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# EFFECT OF NOTOGINSENOSIDE R1 ON THE SYNTHESIS OF COMPONENTS OF THE FIBRINOLYTIC SYSTEM IN CULTURED SMOOTH MUSCLE CELLS OF HUMAN PULMONARY ARTERY

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**Abstract** - We have previously reported that notoginsenoside R1 (NG-R1) increases the synthesis of tissue-type plasminogen activator (t-PA) and decreases plasminogen activator inhibitor-1 (PAI-1) activity in cultured human endothelial cells from different vascular sources. It was the aim of this study to investigate whether the effect of NG-R1 on the synthesis of components of the fibrinolytic system is also operative in another cell type of the blood vessel wall, the smooth muscle cell. Therefore cultured human pulmonary artery smooth muscle cells (HPASMCs) were treated with NG-R1. When the HPASMCs (passage 4 or 5) were conditioned with NG-R1, a dose (0.01-100 μg NG-R1/ml for 24 hrs.) dependent increase in t-PA and u-PA synthesis was observed, which was significant from 1 μg NG-R1/ml on. t-PA antigen increased from 2.4 ± 0.1 to 4.7 ± 0.5 ng/$10^5$ cells/24 hrs.; u-PA antigen increased from 1.8 ± 0.1 to 3.0 ± 0.4 ng/$10^5$ cells/24 hrs. In contrast no change in PAI-1 antigen synthesis was seen in the conditioned media from NG-R1 treated HPASMCs. On Northern blot analysis of mRNA obtained from NG-R1-stimulated and control HPASMCs NG-R1 induced a significant increases in mRNA levels of t-PA and u-PA (180% and 200% of control value, respectively) at 100 μg NG-R1/ml while PAI-1 mRNA decreased slightly. In conclusion our data give evidence that NG-R1 can increase the fibrinolytic potential in cultured HPASMCs *in vitro* by increasing the production of t-PA and u-PA. If operative *in vivo* this effect of NG-R1 on the fibrinolytic system of SMCs might also contribute to the effect of the Chinese herb drug *Panax notoginseng* in the treatment of cardiovascular diseases.

**Key words**: Notoginsenoside R1, human smooth muscle cells, fibrinolytic system, t-PA, u-PA and PAI-1

## INTRODUCTION

The blood vessel wall plays a critical role in regulating the fibrinolytic system. The contribution of vascular smooth muscle cells, the predominant cell type of the blood vessel wall, to the management of the fibrinolytic system has received more attention in recent years. Several studies have demonstrated that vascular smooth muscle cells produce both t-PA and u-PA as well as PAI-1 and PAI-2 (Bell and Madri, 1990; Booyse *et al.*, 1981; Clowes *et al.*, 1990; Laug *et al.*, 1989; Levin and Loskutoff, 1979). Changes in the fibrinolytic capacity of smooth muscle cells might have far reaching implications in processes like cell migration and growth, development of arteriosclerotic lesions, and the shift from an antithrombotic to a prothrombotic environment in case of vascular injury. Several studies have provided insight in the regulation of the components of the fibrinolytic system in vascular smooth muscle cells. The production of components of the fibrinolytic system by vascular smooth muscle cells seems to be highly regulated with tumor necrosis factor-α (TNF-α), interleukin-α

(IL-1α), thrombin, transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF) increasing the production of PAI-1 in these cells (Gallicchio *et al.*, 1995; Noda-Heiny *et al.*, 1993; Reilly and McFall, 1991; Wojta *et al.*, 1993). On the other hand heparin has been shown to inhibit the transcription of t-PA in smooth muscle cells (Au *et al.*, 1992). Using a rat model, increased u-PA expression has been observed in smooth muscle cells during mitogenesis, whereas t-PA expression was increased during migration (Clowes *et al.*, 1990). An increase of t-PA and u-PA induced by PDGF and bFGF was reported recently in the injured rat carotid artery *in vivo* (Jackson and Reidy, 1993; Jackson *et al.*, 1993).

We have previously reported that the saponin notoginsenoside R-1 (NG-R1) purified from the Chinese herb drug *Panax notoginseng* can modulate the fibrinolytic capacity of endothelial cells by stimulating the induction of t-PA and u-PA in cultured human endothelial cells from different vascular beds (Zhang *et al.*, 1994, 1995). It was the aim of this study to investigate whether the effect of NG-R1 on the synthesis of components of the fibrinolytic system is also operative in vascular smooth muscle cells.

## MATERIALS AND METHODS

*Materials*

Chemically pure notoginsenoside R1 was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). NG-R1 was dissolved and diluted in incubation medium to yield final concentrations of 0.01-100 μg/ml. Morpholinopropane sulfonic acid (Serva, Germany), guanidine thiocyanate (Fluka, Switzerland), piperazine-*N*,*N*'-bis(2-ethane sulfonic acid) (PIPES; Sigma, USA), Seakem LE agarose (FMC Bioproducts, USA), alpha-$^{32}$P-dCTP (ICN Radiochemicals, USA), were obtained from the sources indicated. Other materials used in the methods described below have been specified in detail in the respective references.

*Cell Culture*

Human pulmonary artery smooth muscle cells (HPASMCs) were isolated from pieces of normal human pulmonary artery obtained after surgery using the explant technique (Chamley-Campbell *et al.*, 1979). Briefly, the respective tissue specimen was cut into 10 to 20 pieces of 1-2 mm diameter, these pieces were placed in a Petri dish (100 mm; Costar, USA) coated with 1% calf skin gelatine in PBS and covered with a drop of Medium 199 containing 20% supplemented calf serum (SCS; Hyclone, USA), 100 μg/ml streptomycin, 100 IU/ml penicillin, 250 ng/ml amphotericin B, 1 mmol/l glutamine. After 3 to 5 days the explants became adherent and the Petri dish was filled with Medium 199 containing supplements as described above. Smooth muscle cells growing out from the explants were grown to confluence and subcultured using a split ratio of 1:3. Cells were confirmed to be vascular smooth muscle cells by their typical "hill and valley" morphology and by positive immunofluorescence staining with a monoclonal antibody against alpha-smooth muscle cell-actin (Boehringer-Mannheim, Germany). 95% to 98% of the cells tested showed positive staining for alpha-smooth muscle cell-actin (Skalli *et al.*, 1986). Subconfluent cells were allowed to grow to confluence under the same conditions and were harvested-during exponential cell growth-with trypsin-EDTA and frozen in 1 ml aliquots of Medium 199 containing 20% SCS and 10% dimethylsulfoxide (DMSO) in liquid nitrogen. For experiments, vials were thawed at 37°C and cells were grown in six-well plates (9.4 cm$^2$; Costar) in medium M199 containing 20% SCS until confluence was reached. Average cell densities at confluence were 2 x $10^5$ cells/well. All cells used in this study were in passage 4 or 5. The cells were always fed the day before the experiment with fresh medium.

*Preparation of conditioned Media*

Confluent cultures were rinsed twice with Hank's balanced salt solution (HBSS; Sigma) and incubated at 37°C in 1 ml/well medium M199 containing 1.25% SCS and the indicated concentrations of NG-R1. After incubation, the culture supernatant was collected following removal of cell debris by centrifugation and stored at -70°C until used. The total cell number of the respective cultures after trypsinisation was counted with a hemocytometer. The respective experiments were repeated three times; each of the experiments was performed in triplicate.

*Assays for t-PA, u-PA and PAI-1 Antigens in conditioned Media*

t-PA, u-PA and PAI-1 antigen concentrations were determined by specific commercially available enzyme-linked immunosorbent assays (ELISAs) (Technoclone, Austria) according to the manufacturer's instruction. The test ranges for these assays are 0.3-2.5 ng/ml for t-PA, 0.6-10 ng/ml for u-PA and 1.0-30 ng/ml for PAI-1. The t-PA-ELISA detects free t-PA and t-PA in complex with PAI-1. The u-PA-ELISA detects single chain u-PA (scu-PA) and two chain u-PA (tcu-PA), free u-PA and u-PA in complex with PAI-1. The PAI-1 ELISA measures free, complexed and latent PAI-1.

*Quantification of t-PA, u-PA and PAI-1 mRNA Levels by Northern blot Analysis*

Total cellular RNA was isolated from cultured HPASMCs by acid guanidinium thiocyanate/phenol/chloroform extraction as described by Chomczynski and Sacchi (1987). The

final RNA pellet was resuspended in 10-20 µl of 0.5% SDS and the concentration determined at 260 nm. For Northern blot analysis, RNA samples were electrophoresed in a 1.2% agarose gel followed by capillary transfer of the fractionated RNA to a Duralon-UV membrane (Stratagene, USA). RNA blots were placed in Seal-a-Meal bags and prehybridized in 50 mmol/l PIPES, 100 mmol/Ll NaCl, 50 mmol/l sodium phosphate, 1 mmol/L EDTA, containing 5% sodium dodecyl sulphate (SDS) for at least 3 hrs. at 57°C. The prehybridization buffer was then discarded and replaced with fresh prehybridization buffer containing $10^6$ cpm/ml of the $^{32}$P-labelled cDNA probes for human t-PA, human u-PA, human PAI-1 and rat glyceraldehyde-3-phosphate dehydrogenase (GAPD), respectively. Hybridization was carried out in a water bath overnight at 57°C. After hybridization, blots were removed from the bag and rinsed for 10 min. in 100 ml of 5% SDS, 0.2 x standard saline citrate (SSC, 1 x SSC = 0.015 mol/l sodium citrate, 0.15 mol/l NaCl, pH 7.0) at room temperature. Thereafter the blots were washed for 20 min in 400 ml of 5% SDS, 0.2 x SSC at the hybridization temperature. Then the RNA blots were air dried and exposed to XAR-5 X-ray films (Eastman Kodak, USA) at -70°C. In order to quantitate differences in specific mRNA expression, the developed films were scanned with a densitometer (Hirschmann Elscript 400; Hirschmann, Germany). Scanning data for each specific mRNA message were compared with the intensity of the GAPD message.

*cDNA Probes*

The following cDNA fragments were used as probes in the hybridization experiments: a 1.5 kb *SmaI*/*Hin*dIII fragment of human t-PA cDNA (kindly provided by Dr. Pannekoek, University of Amsterdam, The Netherlands), a 1.4 kb *PstI*/*Pst*I fragment of human u-PA cDNA (ACTT, USA), a 1.4 kb *EcoRI*/*Bgl*II fragment of a human PAI-1 cDNA of the 3.2 kb transcript (PCR amplified coding sequence from MJZJ cDNA) and a 1.2 kb *Pst*I fragment of a rat GAPD cDNA (kindly provided by Dr. Busslinger, University of Vienna), which was used as an internal-standard probe. The cDNA fragments were radiolabelled by random-priming using a Random Prime DNA Labelling Kit (Boehringer Mannheim, Germany).

*Statistical Analysis*

The results are reported as means ± standard deviation. Ordinary ANOVA and a Student's unpaired *t*-test was used to determine significance levels.

## RESULTS

As shown in figs. 1A and 1B, treatment of HPASMCs with increasing doses of NG-R1 for 24 hrs. resulted in a dose-dependent increase of t-PA (1A) and u-PA (1B) antigen in conditioned media of such treated cells: Maximum effects were achieved with 100 µg/ml NG-R1 (for t-PA antigen: 4.7 ± 0.5 versus 2.4 ± 0.1 ng/$10^5$ cells/24 hrs. in control; n=9, $p<0.001$; for u-PA antigen: 3.0 ± 0.4 versus 1.8 ± 0.1 ng/$10^5$ cells/24 hrs. in control; n=9, $p<0.001$). At the same concentration of NG-R1 PAI-1 antigen in the conditioned media of HPASMCs did not change significantly when compared with control (2.92 ± 0.32 versus 2.78 ± 0.45 µg/$10^5$ cells/24 hrs. in control; n=9) (Fig. 1C). Also it can be seen in fig. 2A, that t-PA antigen increased time dependently in the conditioned media of HPASMCs treated for 6, 12 or 24 hrs. with 100 µg/ml NG-R1 compared with controls, whereas PAI-1 antigen did not change significantly in such treated cells (Fig. 2B).

As shown in fig. 3, the stimulating effect of NG-R1 on t-PA and u-PA secretion in HPASMCs was also reflected on the level of specific mRNA expression. t-PA and u-PA specific message increased as much as 1.8-fold and two-fold, respectively in HPASMCs treated with 100 µg/ml NG-R1 for 12 hrs., whereas PAI-1 specific mRNA expression was slightly downregulated by NG-R1.

## DISCUSSION

The fibrinolytic and proteolytic capacity of smooth muscle cells seems to be highly regulated by a variety of stimuli (Au *et al.*, 1992; Gallicchio *et al.*, 1995; Jackson and Reidy, 1993; Jackson *et al.*, 1993; Noda-Heiny *et al.*, 1993; Reilly and McFall, 1991; Wojta *et al.*, 1993). Changes in this capacity might have far reaching implications in processes like cell migration and growth, development of arteriosclerotic lesions, and the shift from a profibrinolytic/antithrombotic to an antifibrinolytic/prothrombotic environment in case of vascular injury. A variety of factors which would accumulate at the site of vascular injury such as thrombin, the cytokines IL-1α or TNF-α and the growth factors PDGF and TGF-β have been shown to increase the expression of PAI-1 in smooth muscle cells (Gallicchio *et al*, 1995;

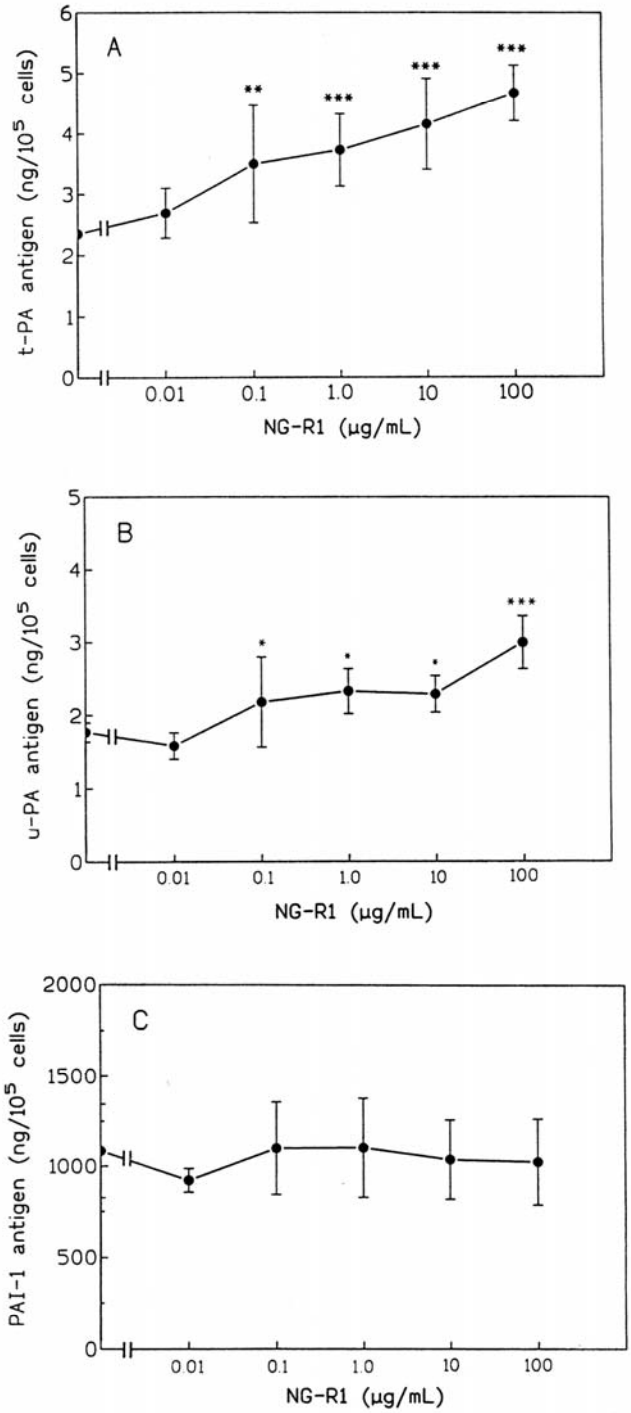


**Fig. 1** *Effect of notoginsenoside R1* (NG-R1) *on tissue-type plasminogen activator* (t-PA) *antigen* (**A**), *urokinase-type plasminogen activator* (u-PA) *antigen* (**B**), *and plasminogen activator inhibitor-1* (PAI-1) *antigen* (**C**) *secretion in cultured human pulmonary artery smooth muscle cells* (HPASMCs). HPASMCs were incubated for 24 hrs. with different concentrations of NG-R1. Conditioned media were harvested and analyzed for t-PA antigen, u-PA antigen and PAI-1 antigen, respectively, as described under Materials and Methods. Results are the mean values ± S.D. of three experiments, each performed in triplicate. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with control.

Noda-Heiny *et al.*, 1993; Reilly and McFall, 1991; Wojta *et al.*, 1993). Such a local increase in PAI-1 could contribute to the above mentioned shift from a profibrinolytic/antithrombotic to an antifibrinolytic / prothrombotic environment which would favour thrombus formation and stability.

Here we provide evidence that, in addition to its effects on endothelial cells (Zhang *et al.*, 1994, 1995), notoginsenoside R-1 (NG-R1) can also modulate the fibrinolytic system of human vascular smooth muscle cells by inducing both t-PA and u-PA on the level of protein as well as on the level

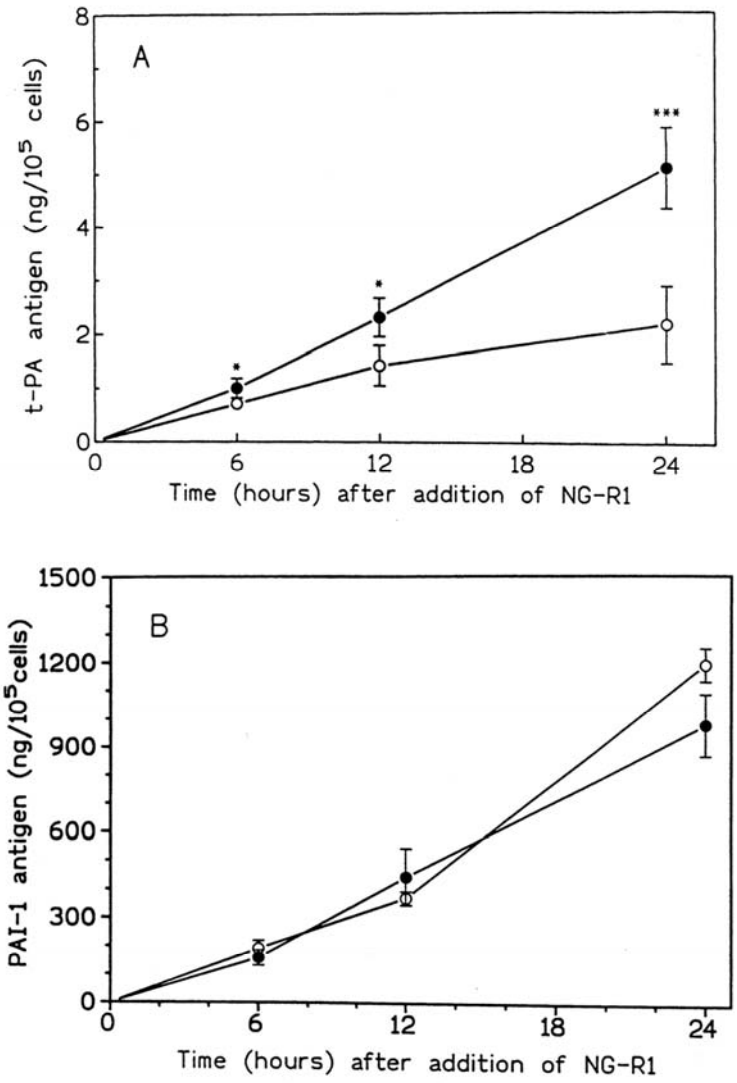


**Fig. 2** *Time course of tissue-type plasminogen activator* (t-PA) *antigen* (**A**) *and plasminogen activator inhibitor-1* (PAI-1) *antigen* (**B**) *production after exposure of cultured human pulmonary artery smooth muscle cells* (HPASMCs) *to notoginsenoside R1* (NG-R1). HPASMCs were incubated for the indicated time periods in the absence (open circles) or presence of 100 µg/ml NG-R1 (full circles). At the specified times, conditioned media were harvested and assayed for t-PA antigen and PAI-1 antigen, respectively, as outlined under Materials and Methods. Results are the mean values ± S.D. of three experiments, each performed in triplicate. *$p<0.05$; ***$p<0.001$ compared with control.

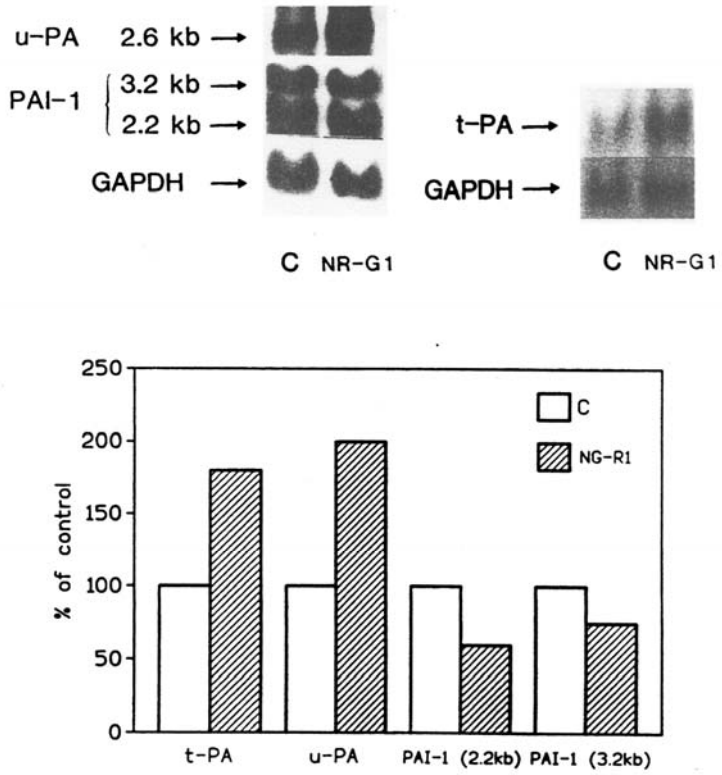


**Fig. 3** *Effects of notoginsenoside R1* (NG-R1) *on tissue-type plasminogen activator* (t-PA), *urokinase-type plasminogen activator* (u-PA) *and plasminogen activator inhibitor-1* (PAI-1) *mRNA levels in cultured human pulmonary artery smooth muscle cells* (HPASMCs). Confluent HPASMCs were incubated for 12 hrs. in the absence (C) or presence (T) of NG-R1 (100 μg/ml). Northern blot analysis of RNA extracts from untreated and NG-R1-treated HPASMCs was performed using $^{32}$P-labelled cDNA probes for t-PA, u-PA, PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPD) mRNA, respectively. Intensity of the bands present on the autoradiogram was assessed by densitometry and specific mRNA for t-PA, u-PA and PAI-1 was normalized against GAPD mRNA to account for differences in loading. Signal intensity was compared as a ratio of signal obtained from NG-R1-treated HPASMCs (hatched bars) to that from untreated control cells (open bars). Data represent results of two separate experiments that gave similar results.

of specific mRNA expression. Such an upregulation of u-PA and t-PA expression would lead to an increase in the fibrinolytic capacity of these cells. One could speculate that this increase in fibrinolytic capacity induced by NG-R1 could serve as a defence mechanism preventing extensive thrombus formation in case of vascular injury. Such an effect of NG-R1 might be also operative *in vivo* and contribute to the effect of the Chinese herb drug *Panax notoginseng* in the treatment of vascular disease. It is however difficult to assess how our results compare to the *in vivo* situation since in most clinical studies *Panax notoginseng* is only one component of a mixture of medical herbs and no data are available on the exact amount of NG-R1 in this mixture (Li *et al.*, 1990; Yu, 1993). Thus *in vitro* studies are needed to evaluate the potential of NG-R1 in the treatment of pathological states described above.

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## REFERENCES


Au, Y.P.T., Kenagy, R.D. and Clowes, A.W., Heparin selectively inhibits the transcription of tissue-type plasminogen activator in primate arterial smooth muscle cells during mitogenesis. *J. biol. Chem.* 1992, **267**: 3438-3444.

Bell, L. and Madri, J.A., Influence of the angiotensin system on endothelial and smooth muscle cell migration. *Am. J. Pathol.* 1990, **137**: 7-12.

Booyse, F.M., Scheinbuks, J., Radek, J., Osikowicz, G., Feder, S. and Quarfoot, A.J., Immunological identification and comparison of plasminogen activator forms in cultured normal human endothelial cells and smooth muscle cells. *Thromb. Res.* 1981, **24**: 495-504.

Chamley-Campbell, J., Campbell, G.R. and Ross, R., The smooth muscle cell in culture. *Physiol. Rev.* 1979, **59**: 1-61.

Chomczynski, P. and Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 1987, **162**: 156-159.

Clowes, A.W., Clowes, M.M., Au, Y.P.T., Reidy, M.A. and Belin, D., Smooth muscle cells express urokinase during mitogenesis and tissue-type plasminogen activator during migration in injured rat carotid artery. *Circ. Res.* 1990, **67**: 61-67.

Gallicchio, M., Wojta, J., Hamilton, J.A. and McGrath, K., Regulation of plasminogen activator inhibitor type 1 in cultured smooth muscle cells by interleukin 1α and tumor necrosis factor-α. *Fibrinolysis* 1995, **9**: 145-151.

Jackson, C.L., Raines, E.W., Ross, R. and Reidy, M.A., Role of endogenous platelet derived growth factor in arterial smooth muscle cell migration after balloon catheter injury. *Arterioscler. Thromb.* 1993, **13**: 1218-1226.

Jackson, C.L. and Reidy, M.A., Basic fibroblast growth factor: Its role in the control of smooth muscle cell migration. *Am. J. Pathol.* 1993, **143**: 1024-1031.

Laug, W.E., Aebersold, R., Jong, A., Rideout, W., Bergman, B.L. and Baker, J., Isolation of multiple types of plasminogen activator inhibitors from vascular smooth muscle cells. *Thromb. Haemost.* 1989, **61**: 517-521.

Levin, E.G. and Loskutoff, D.J., Comparative studies of the fibrinolytic activity of cultured vascular cells. *Thromb. Res.* 1979, **15**: 869-878.

Li, X., Chen, J.X. and Sun, J.J., Protective effects of Panax notoginseng saponins on experimental myocardial injury induced by ischaemia and reperfusion in rat. *Chung-Kuo-Yao-Li-Hsueh-Pao.* 1990, **11**: 26-29.

Noda-Heiny, H., Fujii, S. and Sobel, B.E., Induction of vascular smooth muscle cell expression of plasminogen activator inhibitor-1 by thrombin. *Circ. Res.* 1993, **72**: 36-43.

Reilly, C.F. and McFall, R.C., Platelet-derived growth factor and transforming growth factor-β regulate plasminogen activator inhibitor-1 synthesis in vascular smooth muscle cells. *J. biol. Chem.* 1991, **266**: 9419-9427.

Skalli, O., Ropraz, P., Trzeciak, A., Benzonana, G., Gillessen, D. and Gabbiani, G., A monoclonal antibody against α-smooth muscle actin: A new probe for smooth muscle differentiation. *J. Cell Biol.* 1986, **103**: 2787-2796.

Wojta, J., Gallicchio, M., Zoellner, H., Hufnagl, P., Last, K., Filonzi, E.L., Binder, B.R., Hamilton, J.A. and McGrath, K., Thrombin stimulates expression of tissue-type plasminogen activator and plasminogen activator inhibitor type 1 in cultured human vascular smooth muscle cells. *Thromb. Haemost.* 1993, **70**: 469-474.

Yu, X.J., A prospective study on reversion of 200 precancerous patients with hua-sheng-ping. *Chung-Kuo-Chung-Hsi-I-Chieh-Ho-Tsa-Chih.* 1993, **13**: 147-149.

Zhang, W.J., Fabry, A., Wojta. J. and Binder, B.R., Effect of notoginsenoside R1 on the synthesis of components of the fibrinolytic system in cultured human pulmonary artery endothelial cells and human skin microvascular endothelial cells. *Fibrinolysis* 1995, **9**(Suppl. 1): 133-139.

Zhang, W.J., Wojta, J. and Binder, B.R., Effect of Notoginsenoside R1 on the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor-1 in cultured human umbilical vein endothelial cells. *Arterioscler. Thromb.* 1994, **14**: 1040-1046.