Muscle Cells

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***Aim*: Gambogic acid (GA) is the major active compound of Gamboge, a brownish or orange resin exuded from Garcinia hanburryi tree in Southeast Asia. Previous studies have demonstrated that GA exhibits potent anticancer effects by inducing cell cycle arrest or apoptosis in many types of cancer cell lines and blocking angiogenesis via inhibition of vascular endothelial cell proliferation and migration. Proliferation and migration of vascular smooth muscle cells (VSMCs) are critical steps in the progress of atherosclerosis and restenosis after angioplasty. In the present study, we investigated whether GA has an inhibitory effect on the proliferation and migration of VSMCs and its possible mechanism.**

***Methods*: The inhibitory effect of GA on the proliferation induced by PDGF-BB and EGF was measured by using Cell number counting assay and [^{3}H]-thymidine incorporation. The effects of GA on the cell cycle progression and viability stimulated by PDGF-BB and EGF were also analyzed by flow cytometry analysis. The inhibitory effect of GA on the migration stimulated by PDGF-BB was measured by transwell chamber assay. The effect of GA on the Cell cycle regulatory molecules (cyclinD1, cyclinE, CDK2, CDK4), PDGFR and its downstream signaling molecules including ERK1/2, PLCγ1, AKT and JNK was measured by western blotting. The effect of GA on the Rac1 activity was measured by using GST-pulldown assay. The effects of GA on the tyrosine phosphorylation stimulated by PDGF-BB and EGF and the capacity of GA binding with PDGF-BB and EGF were also measured.**

***Results*: We found that GA significantly inhibited proliferation, migration and DNA synthesis in primary cultured rat aortic VSMCs at concentrations of 0.25, 0.5, 1.0 and 2.0 μmol/L after stimulation of 50 μg/L platelet-derived growth factor-BB (PDGF-BB). GA induced G0/G1 phase arrest in the cell cycle progression of VSMCs. No obvious necrosis or apoptosis was found with GA treatment. The expressions of CDK2, CDK4, cyclin D1 and cyclin E, cell cycle regulatory molecules, were significantly suppressed by GA treatment in a concentration-dependent manner. The phosphorylation of PDGF receptor β (PDGFR-β) and the activities of downstream intracellular signaling molecules including phospho-ERK, phospho-PLCγ1, phospho-AKT, phospho-JNK and GTP-Rac1 were also inhibited by GA pretreatment. GA inhibited PDGFR-β phosphorylation through inhibiting the activity of tyrosine directly, rather than indirectly via binding PDGF-BB.**

***Conclusions*: The results of this study provide preliminary evidence that the inhibitory effects of GA on VSMCs proliferation and migration may be mediated through multiple signal pathways controlled by PDGF-Rβ activation and its downstream intracelluar signaling.**

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Key words; Gambogic acid, Vascular smooth muscle cell, Proliferation, Migration, Platelet-derived growth factor receptor-β (PDGFR-β)

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Introduction

Abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) is a major contributor to atherosclerosis and restenosis after angioplasty.

Platelet-derived growth factor (PDGF) is known to be an important stimulator[1-3]. Much evidence indicates that PDGF-BB and PDGF receptor β (PDGFR-β)-mediated signals are particularly important for vascular remodeling and neointima formation after vascular injury[4-6]. Therefore, it will become a novel strategy for pharmacologic inhibition of PDGF-induced VSMC proliferation and migration during lesion development.

PDGFR-β activation is associated with a number of Src homology region 2-containing signaling enzymes, including RasGAP, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC)γ1[7]. These signaling molecules selectively associate with phosphorylated tyrosine residues within the cytoplasmic domain of the PDGFR, and their activation induces a highly specific signal transduce cascade. ERK, Akt, JNK and small G proteins, including rho and rac-1, are involved in downstream mediators of PDGFR-β, and they all ultimately take part in PDGF-dependent cellular responses, such as cell cycle progression, migration, and survival[8, 9].

It was reported in Chinese traditional medicine documents that Gamboge resin has potent biological effects, such as hemostasis, anti-inflammation, antioxidation and anti-infection. Gambogic acid (GA, C38H44O8) is the major active compound of Gamboge, a brownish or orange resin exuded from the Garcinia hanburryi tree in Southeast Asia. Previous studies have demonstrated that GA has potent anticancer, anti-inflammatory activity and inhibits angiogenesis both *in vivo* and *in vitro* by suppressing the phosphorylation of AKT, ERK, c-Src, FAK and VEGFR2[10-15]. However, the effects of GA on the proliferation and migration of VSMCs are not clearly understood. In this study, we investigated the effects of GA on rat VSMC proliferation and migration stimulated by PDGF-BB and the underlying molecular mechanism. We found that GA significantly inhibited the proliferation and migration of VSMCs by suppressing the phosphorylation of PDGFR-β and its downstream kinases and small G protein rac1 activity. GA may be developed to a potential drug in the prevention and treatment of atherosclerosis.

Materials and Methods

Materials and Reagents

GA (20 mg) was kindly provided by Professor Guo Qinglong at China Pharmaceutical University. PDGF-BB and EGF were purchased from R&D Corporation (Minneapolis, USA). [^{3}H]-thymidine was bought from China Isotope Corporation (Beijing, China). PDGF-Rβ, phospho-PDGFR-β (Tyr751), PLCγ1, phospho-PLCγ1 (Ser1248), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), AKT, phospho-AKT (Ser473), SAPK/JNK, EGFR, Phospho-EGFR (Tyr1068) phospho- SAPK/JNK (Thr183/Tyr185), phospho-tyrosine and Rac1/2/3 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA), PDGF-BB, EGF, cyclin D1, cyclinE, CDK2, and CDK4 antibodies were supplied by Santa Cruz Biotechnology Corporation (Santa Cruz, CA, USA), while the Rac1 activation kit was bought from Assay Designs Inc. (Michigan, USA). The other chemicals were of the highest analytical grade commercially available.

Cell Culture

VSMCs were isolated from Sprague-Dawley rats using the method described previously[16]. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in a humidified 5% CO_2 incubator. VSMC purity was further confirmed by immunocytochemistry staining with alpha-smooth muscle actin monoclonal antibody. VSMCs between passages 4 and 8 were used in experiments and made quiescent by serum starvation in DMEM containing 0.1% FBS for 24 hrs[17].

Measurement of Cell Proliferation and DNA Synthesis Cell Proliferation

Rat aortic VSMCs (2×10^4) were seeded into 24-well culture plates and cultured in DMEM medium containing 10% FBS and grown to 70% confluence. VSMCs were then incubated with serum-free medium for 24 hrs and then treated with GA at final concentrations of 0.25 to 2.0 μmol/L for another 24 hrs. The cells were stimulated with or without PDGF-BB (50 μg/L) or EGF (10 μg/L) for 24 hrs and counted by hemocytometer.

DNA Synthesis

VSMCs that had been grown to 70% confluence were serum-starved for 24 hrs, and then treated with GA for another 24 hrs before PDGF-BB or EGF stimulation. After cells were stimulated by PDGF-BB (50 μg/L) or EGF (10 μg/L) for 8 hrs and [^{3}H]-thymidine (1 μCi/mL) was added and incorporated with VSMCs for another 16 hrs, the medium was aspirated to terminate the reaction. The cultures were washed sequentially with PBS containing 10% trichloroacetic acid and ethanol/ether (1:1, v/v) on ice. Acid-insoluble [^{3}H]-thymidine was extracted by 1.0 mol/L NaOH with 500 μL per well; the extracted solution was then

mixed with 3 mL scintillation cocktail (Ultimagold; Packard Bioscience, CT, USA) and radioactivity was quantified as count per minute (CPM) using a liquid scintillation counter (LS3801; Beckman, Düsseldorf, Germany).

Cell Cycle Analysis

To estimate the proportion of VSMCs in different phases of the cell cycle treated by GA, cellular DNA contents were measured by flow cytometry. VSMCs were starved for 24 hrs and then treated with GA for another 24 hrs. The cells were collected by trypsinization after stimulation with PDGF-BB (50 μg/L) or EGF (10 μg/L) for 24 hrs. They were fixed by 70% ethanol overnight at 4℃. The fixed VSMCs were briefly vortexed and centrifuged at 15,000×g for 5 minutes. The ethanol was discarded and the pellets were stained with 0.5 mL propidium iodide (PI) solution (50 μg/mL PI in buffer containing 100 μg/mL RNase A), and incubated for 1 hr at room temperature. The complexes of PI-DNA were measured using a FACSCalibur (Beckman Coulter Co., USA). The rates of G0/G1, S and G2/M phases were analyzed.

Annexin-V and PI Double-Staining (Apoptosis) Assay

Apoptotic or necrotic cells were detected by double staining with FITC-conjugated annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit (Bipec Biopharma, USA) according to the manufacturer's instructions. VSMCs were incubated with serum-free DMEM medium for 24 hrs, and then treated with GA at an increasing concentrations (0.25 −2.0 μmol/L) for another 24 hrs before being stimulated by PDGF-BB or EGF. After VSMCs were treated by PDGF-BB or EGF for 24 hrs, the cells were harvested and rinsed twice with cold PBS. Flow cytometric analysis (Beckman Coulter Co., USA) was performed immediately after staining. Finally, the percentage of viable cells (annexin V and PI negative), apoptotic cells (annexin V negative and PI positive) and necrotic cells (annexin V and PI positive) were analyzed.

Cell Migration Assay

Cell migration was measured with 24-well Transwell inserts (Corning and Transwell, NY, USA) as previously described[18]. In brief, a polycarbonate membrane (pore size, 8.0 μm) was incubated with 10 mg/mL gelatin (Sigma, USA) overnight before use. The lower chamber was filled with 500 μL DMEM medium with PDGF-BB (10 μg/L)[19] or without PDGF-BB as a control. The upper chamber was filled with the cell suspension (200 μL, 5×10^5/mL) containing GA at increasing concentration. Cells were incubated in the assembled chamber for 24 hrs. Cells which migrated to the lower face of the membrane were fixed with methanol and stained with 0.1% crystal violet in 20% methanol. The inserts were rinsed in PBS 3 times and air-dried overnight. Crystal violet was lysed with 500 μL 50% ethanol containing 0.1 mol/L sodium citrate. The absorption value was measured at 585 nm using a spectrophotometer, Multiskan MK3 (Thermo, Finland)[15].

Western Blotting Analysis

Rat VSMCs seeded in 6-well plates were incubated in serum-free DMEM medium with GA for 24 hrs. VSMCs were then stimulated by 50 μg/L PDGF-BB or EGF (10 μg/L) and lysed in SDS lysis buffer containing a protease inhibitor, PMSF and a phosphotase inhibitor. Lysates were centrifuged at 13000g for 15 minutes, and then the supernatants were collected. Protein concentration was measured using a BCA protein assay reagent (Beyotime Institute of Biotechnology, Shanghai, China). 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed for protein separation. Proteins were transferred to a PVDF membrane (Millipore Corp., USA). The membranes were blocked by 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) at 4℃ overnight and then incubated with 1:1000 dilution of each of the following antibodies: anti-EGFR, anti-phospho-EGFR, anti-phospho-tyrosine, anti-PDGFR-β, anti-phospho-PDGFR-β, anti-ERK1/2, anti-phospho-ERK1/2, anti-PLC-γ1, anti-phospho-PLC-γ1, anti-AKT, anti-phospho-AKT, anti-JNK, anti-phospho-JNK, anti-cyclin D1, anti-cyclin E, anti-CDK2, anti-CDK4 and anti-Rac1/2/3. After membranes were incubated in the primary antibody at 4℃ overnight and then washed with TBST 3 times, 1:5000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin (Bioworld, USA) was applied for 1 hr at room temperature. Specific protein bands were visualized by enhanced chemiluminescence (ECL) detection reagent (Applygen Technologies Inc., Beijing, China). The levels of phospho- PDGFR-β, phospho-PLC-γ1, phospho-ERK1/2, phospho-AKT, and phospho-JNK were normalized to total PDGF-Rβ, PLC-γ1, total ERK1/2, total AKT, total JNK values, respectively. Band intensities were quantified by the Quantity One program[20].

Dot Binding Assay

A nitrocellulose (NC) membrane was soaked in

transbuffer (25 mM Tris, 199.8 mM glycine and 20% methanol) for 30 s. Recombinant PDGF-BB, EGF, BSA (all 10 ng/μL) and GA (1 μg/μL) were directly spotted on the same membrane due to the small volume of samples. The membrane was then blocked with BSA (5% in PBS) for 30 minutes. After being washed with PBS 3 times, the membrane was incubated with PDGF-BB or EGF (0.5 μg/mL) in PBS for 1 hr at room temperature and then washed with PBS 3 times. The membrane was incubated with anti-PDGF-BB antibody or EGF antibody (2 μg/μL in 1% BSA-containing PBS) for 1 hr at room temperature. 1:5000 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin (Bioworld, USA) was applied for 1hr at room temperature. Specific protein dots were visualized by enhanced chemiluminescence (ECL) detect reagent (Applygen Technologies Inc., Beijing, China)[21].

Measurement of Rac-1 Activation

Rac-1 activation was measured using GST-fusion protein containing the P21-binding domain (PBD) of P21-activated kinase 1 (Pak 1) to affinity precipitate active Rac1 (GTP-Rac1) from cell lysates. VSMCs at 70−80% confluence were incubated in serum-free DMEM medium for 24 hrs, followed by GA treatment at the indicated concentrations for an additional 24 hrs. After stimulation of PDGF-BB for 5 minutes[22], culture medium was carefully removed and the cells were rinsed once with ice-cold TBS. Six hundred microliters of lysis/binding/wash buffer was added to the plates. Cells were scraped and transferred to a microcentrifuge tube which was vortexed and incubated on ice for 5 minutes. Cell lysates were centrifuged at 16000g at 4℃ for 15 minutes. One hundred microliters of supernatants were used to compare protein amounts. The remaining 500 μL supernatant was incubated with GST-PAK-CD fusion protein, which bound to glutathione-coupled Sepharose beads at 4℃ for 30 minutes. The beads and proteins bound to the fusion protein were washed three times in excess lysis buffer, eluted in sample buffer, and then analyzed for bound rac-1 by Western blotting.

Statistical Analysis

The experimental results are expressed as the mean ± S.D. Student's *t*-test and ANOVA with multiple comparisons using the Newman-Keuls test were used for statistical analysis. Statistical significance was accepted at $p < 0.05$.

Results

Effects of GA on PDGF-BB-Mediated Aortic Smooth Muscle Cell Proliferation and DNA Synthesis

GA inhibited PDGF-BB-induced proliferation of rat VSMCs in a concentration-dependent manner (**Fig. 1A**). The number of VSMCs was significantly increased after 50 μg/L PDGF-BB treatment ($70.3 \pm 5.3 \times 10^4$/well) compared to the non-stimulated group ($44.5 \pm 4.8 \times 10^4$/well), and the increased cells were significantly reduced to 62.6 ± 4.3, 58.5 ± 5.8, 47.5 ± 4.7, and $42.2 \pm 2.8 \times 10^4$/well after being treated with GA at concentrations of 0.25 μmol/L, 0.5 μmol/L, 1.0 μmol/L, and 2.0 μoml/L, respectively. GA also inhibited EGF-induced proliferation of rat VSMCs in a concentration-dependent manner (**Fig. 1B**).

The effect of GA on DNA synthesis was assayed using [^{3}H] thymidine incorporation. As shown in **Fig. 1C**, the CPM/well value was increased significantly after 50 μg/L PDGF-BB stimulation (15290.0 ± 783.6/well) compared to the non-stimulated group (6759.7 ± 209.8/well). In contrast, GA inhibited PDGF-induced [^{3}H]-thymidine incorporation into DNA on VSMCs in a concentration-dependent manner. The inhibition rates of 0.25, 0.5, 1.0, and 2.0 μmol/L GA were 27.2%, 41.3%, 46.8%, and 58.7%, respectively, with PDGF-BB treatment. GA also inhibited DNA synthesis induced by EGF in rat VSMCs in a concentration-dependent manner (**Fig. 1D**).

Effects of GA on Cell Cycle Analysis

Effects of GA on cell cycle progression were also analyzed by flow cytometry analysis (**Fig. 2A**). The serum deprivation of VSMCs in primary culture for 24 hrs resulted in approximately 94.7 ± 1.6% synchronization of the cell cycle in the G0/G1 phase. The percentage of cells in the S phase increased from 3.5 ± 1.2 to 12.8 ± 2.7% for 24 hrs after PDGF-BB was added. In contrast, cell cycle progression was blocked significantly in GA pre-treated cells. The reduced percentage of cells in the S phase was 10.2 ± 1.8% ($p < 0.05$, $n = 3$, duplicate), 8.4 ± 1.7%, 5.6 ± 1.2% and 3.9 ± 1.0% ($p < 0.01$, $n = 3$, duplicate) at concentrations of 0.25, 0.5, 1.0 and 2.0 μmol/L, respectively. These findings indicated that GA might act in the early events of the cell cycle to be effective against DNA synthesis induced by PDGF-BB. GA also blocked cell progression induced by EGF (**Fig. 2B**).

Effect of GA on Viability of Rat Aortic VSMCs

As shown in **Fig. 3**, apoptotic cells were double-stained with annexin V and propidium iodide 24 hrs

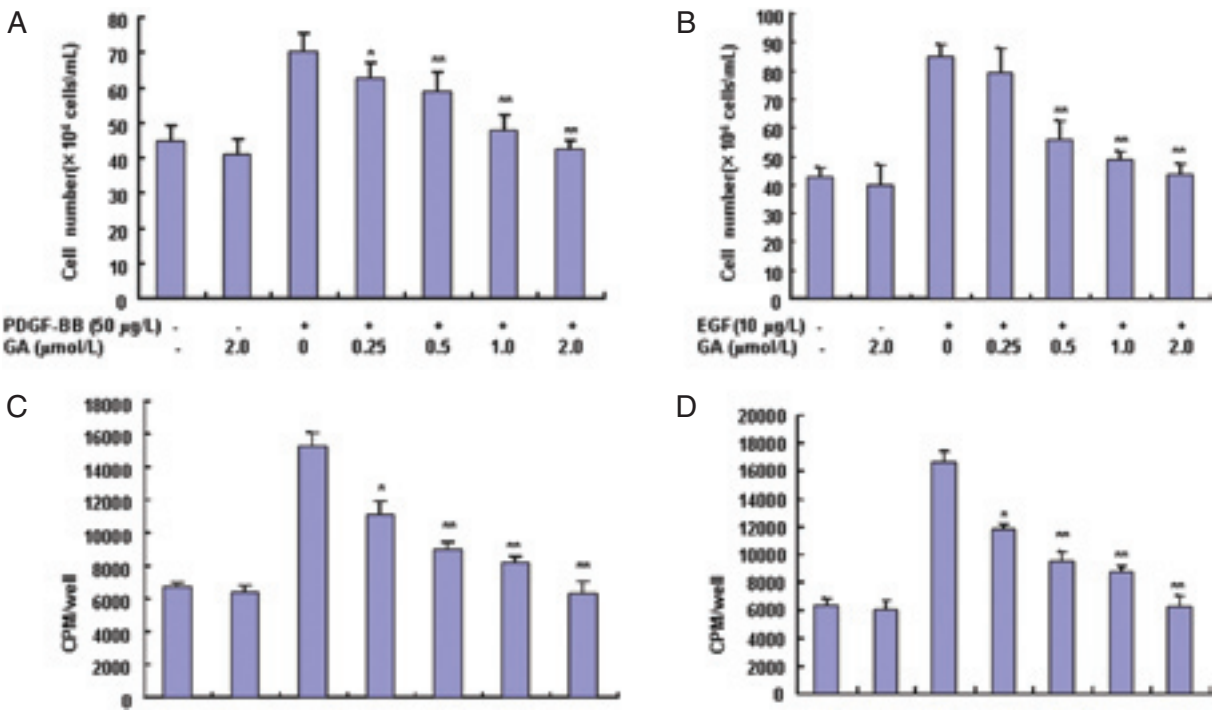

Fig. 1. Effects of GA on proliferation and DNA synthesis induced by PDGF-BB in rat VSMCs. (1A) GA inhibited the proliferation of VSMCs stimulated with PDGF-BB (50 μg/L) in a concentration-dependent manner. Cell number counted by hemocytometer is presented as the mean ± S.D. of 3 separate experiments. (1B) GA inhibited the proliferation of VSMCs stimulated by EGF (10 μg/L) in a concentration-dependent manner. Cell number counted by hemocytometer is presented as the mean ± S.D. of 3 separate experiments. (1C) GA suppressed the incorporation of [^{3}H] thymidine into VSMCs in the presence of PDGF-BB (50 μg/L). CMP value is expressed as the mean ± S.D. of 3 separate experiments. $^*p<0.05$, and $^{**}p<0.01$, vs PDGF-BB stimulation group, respectively. (1D) GA suppressed incorporation of [^{3}H] thymidine into VSMCs in the presence of EGF (10 μg/L). CMP value is expressed as the mean ± S.D. of 3 separate experiments. $^*p<0.05$, and $^{**}p<0.01$, vs EGF stimulation group respectively.

after stimulation with PDGF-BB or EGF in the presence or absence of various concentrations of GA (0.25, 0.5, 1.0 and 2.0 μmol/L). The proportions of apoptotic cells and necrotic cells were not significantly different in PDGF-BB and EGF-stimulated rat VSMCs pretreated with various concentrations of GA, suggesting that the antiproliferative effect of GA was not due to the induction of apoptosis and necrosis in rat VSMCs.

Effects of GA on PDGF-BB Induced Cell Migration

As shown in **Fig. 4A**, PDGF-BB stimulation significantly induced the migration of rat VSMCs by 1.7-fold (data shown as absorption value of crystal violet). GA pretreatment in the upper chamber significantly reduced the increasing cell migration stimulated by PDGF-BB. Compared with the DMSO control group without GA after PDGF-BB stimulation, the number of cells that passed through the membrane was reduced by 17.2%, 32.2%, 35.5% and 45.8% after pretreatment with GA at concentrations of 0.25, 0.5, 1.0 and 2.0 μmol/L respectively. **Fig. 4B** showed that VSMCs stained with crystal violet migrated through the polycarbonate membrane, indicating that GA inhibited cell migration stimulated by PDGF-BB in a concentration-dependent manner.

Effects of GA on PDGF-BB-Induced PDGFRβ and Tyrosine Phosphorylation

Rat VSMCs were incubated with serum-free DMEM medium for 24 hrs, and then treated with GA for an additional 24 hrs. Protein was harvested at 1 minute and 5 minutes after stimulation by PDGF-BB (50 μg/L)[3, 23, 24]. As shown in **Fig. 5**, pretreatment with GA at 0.25, 0.5, 1.0, and 2.0 μmol/L significantly inhibited PDGFRβ and tyrosine phosphorylation stimulated by PDGF-BB compared with the control group without GA in a concentration-dependent manner. In the EGF stimulation group, GA

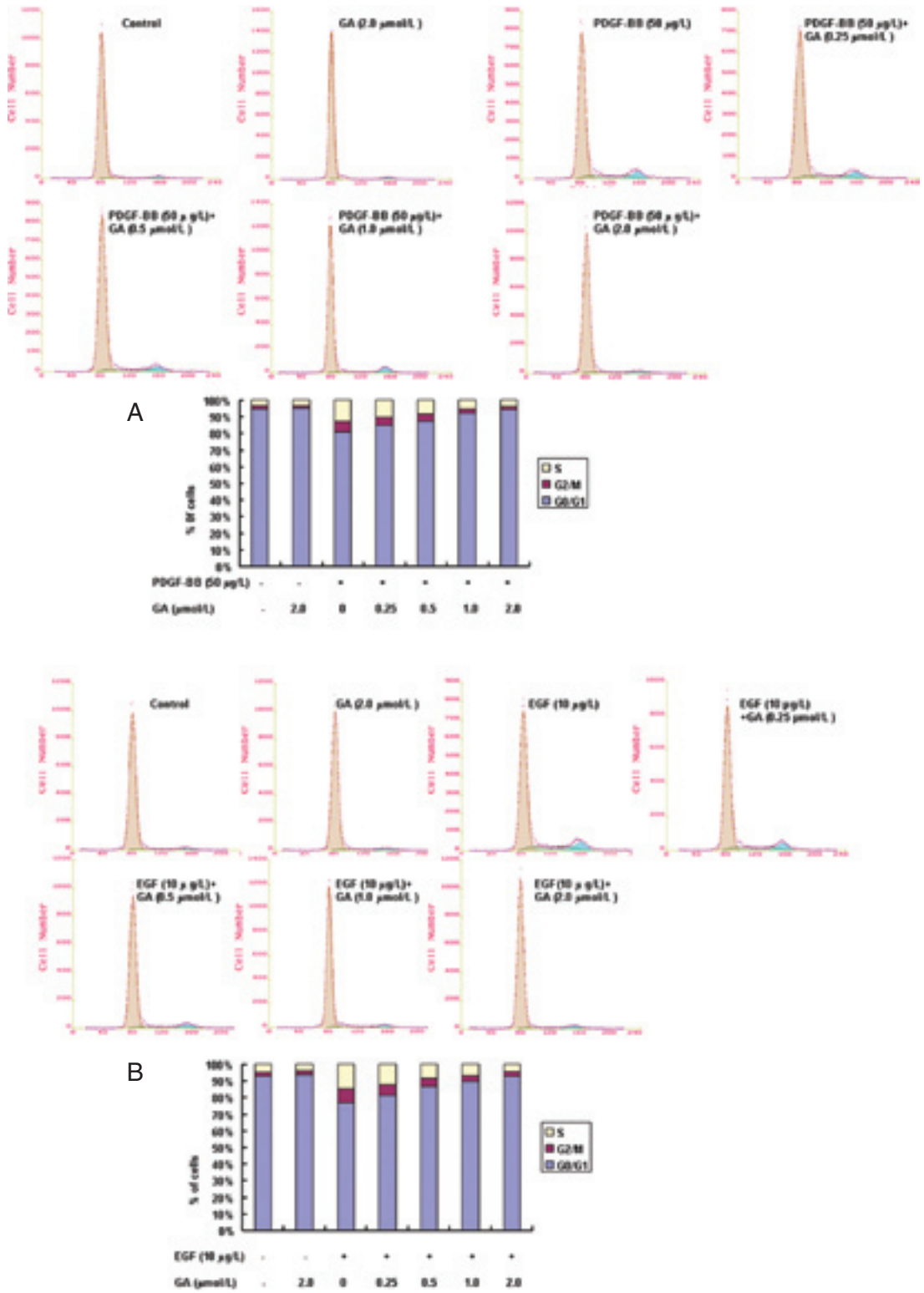

Fig. 2. Effects of GA on PDGF-BB and EGF-induced cell cycle progression in VSMCs. Individual nuclear DNA content as measured by the fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment where data from at least 10,000 events were obtained. Data are representative of at least three independent experiments with similar results. (A) Effect of GA on PDGF-BB-induced cell cycle progression in VSMCs. (B) Effect of GA on EGF-induced cell cycle progression in VSMCs.

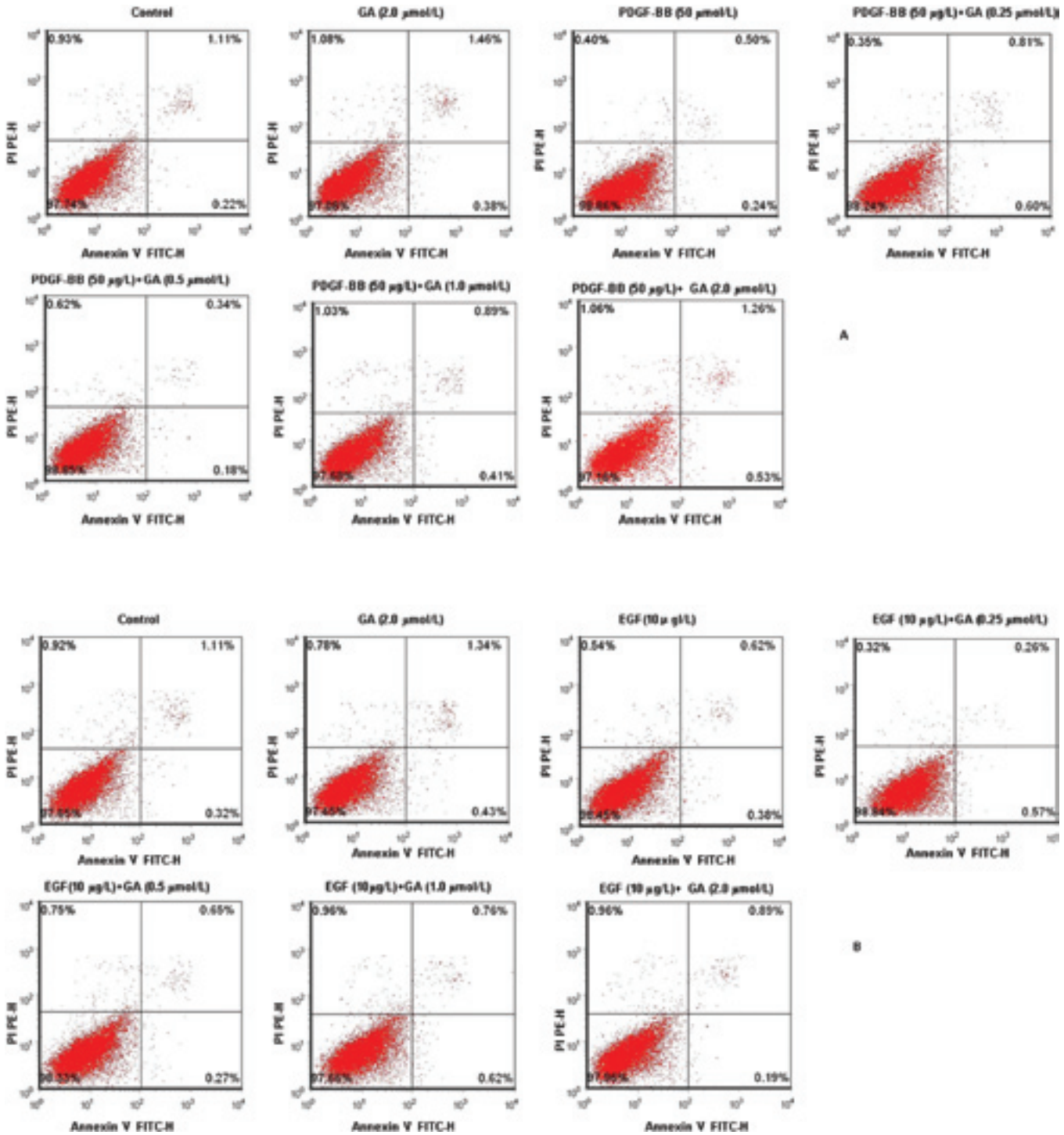

Fig. 3. Dual parameter flow cytometry analysis of rat aortic VSMCs at 24 hrs after PDGF-BB stimulation and EGF stimulation in the presence of GA. Cells were double-stained with annexin V (shown on the x-axis, logarithmic scale) and propidium iodide (shown on the y-axis, logarithmic scale) at 24 hrs after 50 μg/L PDGF-BB or 10 μg/L EGF stimulation in the presence or absence of various concentrations of GA (0.25, 0.5, 1 and 2 μmol/L). Early apoptotic cells (annexin V+/PI−) are shown in the lower right panel, while necrotic cells/late apoptosis (annexin V+/PI+) are shown in the upper right panel. The results are representative of three independent experiments. (A) Dual parameter flow cytometry analysis of rat aortic VSMCs 24 hrs after PDGF-BB stimulation in the presence of GA. (B) Dual parameter flow cytometry analysis of rat aortic VSMCs 24 hrs after EGF stimulation in the presence of GA.

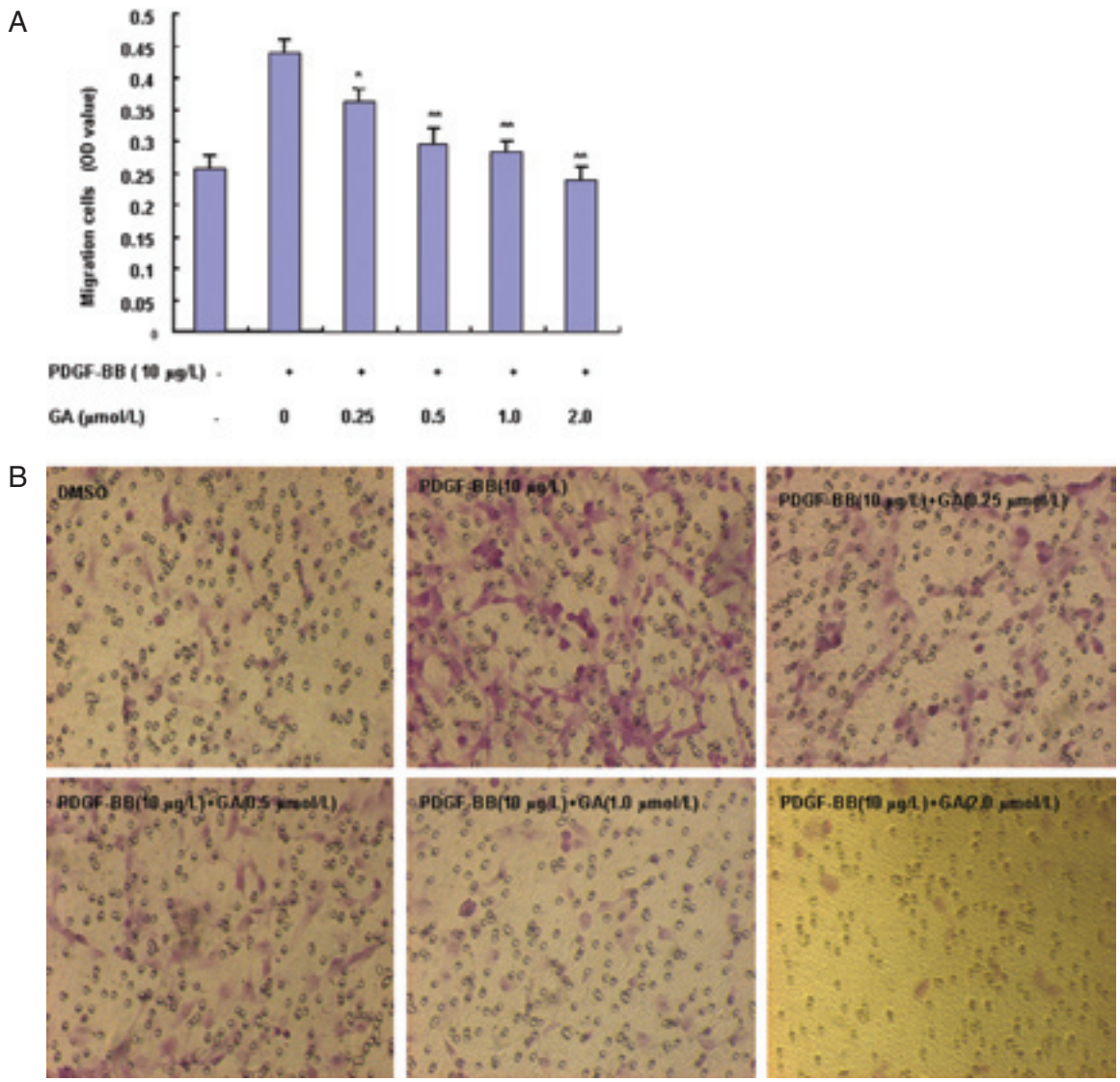

Fig. 4. Effects of GA on VSMC migration *in vitro*. (A) The chamber migration assay showed that GA inhibited VSMC migration treated by PDGF-BB (10 μg/L) in a concentration-dependent manner. The absorption values of crystal violet are presented as the mean ± S.D. of 3 separate experiments. $^{*}p<0.05$, and $^{**}p<0.01$ vs PDGF-BB stimulation group, respectively. (B) Photographs show cells stained by crystal violet, which had migrated through the polycarbonate membrane to the opposite surface. The inhibitory effect of GA on cell migration stimulated by PDGF-BB (10 μg/L) was in a concentration-dependent manner.

also inhibited EGFR phosphorylation and tyrosine phosphorylation in a concentration-dependent manner.

Effects of GA on PLC-γ1, ERK1/2, PI3K/Akt and JNK Phosphorylation

The downstream intracelluar signaling of PDGFR-β has been relatively well characterized[25-27]. These signaling molecules, including ERK1/2, PLC-γ1, AKT, and JNK are involved in the PDGFRβ pathway. After quiescent VSMCs had been treated with GA for an additional 24 hrs, the cell lysis was harvested at 5 minutes (ERK1/2, PLC-γ1)[28] and 15 minutes (AKT, JNK)[22, 29] after stimulation with PDGF-BB (50 μg/L). Western blotting results are shown in **Fig. 6** and **7**, and the increased phosphorylation of ERK1/2, PLC-γ1, Akt and JNK stimulated with PDGF-BB was significantly inhibited by pretreatment with GA in a concentration-dependent manner.

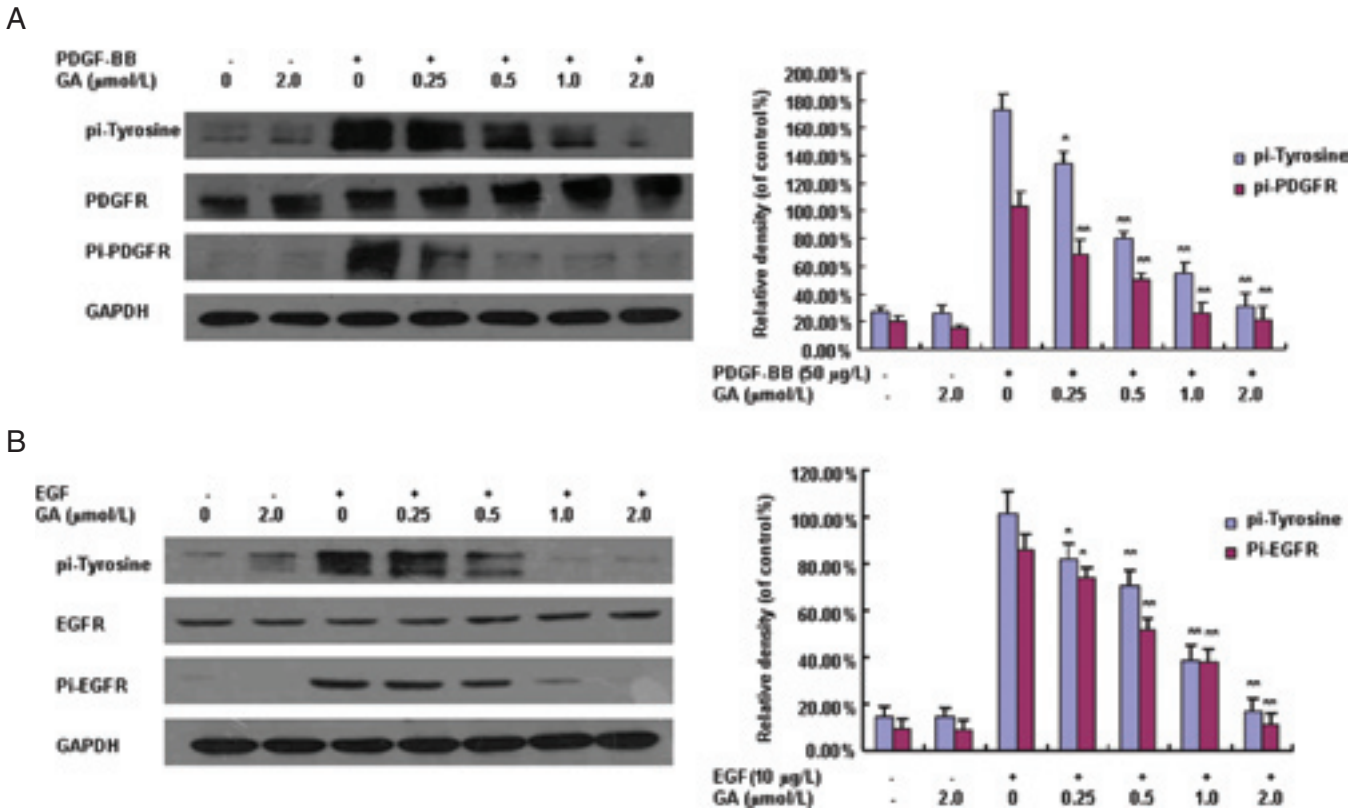

Fig. 5. Effects of GA on pi-PDGFR-β, pi-EGFR and pi-tyrosine level induced by PDGF-BB or EGF. Confluent cells were pre-cultured in the presence or absence of GA (0.25−2 μmol/L) in serum-free medium for 24 hrs, and then stimulated by 50 μg/L PDGF-BB for 1 minute or 10 μg/L EGF for 5 minutes. The cells were lysed, and the proteins were then analyzed using 9.0% SDS-PAGE and immunoblotting. Relative activities were quantified by scanning densitometry and shown as relative value of the GAPDH. Data are expressed as the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ vs PDGF-BB stimulation group or EGF stimulation group. (A) Effect of GA on pi-PDGFR-β and pi-tyrosine level induced by PDGF-BB. (B) Effect of GA on pi-EGFR and pi-tyrosine level induced by EGF.

Effects of GA on Cyclin D1, Cyclin E, CDK4 and CDK2 Expression

As shown in **Fig. 8**, there was an increase in the expression of Cyclin D1, Cyclin E, CDK4 and CDK2 after stimulation with 50 μg/L PDGF-BB for 24 hrs. While being pretreated with GA (0.25−2.0 μmol/L) for 24 hrs, the expressions of Cyclin D1, Cyclin E, CDK4 and CDK2 were significantly inhibited in a concentration-dependent manner.

GA Binds PDGF-BB Assay

As shown in **Fig. 9**, recombinant rat PDGF-BB, EGF and GA were immobilized on the membrane. After incubation with or without PDGF-BB or EGF, the membrane was incubated with antibody directed against PDGF-BB or EGF and then developed. It was shown that immobilized PDGF-BB and EGF could be recognized by the anti-PDGF-BB antibody and anti-EGF antibody. A negative binding signal was detected on the GA and BSA spot incubated with PDGF-BB and EGF.

Effects of GA on Rac1 Activation

Rac-1 activity was measured with GST pull-down assays. As shown in **Fig. 10**, there was transient activation of Rac-1 after stimulation with 50 μg/L PDGF-BB for 5 minutes[22], while pretreatment with GA (0.25 μmol/L−2.0 μmol/L) resulted in the concentration-dependent inhibition of Rac-1 activity.

Discussion

It has been well established that abnormal proliferation of VSMCs and migration of VSMCs from the media to the intima play a central role in the development of atherosclerosis and restenosis after angioplasty[30]. PDGF is known to be an important cytokine which stimulates cell growth and migration. Much

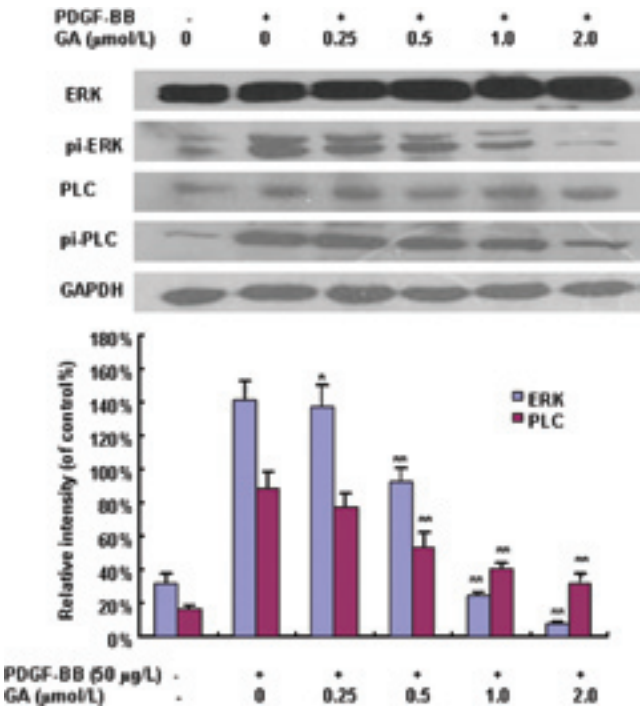

Fig. 6. Effects of GA on pi-ERK and pi-PLC levels induced by PDGF-BB. VSMCs were treated with GA (0.25–2.0 μmol/L) in FBS-free medium for 24 hrs, and then stimulated by 50 μg/L PDGF-BB for 5 minutes. The cells were lysed, and the proteins were then analyzed using 10% SDS-PAGE and immunoblotting. Relative activities were quantified by scanning densitometry and shown as the relative value of the GAPDH. Data are expressed as the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ vs PDGF-BB stimulation group.

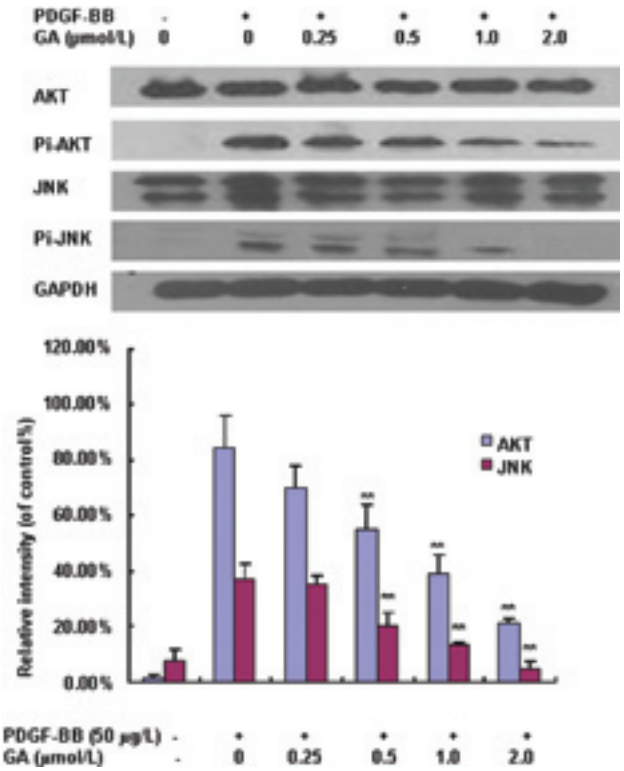

Fig. 7. Effects of GA on pi-AKT and pi-JNK levels induced by PDGF-BB. VSMCs were treated with GA (0.25–2.0 μmol/L) in FBS-free medium for 24 hrs, and then stimulated with 50 μg/L PDGF-BB for 15 minutes. The cells were lysed, and the proteins were then analyzed using 10% SDS-PAGE and immunoblotting. Relative activities were quantified by scanning densitometry and shown as the relative value of the GAPDH. Data are expressed as the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ vs PDGF-BB stimulation group.

evidence indicates that PDGF-BB and its receptor PDGFR-β-mediated signals are particularly important for vascular remodeling and neointima formation after vascular injury. Moreover, inhibition of PDGF signaling, which potentially prevents atherogenesis, has been confirmed in various models[5, 31, 32]. Therefore, inhibition of PDGF-induced VSMC proliferation and migration represents a key pharmacologic strategy during atherogenesis development.

In the present study, our data confirmed that the proliferation and DNA synthesis of rat VSMCs induced by PDGF-BB were suppressed by pretreatment with GA in a concentration-dependent manner. Previous studies have shown that GA can induce apoptosis in some tumor cells[13, 33, 34]. Our results suggested that the antiproliferative effect of GA was not due to apoptosis or necrosis (**Fig. 3**). Our further investigation indicated that the antiproliferative effects of GA were associated with the increased accumulation of cells in the G0/G1 phase of the cell cycle (**Fig. 2**); however, our findings also showed that GA inhibited proliferation, DNA synthesis and cell progression induced by EGF in a concentration-dependent manner, implying that the inhibition effects of GA were not specific to PDGF-BB stimulation.

Cell cycle regulatory molecules, such as CDKs and cyclins, are related to the G1 phase of the cell cycle[35-37]. In the present study we found that VSMC cell cycle arrest in G0/G1 phase was induced by GA pretreatment; therefore, the effects of GA on expressions of CDK2, CDK4, cyclin E and D1 were further examined. Our data showed that increasing expressions of CDK2, cyclin E, CDK4 and cyclin D1 were inhibited by pretreatment with GA in a concentration-dependent manner, indicating that the effects of GA on cell cycle arrest might be due to the downregulation of CDK/cyclin complex expression.

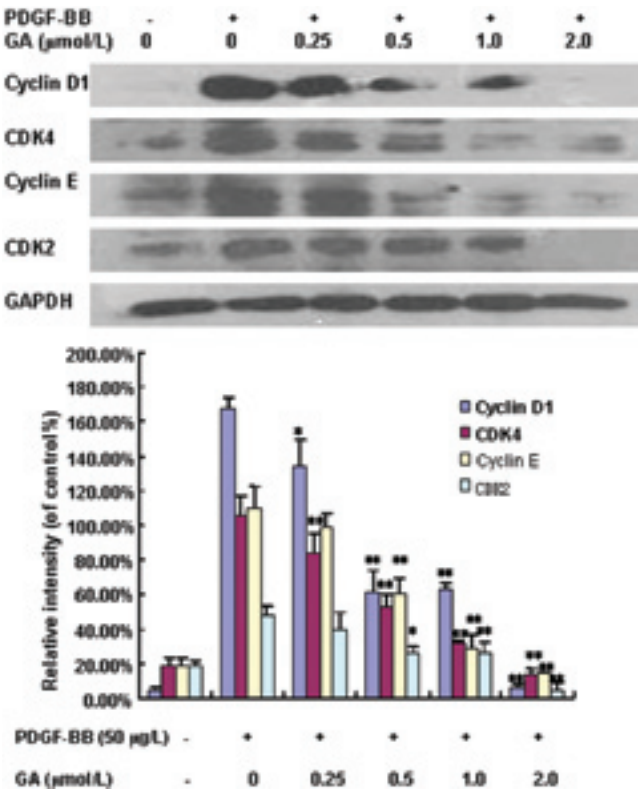

Fig. 8. Effects of GA on cyclin D1, CDK4, cyclin E and CDK2 expression induced by PDGF-BB. Quiescent VSMCs were stimulated with 50 μg/L PDGF-BB either in the absence or presence of GA (0.25–2.0 μmol/L) for 24 hrs. The cells were lysed, and the proteins were then analyzed using 10% SDS-PAGE and immunoblotting. Relative activities were quantified by scanning densitometry and shown as the relative value of the GAPDH. Data are expressed as the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ vs PDGF-BB stimulation group.

Migration is another important factor in vascular remodeling and neointima formation after vascular injury. Our data showed that GA markedly suppressed rat VSMC migration induced by PDGF-BB in a concentration-dependent manner.

Rac-1 (21 kDa) is a member of the Rho family of small GTPases. Rho proteins are very important regulators of the actin cytoskeleton, serve as transducers between mechanical forces, cell morphology, and gene regulation, and thus regulate cellular motility. Ryu and coworkers demonstrated that the suppression of rac-1 activation resulted in the significant negative regulation of cell migration[38]. It has been well established that rac1 is dependent on the activation of PDGF-Rβ and is marked at low PDGF concentrations[19, 39]. Our findings showed an increase in Rac-1

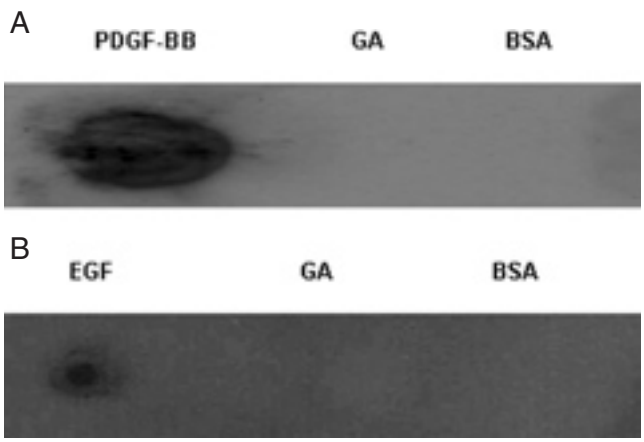

Fig. 9. GA directly binds with PDGF-BB and EGF. (A) Rat PDGF-BB (100 ng) and GA (GA, 10 μg) were applied to the NC membrane. The membrane was incubated with PDGF-BB, followed by incubation with anti-PDGF-BB antibody, and developed. (B) Rat EGF (100 ng) and GA (GA, 10 μg) were applied to the NC membrane. The membrane was incubated with EGF, followed by incubation with anti-EGF antibody, and developed.

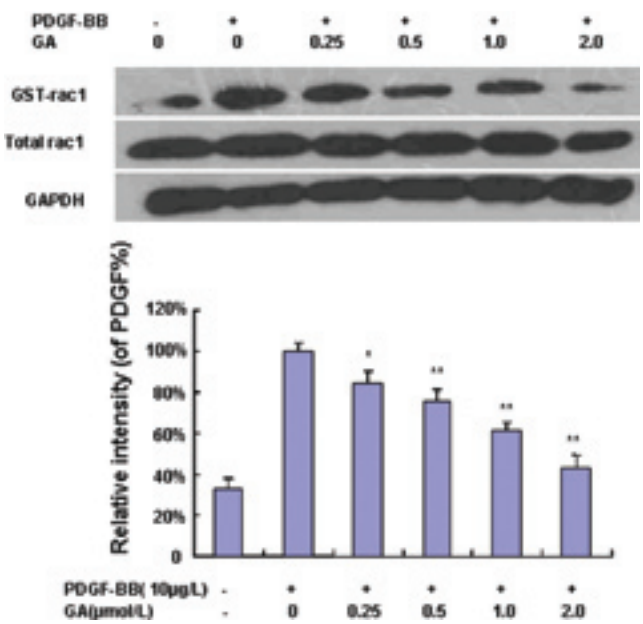

Fig. 10. Effects of GA on PDGF-dependent rac1 activation. VSMCs were treated with GA (0.25–2.0 μmol/L) in FBS-free medium for 24 hrs, stimulated with 10 μg/L PDGF-BB for 5 minutes, followed by glutathione S-transferase (GST) pull-down assays. Relative activities were quantified by scanning densitometry and shown as the relative value of the PDGF-BB stimulation group. Data are expressed as the mean ± S.E.M. (n=3). $^{*}p<0.05$, $^{**}p<0.01$ vs PDGF-BB stimulation group.

activity after PDGF-BB (10 μg/L) stimulation for 5 minutes, while pretreatment with GA (0.25–2.0 μmol/L) resulted in concentration-dependent inhibition, which indicated that the inhibition effect of GA on rat VSMCs migration was related to Rac-1 activity inhibition.

Rac-1 activity is crucial for PDGF-dependent migration of VSMCs and the tyrosine phosphorylation of PDGF-Rβ is part of the early signaling cascade of rat aortic VSMC proliferation[7, 37, 40]. GA actually inhibited Rac-1 activity and tyrosine phosphorylation of PDGF-Rβ stimulated by PDGF-BB in the same concentration range that inhibited rat aortic VSMC proliferation, DNA synthesis, cell cycle progress and migration (**Fig. 5**). It was suggested that PDGF-Rβ was the upstream block point for GA. Interestingly, our findings also showed that GA inhibited EGFR phosphorylation in a concentration-dependent manner. Because the activation of PDGFR and EGFR depends on tyrosine phosphorylation, the level of tyrosine phosphrylation was also examined. The data showed that tyrosine phosphorylation stimulated by PDGF-BB and EGF was inhibited significantly in the same manner as PDGFR and EGFR phosphorylation, which indicated that GA inhibition effects were due to the inhibition of tyrosine phosphorylation. Dot-binding assay also showed that GA did not bind with PDGF-BB or EGF.

To understand that inhibition of PDGF-BB-induced phosphorylation of PDGF-Rβ by GA also resulted in the inhibition of the downstream intracellular signaling transduction pathway, the levels of p-ERK, p-PLC, p-AKT and p-JNK in GA-treated VSMCs were also examined. As shown in **Fig. 6**, **7** and **8**, pretreatment of VSMCs with GA for 24 hrs resulted in a marked decrease of p-ERK and p-PLC (5 minutes after stimulation with PDGF-BB), and p-AKT and pi-JNK (15 minutes after stimulation with PDGF-BB) in a concentration-dependent manner.

Taken together, these observations show that inhibition effects on the proliferation and migration of GA are mediated by PLC-γ1, MEK-ERK1/2, PI3/AKT, SAPK/JNK and Rac-1 activation through the inhibition of PDGF-Rβ tyrosine kinase in VSMCs. Furthermore, GA arrests cell cycle progression in the G0/G1 phase by downregulating the expression of cyclin D1, cyclin E, CDK4 and CDK2 proteins. Our study suggests that GA could be used as a preventive agent for the progression of vascular complications, such as restenosis after angioplasty and atherosclerosis.

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