Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmacology 517 (2005) 1 – 10
www.elsevier.com/locate/ejphar

YC-1-inhibited proliferation of rat mesangial cells through suppression of cyclin D1—Independent of cGMP pathway and partially reversed by p38 MAPK inhibitor

Wen-Chih Chiang[a], Che-Ming Teng[b], Shuei-Liong Lin[a], Yung-Ming Chen[a], Tun-Jun Tsai[a,*], Bor-Shen Hsieh[a]

[a]*Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, 10016, Taiwan*
[b]*Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan*

Received 8 November 2004; received in revised form 28 April 2005; accepted 29 April 2005
Available online 13 June 2005

Abstract

This study was designed to investigate the effect of 1-benzyl-3-(5′-hydroxymethyl-2′-furyl) indazole (YC-1), a guanylate cyclase activator, upon the proliferation of rat mesangial cells and its underlying mechanism. YC-1 inhibited cell proliferation and DNA synthesis in a dose- and time-dependent manner. Flow cytometry cell-cycle studies revealed that YC-1 prevented the entry of cells from G1 into S phase. The expression of cyclin D1 and the kinase activity of cyclin D1/cyclin-dependent kinase (CDK)4 were lower within YC-1-treated cells, revealed by Western blotting, Northern blotting and kinase assays. YC-1 did not increase the intracellular cGMP concentration in mesangial cells. Inhibitors of soluble guanylate cyclase, protein kinase G, or protein kinase A also did not reverse the inhibitory effect elicited by YC-1, while co-treatment with p38 mitogen-activated protein kinase (MAPK) inhibitor could partially reverse the suppressive effect. YC-1 inhibited proliferation of mesangial cells and induced cell-cycle arrest by the reduction of cyclin D1 synthesis and cyclin D1/CDK4 kinase activity. This effect acts partially through p38 MAPK signal transduction activation and is independent of cGMP-signaling pathways.

Keywords: YC-1; Mesangial cells; Cyclin D1; Proliferation; p38 mitogen-activated protein kinase

1. Introduction

For many examples of glomerular disease, mesangial cell proliferation is the important pathological change following the initiation, by the primary insult, of an inflammatory reaction and intraglomerular thrombosis (Pesce et al., 1991; Floege et al., 1992). The increased mesangial cell numbers that results could be responsible for the secretion of a greater quantity of various growth factors, inflammatory cytokines and extracellular matrix substance which will further damage the glomeruli. Such an outcome may result in a vicious circle incorporating the ultimate outcome of significant renal damage. The administration of certain reagents which elicit a growth-inhibition effect upon proliferating mesangial cells, such as a platelet-derived growth factor (PDGF) antagonist, could attenuate the proliferation of mesangial cells and the extent of glomerular damage for many cases of proliferative glomerulonephritis (Lehrke et al., 2002; Floege et al., 1999; Johnson et al., 1992).

1-benzyl-3-(5′-hydroxymethyl-2′-furyl) indazole (YC-1), a soluble guanylate cyclase activator, facilitates and increases the rate of nitric oxide-mediated cyclic GMP formation (Ko et al., 1994; Mulsch et al., 1997). Several earlier studies have demonstrated that YC-1 was able to inhibit the proliferation of certain cells including vascular smooth muscle cells in vivo (Wu et al., 2004; Tulis et al., 2002) and human umbilical vein endothelial cells in vitro (Hsu et al., 2003). In addition to its effect upon cell

* Corresponding author. Tel.: +886 2 23123456x3953; fax: +886 2 23222955.

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.04.046

proliferation, YC-1 also demonstrates an inhibitory effect upon platelet function and, therefore, also thrombus formation (Wu et al., 1995; Teng et al., 1997), a stimulatory effect upon the synthesis of endothelial nitric oxide within the endothelium (Wohlfart et al., 1999), and an inhibitory effect upon white blood cell function (Hwang et al., 2003) and the generation of superoxide anions by neutrophils (Wang et al., 2002). All these effects might be construed to be beneficial for cases of renal disease. To evaluate the possible application of YC-1 to cases of renal disease, we first examined the effect of YC-1 upon the proliferation of mesangial cells in vitro. The transition between different phases of the normal cell cycle depends upon changes to and behavior of many cell-cycle proteins. In this study, we also evaluated whether these changes occurred for YC-1 treated cells. The possible mediating signaling pathways involved, especially a cyclic GMP-mediated pathway, were also evaluated.

2. Materials and methods

2.1. Cell cultures

Mesangial cell cultures were established as has been described previously (Tsai et al., 1995). Following the first cell passage, mesangial cells were maintained in medium 199 (M199) with 10% fetal calf serum at 37 °C in a 5%-CO_2-in-air humidified incubator. Cells deriving from passages 8–15 were used for the following experiments. Vascular smooth cells were prepared and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum as previously prescribed (Chen et al., 1999). Cells from passages 8–15 were used for study. The cells were characterized as smooth muscle cells by morphology and immunostaining with monoclonal antibody specific for smooth muscle α-actin. In these experiments, cells were subcultured into tissue culture grade 10-cm dishes and/or six- or 96-well plates. Sub-confluent cells were cultured in serum-free M199 or DMEM for 24 h in order to achieve cell quiescence. Cells were stimulated with 10% fetal calf serum in the cell-proliferation experiments or 20 ng/ml of rat platelet-derived growth factor BB for all other experiments. Stimulated cells were treated simultaneously with stimulation with test agents or vehicle. For experiments that lasted longer than 48 h, the media, platelet-derived growth factor (PDGF) and/or test agents were changed completely in order to ensure an adequate culture environment for cells to proliferate. Pentobarbital was used as the anesthetic for all procedures conducted with test rats. The management of rats fulfilled the laws of the Republic of China and all animal management and experimental protocols were approved by the Committee of Experimental Animal Management, the College of Medicine, National Taiwan University. For all experiments, the final concentration at which dimethyl sulfoxide (DMSO) was used for both the control and treatment groups was purposely adjusted to be at the same value (0.1%).

2.2. Assessment of cell proliferation and DNA synthesis

Twenty thousand mesangial cells were seeded in each well of the six-well culture plates. Subsequent to 24 h of quiescence, cells were stimulated with 10% fetal calf serum in the presence of various concentrations of YC-1 or vehicle (DMSO). At the following time points (24, 48, 72, or 96 h subsequent to stimulation) culture medium was removed and cells were trypsinized. The trypsinized cells were centrifuged, supernatant was aspirated and the cells were suspended with 3 ml of culture medium. Twenty microliters of this cell suspension was removed and mixed with the same volume of trypan blue. An aliquot of the mixture was applied to a hemocytometer and the density of surviving cells (cells the cytoplasm which was devoid of the characteristic trypan blue stain) and dead cells (cells featuring trypan blue staining of the cytoplasm) were enumerated under a microscope. The total number of surviving cells per well was estimated by the estimated cell density multiplied by the quantity of diluting medium. The proportion (percentage) of dead cells was calculated relative to the total cell count. To evaluate the toxicity of YC-1, lactate dehydrogenase (LDH) levels present in the collected supernatant were determined on day 4 subsequent to YC-1 stimulation and compared with those cells not treated with YC-1. In order to evaluate cell loss by detachment from the culture surface, the number of cells present in the supernatants was determined daily.

5-Bromo-2-deoxyuridine (BrdU) incorporation analysis was performed to attempt to assess the level of DNA synthesis. Three thousand mesangial cells were plated in each well of the 96-well culture plates. Cells were stimulated with PDGF (20 ng/ml) containing various concentrations of YC-1 or vehicle. BrdU was added 18 h subsequent to PDGF stimulation. The quantity of incorporated BrdU was analyzed using an enzyme-linked immunosorbent assay kit according to the protocol provided by the manufacturer (Amersham, Piscataway, NJ, USA).

2.3. Flow cytometry

One hundred and fifteen thousand mesangial cells were seeded into 10 cm-diameter tissue culture dishes. Cells were allowed to proliferate to a level of subconfluency, following which cells were treated with PDGF and YC-1 or DMSO subsequent to 24 h of cell quiescence. Flow cytometry analysis of the cell-cycle distribution of mesangial cells was performed before and 18 h subsequent to treatment of cells with the various test agents. Briefly, cells were washed twice with phosphate buffered saline (PBS), harvested by trypsinization, centrifuged, and suspended with 1 ml of cold PBS and then fixed in methanol for 30 min on ice. Following two washes with PBS, fixed cells were incubated in RNase (1 mg/ml) at 37 °C for 30 min, followed by staining of the DNA with propidium iodide (1 μg/μl) at 4 °C for 30 min in the dark; each sample was analyzed with a Coulter EPICS 753 flow cytometer and the proportion (percentage) of cells within the G1, S, and G2/M phases of the cell cycle were determined (Matsushime et al., 1991).

2.4. Western blot analysis

Western blot analysis was performed using a protocol that has been previously described (Chen et al., 2004). Mesangial cells were washed and lysed in RIPA buffer. Forty micrograms of cell lysate were heated at 100 °C for 10 min, applied to sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and electrophoresed, following which, the electrophoresed proteins in gels were transferred onto polyvinylidine difluoride membranes (Millipore, Bed-

ford, MA, USA) using a transblot chamber with Tris buffer. Subsequent to blocking, Western blots were incubated at 4 °C overnight with the appropriate primary antibody and, in the morning of the following day, membranes were washed with PBS/Tween-20 and incubated for 1 h with peroxidase-conjugated secondary antibodies at room temperature. The dilution for both the primary and secondary antibody was 1 : 1000 (or 1 : 500 for the primary anti-phospho-p38 mitogen-activated protein kinase (MAPK) and anti-total p38 MAPK antibody). Subsequent to washing with PBS/Tween-20, membranes were developed with enhanced chemilumination (Amarshem, Piscataway, NJ, USA).

2.5. Northern blot analysis

Total RNA was isolated using the acid quanidinium thiocyanate–phenol–chloroform method and sample concentration as has been described previously (Tsai et al., 1995). Total RNA was electrophoresed on formaldehyde-denatured 1% agarose gels in MOPS buffer (morpholinopropanesulfonic acid [0.2 M], sodium acetate [0.05 M], and EDTA [0.01 M]) and transferred to nylon membranes (Genescreen, Boston, MA, USA). Hybridization was performed with digoxigenin-labeled RNA probes. The blots were developed using CSPD® (Roche Molecular Biochemicals, Mannheim, Germany) as the substrate for alkaline phosphatase and results normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages.

Rat cyclin D1 RNA probe was synthesized as described below. The cDNA fragments were generated by reverse transcription-polymerase chain reaction from mesangial cell DNA using the specific primer pairs: 5′-CTGACACCAATCTCCTCAAC-3′ (corresponding to bases 178–197) and 5′-GTAGATGCACAACTTC-TCGG-3′ (complementary to bases 506–487) (Tamura et al., 1993). The products were subsequently subcloned into the pGEM-dT vectors (Promega; Madison, WI, USA). The cloned cDNA was then linearized and used as the templates for the in vitro transcription of antisense digoxigenin-conjugated riboprobes, according to the supplier's instructions (Roche, Mannhein, Germany).

2.6. Immunoprecipitation and cyclin D1/CDK4 kinase assay

Mesangial cells were stimulated with PDGF and the various appropriate test agents or vehicle, following which cell lysates were collected with specific lysis buffer (Tris [20 mM; pH = 7.5], NaCl [150 mM], EDTA [1 mM], EGTA [1 mM], Triton-100 [1%], $Na_2H_2P_2O_4$, [2.5 mM], β-glycerophosphate [1 mM], Na_3VO_4 [1 mM], leupeptin [1 μg/ml], and PMSF [1 mM]). Immunoprecipitating antibody (anti-CDK4 [2.5 μg]) was added to cell lysate protein (500 μg/500 μl) and rocked on a shaking table for 2 h at 4 °C. The immunocomplex was captured using 25 μl of protein A/G plus agarose beads. Following subsequent centrifugation, the complex was washed twice with lysis buffer and then twice with kinase buffer (Tris [25 mM; pH = 7.5], β-glycerophosphate [2.5 mM], DTT [2 mM], Na_3VO_4 [0.1 mM], and $MgCl_2$ [10 mM]). The immunoprecipitation bead pellet was suspended in 40 μl kinase buffer supplemented with ATP (200 μM) and 1 μg of substrate Rb fusion protein fragment (amino acid 769–921) and then incubated for 30 min at 30 °C. The reaction was terminated using 10 μl of 5× SDS sample buffer. The mixture was then boiled at 100 °C for 5 min then cooled on ice and then pellet down. The supernatant was then used for SDS-PAGE electrophoresis and subsequently analyzed by a Western blot technique using an anti-pRb antibody (Ser 780).

2.7. Measurement of intracellular cGMP

Three thousands mesangial cells were plated onto 96-well plates. After 24 h of quiescence, cells were stimulated with PDGF and various concentration of YC-1. Intracellular cGMP was measured 1 h after PDGF stimulation as protocol provided (Amarshem, Piscataway, NJ, USA). The relative intracellular cGMP level to vehicle-treated group was used to compare cGMP level between groups. Vascular smooth cells were used as positive control cell which YC-1 could stimulate to generate cGMP in previous study (Pan et al., 2004).

2.8. Reagents

Medium 199, fetal calf serum, and other tissue culture reagents were purchased from Gibco BRL (Rockville, MD, USA). Culture flasks and plates were purchased from Costa Corning (Cambridge, MA, USA). Rat recombinant PDGF-BB was purchased from R and D Systems (Minneapolis, MN, USA). Antibody against β-actin and 8-Br-cGMP were obtained from Sigma (The Sigma Chemical Co., Saint Louis, MO, USA). Antibodies against cyclin D1, cyclin E, total Akt, CDK2, $p21^{waf1}$, and $p27^{Kip1}$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosphorylated extracellular signal-regulated kinase (ERK)1/2 [p-ERK], total ERK1/2, phosphorylated-p38 MAPK [p-p38 MAPK], total p38 MAPK, Ser473-phosphorylated Akt [p-Akt], Ser780-phosphorylated retinoblastoma [p-Rb], and Ser9-phosphorylated glycogen synthase kinase (GSK)3β [p-GSK3β] were acquired from Cell Signaling Technology (Beverly, MA, USA). Antibody against CDK4 was purchased from BD Transduction Laboratories (Lexington, KY, USA). Protein A/G plus agarose beads and retinoblastoma fusion protein was a product of Santa Cruz Biotechnology. RNase was purchased from Calbiochem (San Diego, CA, USA). YC-1 was generously provided by Yung-Shin Pharma Ind. Co. (Taipei, Taiwan). *N*-[2-(4-bromocinnamylamino)-ethyl]-5-isoquinoline (H89), (9*S*,10*R*,12*R*)-2,3,9,10,11,12, hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9.12-epoxy-1H-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-I][1,6]benzodiazocine-10-carboxylic acid methyl ester (KT5823), [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole] (SB203580) and 1H-[1,2,4]oxadiazole[4,3-a]quinoxaline-1-one (ODQ) were obtained from Calbiochem (San Diego, CA, USA). All chemicals used for total RNA isolation, reverse transcription-polymerase chain reaction, Northern blot analysis, whole-cell lysate extraction, and Western blot analysis were of molecular grade and were obtained from either Sigma or Roche unless otherwise specified. YC-1, ODQ and SB203580 were dissolved in dimethyl sulfoxide (DMSO).

2.9. Statistical analysis

All values described in the text and figures are expressed as mean ± S.E.M. All experiments were performed as a minimum of three replicates. Statistical significance was evaluated by application of the Student's *t*-test or one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. A P value of less than 0.05 was considered to represent statistically significant difference between test populations.

3. Results

3.1. YC-1-inhibited cell growth, BrdU incorporation and induced G1 arrest for mesangial cells

Treatment of mesangial cells with YC-1 in the concentration range of 1~30 μM resulted in a time- and dose-dependent inhibition of cell growth for cultured cells (Fig. 1A). The synthesis of DNA by PDGF-stimulated mesangial cells was also inhibited by a similar concentration of YC-1 as revealed by the BrdU-incorporation assay (Fig. 1B). We then analyzed the effect of YC-1 upon cell-cycle profiles by flow cytometry. Mesangial cells commenced entry into S phase from G1 phase during the period of from 8~12 h subsequent to PDGF stimulation. The cell number in S phase reached its greatest level 18 h subsequent to PDGF stimulation. Fig. 2 reveals representative cell-cycle profiles for cells stained with propidium iodide both prior to and 18 h following PDGF stimulation. The mesangial cells treated with YC-1 exhibited a decreased fraction of S-phase cells and an increased accumulation of G1-phase cells. Such a result indicated that treatment with YC-1 inhibited cellular growth through cell-cycle arrest between G1 and S phases within mesangial cells.

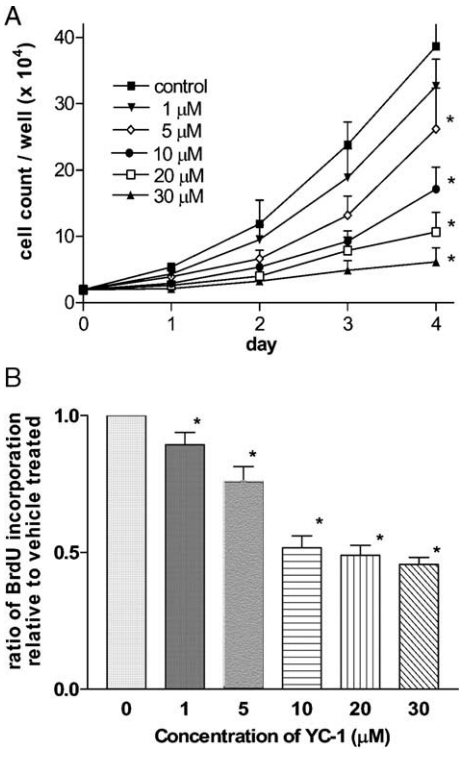

Fig. 1. Effect of YC-1 upon the growth and BrdU incorporation of cultured rat mesangial cells. A) The same numbers of mesangial cells stimulated with 10% serum were cultured with various concentrations of YC-1. Cell number was determined at the indicated time points. The proliferation of mesangial cells was inhibited by treatment with YC-1 in a dose- and time-dependent manner. (*$P<0.05$ versus vehicle [DMSO] treated on days 2, 3, and 4). B) Mesangial cells were stimulated with PDGF (20 ng/ml) following deprivation of growth stimulation and simultaneous treatment with YC-1 at a variety of concentrations. Cellular incorporation of BrdU was determined 18 h subsequent to PDGF stimulation. A greater dose of YC-1 exhibited a more pronounced level of suppression of BrdU incorporation (*$P<0.05$ versus vehicle-treated).

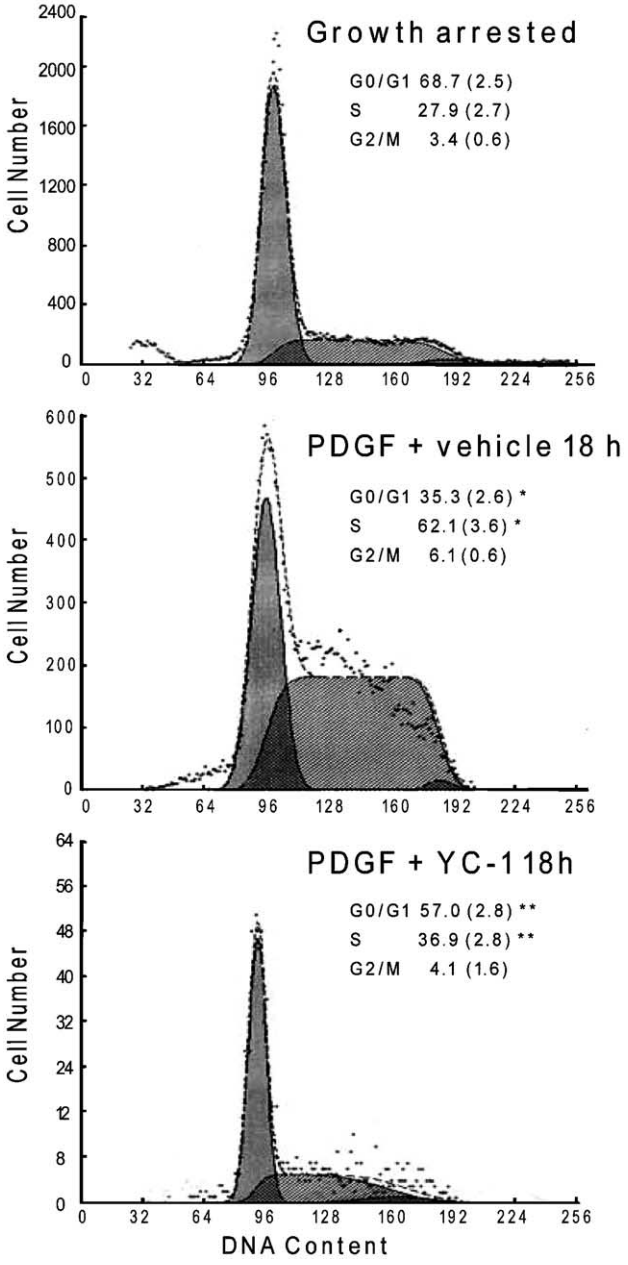

Fig. 2. Effect of YC-1 upon mesangial cell cell-cycle profile. Mesangial cells were collected and cell-cycle analyses performed with propidium iodide and flow cytometry. The proportion (percentage) of cells in each phase of the cell cycle is presented as the mean (± S.E.M.). A higher proportion of mesangial cells in G1 phase was found prior to PDGF stimulation. Eighteen hours subsequent to PDGF stimulation, a significantly greater number of mesangial cells had entered into S phase from G1 phase than was the case prior to such stimulation. The transition between the two phases was inhibited by the administration of YC-1 (30 μM). There were fewer cells in S phase and more cells in G1 phase for the YC-1-treated group than was the case for the group devoid of YC-1 treatment (*$P<0.05$ versus growth-arrested and **$P<0.05$ versus vehicle-treated).

The levels of LDH in the cultured-cell supernatant did not increase following 4 days of incubation for the YC-1-treated groups compared to the vehicle group (vehicle, 107.4±3.5; YC-1 [1 μM], 109±5.0; YC-1 [5 μM], 111.1±6.2; YC-1 [10 μM], 107.0±5.1; YC-1 [20 μM], 114.0±4.4; YC-1 [30 μM],

117.0±6.8 IU/l). The proportion (percentage) of detached cells in the supernatant was less than 5% for all groups and did not differ between YC-1-treated cells and control cells. The proportion of membrane-damaged adherent cells stained with trypan blue was less than 1% for all groups and did not differ between cells featuring YC-1 treatment and those that did not. Therefore, on the basis of such results, the antiproliferative effect of YC-1 upon cultured mesangial cells was not able to be attributed to YC-1's cytotoxicity.

3.2. YC-1 suppressed the expression of cyclin D1 protein and mRNA but had no effect upon protein expression of cyclin E, $p21^{waf1}$, $p27^{kip1}$, CDK2 and CDK4

For mammalian cells, the major cyclin/CDK complexes involved in the G1–S cell-cycle transition are cyclin D1/CDK4 and cyclin E/CDK2 (Sherr, 1993). $P21^{waf1}$ and $p27^{kip1}$ are the two CDK inhibitors for these two major cyclin/CDK complexes. We examined the effect of YC-1 upon the protein expression of these cell-cycle proteins for mesangial cells. Following PDGF stimulation, cyclin D1 protein expression achieved its greatest level approximately 6~9 h later. Such expression was inhibited by the simultaneous administration of YC-1. Treatment with YC-1 simultaneously with PDGF stimulation, however, did not affect the protein expression of cyclin E, $p21^{waf1}$, $p27^{kip1}$, CDK2 or CDK4 on the same period compared to vehicle-treated cells (Fig. 3A). Such similar effects of YC-1 upon protein expression for the above-mentioned cell-cycle proteins were also observed beyond 9 h and extending to 24 h subsequent to growth-factor stimulation (data not shown). Northern blot analysis revealed that the mRNA expression of cyclin D1 was also inhibited by treatment with YC-1 which meant that the inhibition of protein expression proceeded through the down-regulation of mRNA expression (Fig. 3B).

3.3. YC-1 did not increase intracellular GMP concentration in mesangial cells and inhibition of cyclin D1 expression by YC-1 was not reversed by inhibitors of soluble guanylate cyclase, protein kinase G, or protein kinase A

YC-1, a soluble guanylate cyclase activator, has been demonstrated to be able to increase cGMP synthesis for a number of different cell types. YC-1 is also a weak phosphodiesterase inhibitor (Galle et al., 1999) which has been shown to be able to increase cAMP levels in neutrophil (Hwang et al., 2003) and possibly also cGMP levels through inhibition of phosphodiesterase activity. To investigate whether the inhibition of cyclin D1 protein expression was related to the activation of the cGMP/cyclic GMP-dependent protein kinase (PKG)-signaling pathway, we measured the intracellular cGMP level after stimulation with various concentration of YC-1 and treated cultured mesangial cells with ODQ, a soluble guanylate cyclase inhibitor, in order to counteract the activation of soluble guanylate cyclase by YC-1. KT5823, a PKG inhibitor, was used to block the downstream action of cGMP once it was up-regulated by YC-1. One hour after PDGF stimulation the intracellular cGMP concentration did not increase in mesangial cells which were stimulated with the concentration of 1, 10, or 30 μM of YC-1. This was different from that 30 μM of YC-1 could increase the cGMP level up to three-fold in vascular smooth muscle cells (Fig. 4A). Similar effect was also observed 15 min or 2 h after PDGF stimulation in

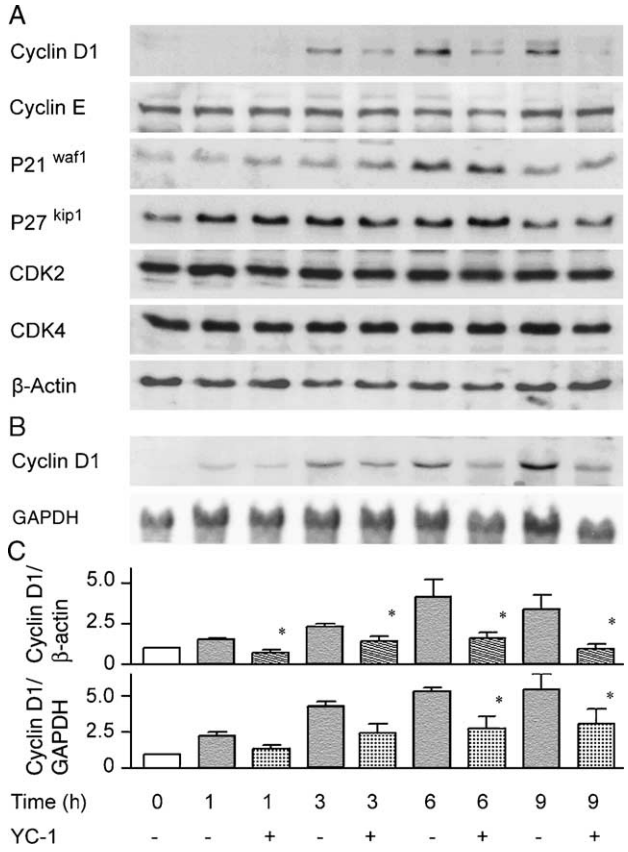

Fig. 3. Effect of YC-1 on the expression of cyclin D1, cyclin E, CDK2, CDK4, $p21^{waf1}$, and $p27^{kip1}$ within 9 h from growth stimulation. A) Subsequent to stimulation with PDGF, protein expression for cyclin D1 increased, this increment being inhibited by the treatment of cells with YC-1 (30 μM). The protein expression of cyclin E, CDK2, CDK4, $p21^{waf1}$, and $p27^{Kip1}$ were not different between the cells treated with YC-1 and vehicle on the same time point, although the $p21^{waf1}$ protein amount changed after PDGF stimulation. B) The mRNA expression of cyclin D1 was also inhibited by YC-1 (30 μM). C) Densitometric analysis of cyclin D1 expression treated with YC-1 or vehicle on various time points in Western/Northern blotting assays shown in Fig. 3A and B. The number below each lane indicates the time point subsequent to PDGF stimulation (*$P<0.05$ versus vehicle-treated on the same time point).

mesangial cells (data not shown). Besides, the inhibition of cyclin D1 protein expression by YC-1 was also not reversed by the co-treatment with either ODQ (30 μM) or KT5823 (1 μM). The application of either ODQ or KT5823 to cultured cells did not, by themselves, elicit any effect upon the expression of cyclin D1 protein (Fig. 4B). The addition of 8-Br-cGMP, a cGMP analogue, in the concentration of 10 nM~1 μM to mesangial cells also did not suppress cyclin D1 expression (Fig. 4C). This indicated that the cGMP, once elevated by YC-1 (actually no significant change was seen in this study), was not adequate to suppress cyclin D1 expression in mesangial cell. We therefore concluded that the suppressive effect of YC-1 upon cyclin D1 protein expression was not related to cGMP/PKG-signaling pathway. The addition of H89, in the concentration of 1 μM adequate to inhibit protein kinase A (PKA) activity in mesangial cells (Lin et al., 2003), also did not reverse the inhibitory effect upon cyclin D1 expression by YC-1 (Fig. 4D), suggesting that the cyclin D1 expression inhibition by YC-1 also did not proceed through the cAMP/PKA-signaling pathway.

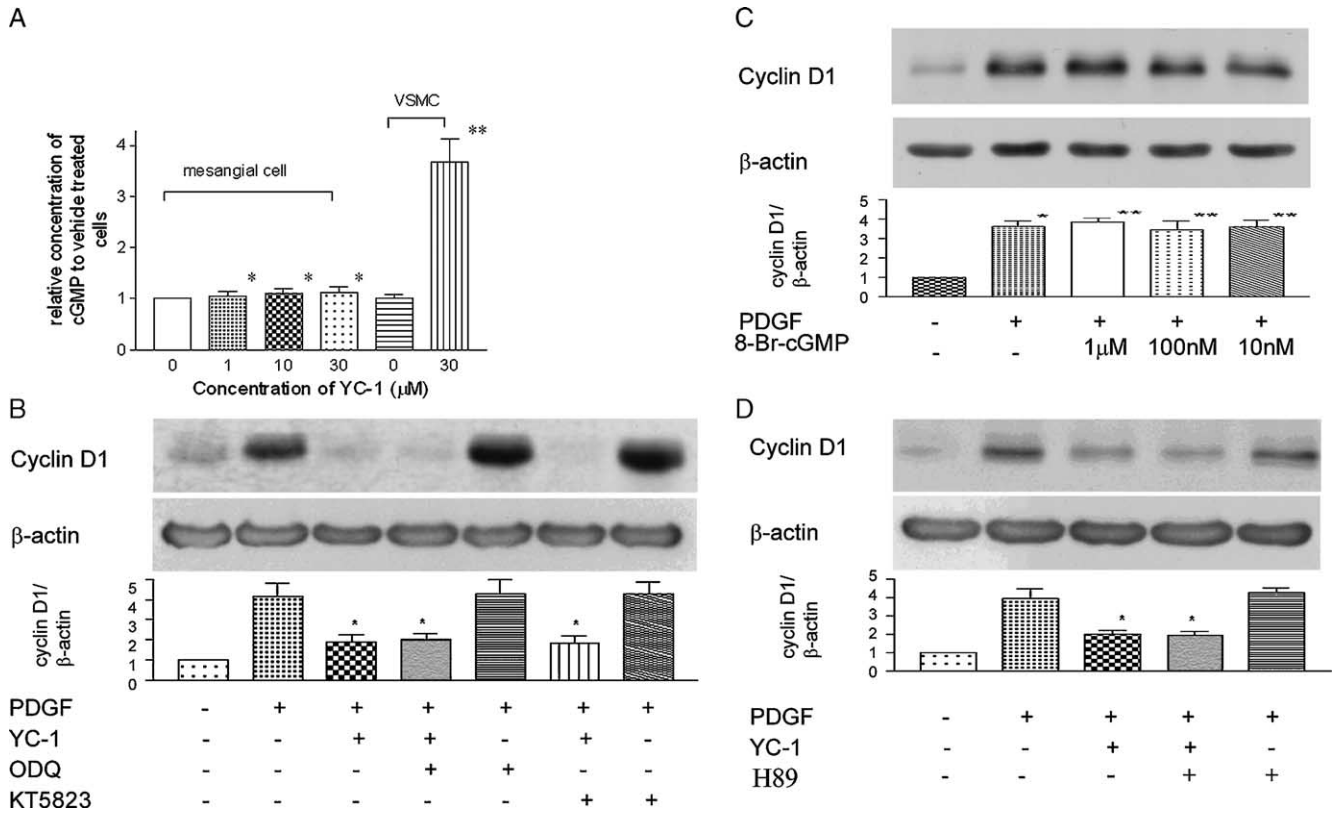

Fig. 4. A) Intracellular cGMP was measured 1 h after PDGF stimulation. The cGMP level was not different significantly between YC-1 treated or vehicle-treated mesangial cells, while the cGMP level was significantly higher in YC-1 (30 μM)-treated vascular smooth muscle cells compared to vehicle-treated cells (*$P>0.05$ for YC-1-treated versus vehicle-treated mesangial cells; **$P<0.05$ for YC-1-treated versus vehicle-treated vascular smooth muscle cells). B) Cell lysate was collected before and 6 h subsequent to PDGF stimulation. Protein expression of cyclin D1 was inhibited by YC-1 (30 μM) stimulation. This inhibition was not reversed by co-treatment of cells with ODQ (30 μM) or KT5823 (1 μM). Neither ODQ nor KT5823 themselves affected the expression of cyclin D1. C) Cell lysate was collected before and after 6 h subsequent to PDGF stimulation. The cyclin D1 expression was not suppressed by treatment with 8-Br-cGMP (10 nM∼1 μM) in mesangial cells (*$P<0.01$ versus cells without PDGF stimulation; **$P>0.05$ versus vehicle-treated cells). D) The inhibition of cyclin D1 by YC-1 was also not reversed by the co-treatment of cells with H89 (1 μM) (*$P<0.05$ versus vehicle-treated).

3.4. YC-1 activated the p38 MAPK but had no effect upon the ERK1/2 MAPK or PI3-Akt-GSK3β-signaling pathway

In the regulation signaling transduction pathways, cyclin D1 synthesis is positively regulated by ERK 1/2 MAPK (Lavoie et al., 1996) and negatively regulated by p38 MAPK pathway (Lavoie et al., 1996; Page et al., 2001). The PI3K-Akt-GSK3β pathway is the inhibitory signaling process related to the destruction of the cyclin D1 protein (Cross et al., 1995; Diehl et al., 1997). In order to investigate which signaling pathway is involved in the cyclin D1 suppression by YC-1, we evaluated the active form (phosphorylated form) of these signaling proteins by Western blotting. Within a 1-h period subsequent to PDGF stimulation of mesangial cells, a prominent increase in the intracellular level of the phosphorylated form of p38 MAPK was observed for YC-1-treated mesangial cells compared to the vehicle-treated cells. The levels of the phosphorylated form of ERK1/2 MAPK, Akt and GSK3β did not alter as a consequence of the treatment of such cells with YC-1. Such an observation suggests that YC-1 probably affects only the activity of the p38 MAPK-signaling pathway and not the ERK1/2 MAPK and PI3K/Akt pathways (Fig. 5A).

3.5. Inhibition of cyclin D1 expression and cyclin D1/CDK4 kinase activity by YC-1 were partially reversed by the p38 MAPK inhibitor SB203580

The inhibition of both mRNA and protein expression for cyclin D1, as elicited by mesangial cell exposure to YC-1, could be partially reversed by the co-treatment of the cells with SB203580 (10 μM), a p38 MAPK inhibitor, in a concentration known to inhibit p38 MAPK kinase activity in mesangial cells (Inui et al., 2000). When mesangial cells were treated with only SB203580 (10 μM), the level of mRNA and protein expression for cyclin D1 did not change (Fig. 5B). Immunoprecipitation of the cyclin D1/CDK4 complex in the cell lysate was performed with mouse anti-CDK4 antibody and the level of kinase activity was evaluated in order to determine the ability of cyclin D1/CDK4 complex to phosphorylate the retinoblastoma fusion protein fragment in vitro. We found that treatment of mesangial cells with YC-1 inhibited the ability of the cyclin D1/CDK4 complex to phosphorylate the retinoblastoma protein fragment at the serine − 780 site which has long been known to be the target of cyclinD1/CDK4 kinase activity (Kitagawa et al., 1996). The suppression of such phosphorylation by YC-1 was also partially reversed by co-treatment with SB203580 (Fig. 5C).

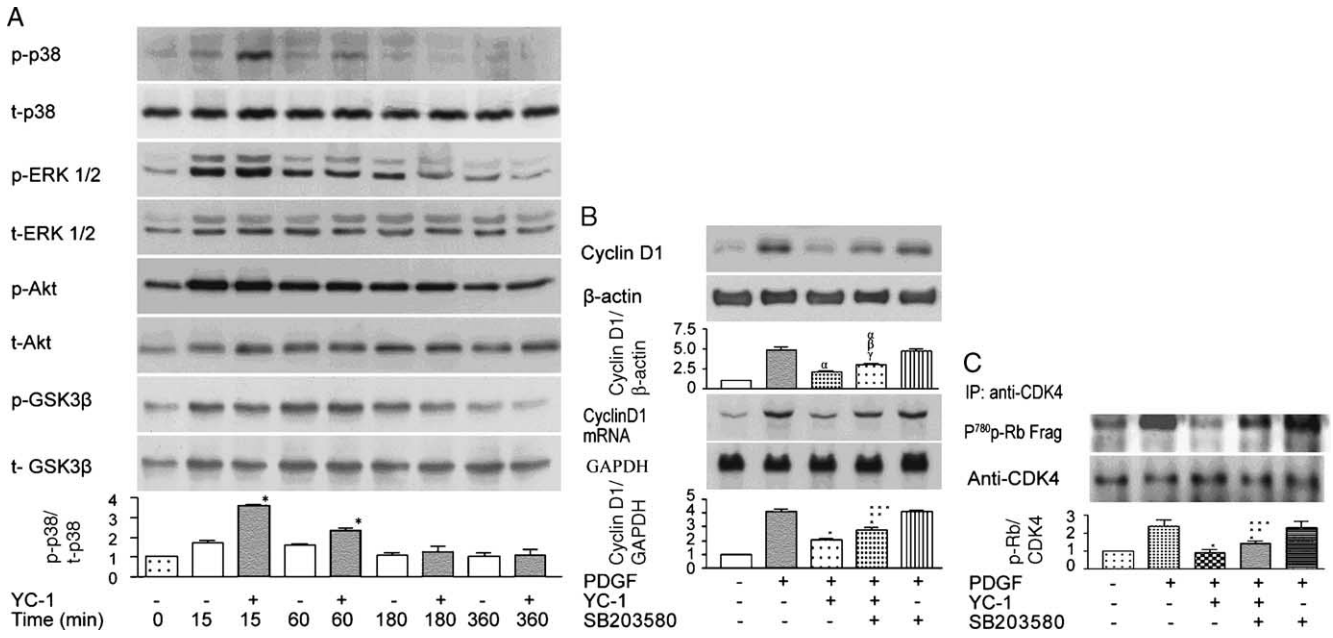

Fig. 5. A) The effect of YC-1 (30 μM) upon the phosphorylation of relevant signaling proteins within 6 h following PDGF stimulation, including ERK1/2, p38 MAPK, Akt, and GSK3β, with only the activation of p38 MAPK being apparent. For each signaling protein, the upper lane represented the phosphorylated form of the protein and the lower lane represented the total form. The number below each lane indicates the time point following PDGF stimulation (*$P<0.05$ versus vehicle-treated at the same time point). B) Cell lysate was collected both prior to and 6 h following PDGF stimulation. Protein expression of cyclin D1 was inhibited by cellular exposure to YC-1 (30 μM). This inhibition was partially reversed by co-treatment of cells with SB203580 (10 μM), a p38 MAPK inhibitor. SB203580 itself did not significantly alter the expression of cyclin D1. Analogous changes to the expression of mRNA were also seen as a consequence of the treatment of mesangial cells with these drugs (α, $P<0.05$ versus vehicle-treated; β, $P<0.05$ versus YC-1-treated only; γ, $P<0.05$ versus SB203580-treated only; *$P<0.01$ versus vehicle-treated only; **$P<0.05$ versus YC-1-treated only; and ***$P<0.01$ versus SB203580-treated only). C) Cyclin D1/CDK4 complex in cell lysate was immunoprecipitated with anti-CDK4 antibody. The kinase activity of the precipitated complex was evaluated by its ability to phosphorylate the retinoblastoma fusion protein fragment at the serine 780 site in vitro. For YC-1 treated cells, the kinase activity was lower than for the controls. This inhibition was able to be partially reversed by co-treatment with SB203580 (10 μM). SB203580 did not affect the kinase activity of cyclin D1/CDK4 complex (*$P<0.05$ versus vehicle-treated; **$P<0.05$ versus YC-1-treated only; and ***$P=0.09$ versus SB203580-treated only).

Such results suggested that YC-1 inhibited cyclin D1 expression and related kinase activity, at least partially, through activation of the p38 MAPK-signaling transduction pathway.

4. Discussion

In this study, we have demonstrated that YC-1, within the concentration range of 1∼30 μM, is able to inhibit the proliferation of cultured mesangial cells. The inhibitory effect of such YC-1 exposure proceeded via the inhibition of DNA synthesis and the arrest of the cell-cycle transition from G1 to S phase. The suppression of cyclin D1 synthesis and related CDK4 kinase activity resulted in this cell-cycle arrest. Such findings suggest that the p38 MAPK pathway might play some role in the signaling transduction process leading to the inhibition of DNA synthesis and cell proliferation.

Cyclin D1 is a key regulator for early G1-phase progression in mesangial cells (Terada et al., 1998; Lang et al., 2000). The inhibition of cyclin D1 by YC-1 was first seen at around 3 h post-exposure and was observed to be greatest at around 6∼9 h post-stimulation. It has previously been reported that cyclin D1 forms complexes with CDK4 and then stimulates kinase activity within mesangial cells (Terada et al., 1998). We have demonstrated that the decrease in the intracellular cyclin D1 level elicited by YC-1 stimulation of mesangial cells resulted in an overall decrease in the kinase activity of cyclin D1/CDK4 present in the whole-cell lysate 6 h subsequent to stimulation. Cyclin D-dependent kinase executes this critical function during middle-to-late G1 phase (Baldin et al., 1993; Quelle et al., 1993). In our study, mesangial cells began to enter cell-cycle S phase from G1 phase at around 8∼12 h subsequent to PDGF stimulation. The specific time point at which cyclin D1 levels were suppressed by YC-1 was noted to be prior to the onset of the G1/S transition. Therefore, it would appear quite reasonable to conclude that the inhibition of cyclinD1/CDK kinase activity by YC-1 led to cellular growth arrest in G1/S phase. This finding appears to contrast an earlier observation by other workers that YC-1 induced cell-cycle G1/S arrest for endothelial cells via the augmentation of $p21^{waf1}$ and $p27^{kip1}$ protein levels, 18 h subsequent to growth stimulation (Hsu et al., 2003). The average protein level of these CDK inhibitors did not appear to change as a consequence of treatment with YC-1 in our study.

Increasing intracellular cGMP level or activating the PKG pathway could inhibit the proliferation of mesangial cells. In 2000, Pandey et al. (2000) reported that activation of PKG by natriuretic peptide receptor A could result in

inhibition of the proliferation of mesangial cells. A previous study has revealed that cGMP was able to delay the normal cell-cycle transition through the suppression of cyclin D1 and CDK4 activation within vascular smooth muscle cells (Fukumoto et al., 1999). Herein, we considered it was necessary to attempt to evaluate whether YC-1 inhibited cyclin D1 synthesis through the activation of some form of cGMP/PKG-signaling system. However, in this study YC-1 failed to increase intracellular cGMP level significantly in mesangial cells. Similar phenomenon was also seen in hepatoma cell (Wang et al., 2005). The reason was unknown. One of the hypothesis is that YC-1 (within a concentration of 1∼30 μM) may only facilitate cGMP production under the co-stimulation with other soluble guanylate cyclase activator (such as nitric oxide) in mesangial cell. The other hypothesis is that the activity of soluble guanylate cyclase is less in this mesangial cell culture as found in some hepatoma cell (Kimura and Murad, 1975). So YC-1 failed to increase cGMP level significantly in the experiment. These hypotheses need further studies to uncover the truth. To exclude the influence by minor change of intracellular cGMP, we further blocked the possible effect of YC-1 upon soluble guanylate cyclase by co-treatment of cells with ODQ, a soluble guanylate cyclase inhibitor, and blocked the activity of PKG with KT5823 to block the downstream-effect machinery cGMP pathway, once activated by intracellular cGMP. Both of these consecutive treatments failed to reverse the inhibition of cyclin D1 expression by YC-1. Administration of cGMP analogue, 8-Br-cGMP, also did not suppress cyclin D1 expression as seen in YC-1 treated cells. We therefore concluded that the suppression of cyclin D1 expression by YC-1 in our study was independent of the cGMP/PKG-signaling pathway. In the 2003 study of Hsu et al. (2003), the growth inhibitory effect of YC-1 upon human umbilical endothelial cells was also not related to cGMP-pathway activation. Wang et al. (2005) also demonstrated that the anti-proliferative effect of YC-1 in cancer cell was not dependent on cGMP pathway.

From the Northern blotting results, the mRNA expression of cyclin D1 proved to be lower for YC-1-treated cells than it was for their untreated controls. The quantity of intracellular mRNA is one of the principal determinants of the extent of protein expression; in effect, a lower level of mRNA expression will lead to a lower level of protein expression and conversely so. Therefore, it could be concluded that YC-1 inhibits cyclin D1 protein expression through the mechanism of eliciting a reduction to mRNA expression. We did observe that treatment of cultured mesangial cells with YC-1 was able to increase the intracellular level of the phosphorylated form of p38 MAPK. By co-treatment of such cells with SB203580, a p38 MAPK inhibitor, the cyclin D1 protein and mRNA expression inhibited by cell exposure to YC-1 was able to be partially reversed. The analogous observation was also made for the kinase activity of the cyclin D1/CDK4 complex. Such a result suggests that there was a possible role for YC-1 as regards inhibiting the cyclin D1 expression of mesangial cells through the activation of p38 MAPK. The suppression of cyclin D1 mRNA expression by YC-1 first occurred 3 h after treatment, yet the p38 phosphorylation occurred much earlier (within 1 h). We hypothesized that it was the time necessary for the activated p38 MAPK by YC-1 to exert the effect on cyclin D1 mRNA transcription. Similar phenomenon was also seen in the up-regulation of cyclin D1 mRNA synthesis by activated ERK1/2 MAPK 3∼6 h after PDGF stimulation. In the studies of Lavoie et al. (1996) and Page et al. (2001) activation of p38 MAPK cascade inhibited cyclin D1 expression and promoter activity. These studies further gave the clues of the possible role of the activated p38 MAPK cascade by YC-1 in mRNA synthesis of cyclin D1. However, the reversal of cyclin D1 suppression in YC-1 treated cells by SB203580 did not appear to be complete in our study. Thus, we speculate that there might be another possible mechanism for the inhibition of cyclin D1 expression by YC-1. The ERK1/2 MAPK pathway was the stimulatory signaling mechanism for cyclin D1 mRNA synthesis (Cheng et al., 1998; Peeper et al., 1997; Aktas et al., 1997) whilst the PI3K-Akt-GSK3β pathway was the mechanism capable of stabilizing cyclin D1 protein expression (Cross et al., 1995; Diehl et al., 1997). The collective activities of these two pathways, which were represented by the phosphorylated form of the kinase protein as shown in Western blotting, were not affected by the exposure of cultured mesangial cells to YC-1. So, the possibility was lower that the expression of cyclin D1 regulated by YC-1 was through one of the two pathways. It is still possible that YC-1 also inhibits cyclin D1 expression through another mechanism, such as YC-1's influence upon protein kinase C signaling transduction, such a possibility clearly warranting further investigation.

It was interesting to recognize that YC-1 inhibits cyclin D1 expression through activation of p38 MAPK as shown here. Browning et al. (1999) reported in 1999 that YC-1 was able to activate p38 MAPK within cultured human neutrophils. From these authors' study, the stimulation of lipopolysaccharide-activated p38 MAPK occurred through nitric oxide-dependent cGMP activation, which was blocked with KT5823. In 2000, Browning's group subsequently confirmed that the activation of p38MAPK within human fibroblasts by nitric oxide and a cGMP analogue required the presence of PKG (Browning et al., 2000). Whether YC-1 activates p38 MAPK through activation of PKG or not will remain as an interesting point of speculation. From the results of our study, the intracellular cGMP level was not elevated and blocking the activity of PKG with KT5823 did not correspondingly block the inhibition of cyclin D1 expression elicited by the exposure of mesangial cells to YC-1. The addition of H89, a PKA inhibitor, to the culture also failed to block inhibition of cyclin D1 by YC-1. Thus it remains difficult to say that the cGMP-PKG- or cAMP-PKA-signaling pathway played any substantial role in the

activation of p38 MAPK which subsequently led to cyclin D1 suppression for cultured mesangial cells. Therefore, there must remain the possibility that YC-1 activates P38 MAPK via another different mechanism.

The proliferation of mesangial cells is one of the most important pathological changes that arise for many different examples of proliferative glomerulonephritis (Pesce et al., 1991). Although YC-1 was observed to have been able to inhibit the proliferation of mesangial cells, it is possible that YC-1 may demonstrate other biological effects within cells, such as eliciting p38 MAPK activation independently of cGMP- or cAMP-signaling pathways, as appeared to have been the case in this study. Such in vitro effects might be associated with rather unexpected pharmacological effects in vivo. Before extending the results of such investigation to any clinical application for human disease, it is necessary to disclose the possible range of the pharmacological effects of YC-1, including its influence upon inflammation, matrix synthesis and vascular hemodynamic change in vivo. The underlying effect and mechanism of YC-1 upon signaling transduction for pathways other than the cGMP pathway should also be evaluated.

Acknowledgments

This work was supported by grants from the National Science Council (92-2314-B-002-363), National Taiwan University Hospital Yun-Lin branch (NTUHYLS007), the Mrs. Hsiu-Chin Lee Kidney Research Fund and Ta-Tung Research Fund, Taipei, Taiwan. This work was also supported in part by Department of Medical Research in NTUH. We also would like to thank Huang Kuo-Don for his technical support for this study.

References

Aktas, H., Cai, H., Cooper, G.M., 1997. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. Mol. Cell. Biol. 17, 3850–3857.

Baldin, V., Lukas, J., Macorte, M.J., Pagano, M., Draetta, G., 1993. Cyclin D1 is a nuclear protein required for cell cycle protein progression in G1. Genes Dev. 7, 812–821.

Browning, D.D., Windes, N.D., Ye, R.D., 1999. Activation of p38 mitogen-activated protein kinase by lipopolysaccharide in human neutrophils requires nitric oxide-dependent cGMP accumulation. J. Biol. Chem. 274, 537–542.

Browning, D.D., McShane, M.P., Marty, C., Ye, R.D., 2000. Nitric oxide activation of p38 mitogen-activated protein kinase in 293T fibroblasts requires cGMP-dependent protein kinase. J. Biol. Chem. 275, 2811–2816.

Chen, Y.M., Wu, K.D., Tsai, T.J., Hsieh, B.S., 1999. Pentoxifylline inhibits PDGF-induced proliferation of and TGF-beta-stimulated collagen synthesis by vascular smooth muscle cells. J. Mol. Cell. Cardiol. 31, 773–783.

Chen, Y.M., Chiang, W.C., Lin, S.L., Wu, K.D., Tsai, T.J., Hsieh, B.S., 2004. Dual regulation of TNF-{alpha}-induced CCL2/monocyte chemoattractant protein-1 expression in vascular smooth muscle cells by NF-{kappa}B and AP-1: modulation by type III phosphodiesterase inhibition. J. Pharmacol. Exp. Ther. 309, 978–986.

Cheng, M., Sexl, V., Sherr, C.J., Roussel, M.F., 1998. Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1). Proc. Natl. Acad. Sci. U. S. A. 95, 1091–1096.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378, 785–789.

Diehl, J.A., Zindy, F., Sherr, C.J., 1997. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev. 11, 957–972.

Floege, J., Burns, M.W., Alpers, C.E., Yoshimura, A., Pritzl, P., Gordon, K., Seifert, R.A., Bowen-Pope, D.F., Couser, W.G., Johnson, R.J., 1992. Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. Kidney Int. 41, 297–309.

Floege, J., Ostendorf, T., Janssen, U., Burg, M., Radeke, H.H., Vargeese, C., Gill, S.C., Green, L.S., Janjic, N., 1999. Novel approach to specific growth factor inhibition in vivo: antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. Am. J. Pathol. 154, 169–179.

Fukumoto, S., Koyama, H., Hosoi, M., Yamakawa, K., Tanaka, S., Morii, H., Nishizawa, Y., 1999. Distinct role of cAMP and cGMP in the cell cycle control of vascular smooth muscle cells: cGMP delays cell cycle transition through suppression of cyclin D1 and cyclin-dependent kinase 4 activation. Circ. Res. 85, 985–991.

Galle, J., Zabel, U., Hubner, U., Hatzelmann, A., Wagner, B., Wanner, C., Schmidt, H.H., 1999. Effects of the soluble guanylyl cyclase activator, YC-1, on vascular tone, cyclic GMP levels and phosphodiesterase activity. Br. J. Pharmacol. 127, 195–203.

Hsu, H.K., Juan, S.H., Ho, P.Y., Liang, Y.C., Lin, C.H., Teng, C.M., Lee, W.S., 2003. YC-1 inhibits proliferation of human vascular endothelial cells through a cyclic GMP-independent pathway. Biochem. Pharmacol. 66, 263–271.

Hwang, T.L., Hung, H.W., Kao, S.H., Teng, C.M., Wu, C.C., Cheng, S.J., 2003. Soluble guanylyl cyclase activator YC-1 inhibits human neutrophil functions through a cGMP-independent but cAMP-dependent pathway. Mol. Pharmacol. 64, 1419–1427.

Inui, D., Yoshizumi, M., Suzaki, Y., Kirima, K., Tsuchiya, K., Houchi, H., Kagami, S., Tamaki, T., 2000. Effect of endothelin-1(1–31) on p38 mitogen-activated protein kinase in cultured human mesangial cells. Life Sci. 68, 635–645.

Johnson, R.J., Raines, E.W., Floege, J., Yoshimura, A., Pritzl, P., Alpers, C., Ross, R., 1992. Inhibition of mesangial cell proliferation and matrix expansion in glomerulonephritis in the rat by antibody to platelet-derived growth factor. J. Exp. Med. 175, 1413–1416.

Kimura, H., Murad, F., 1975. Increased particulate and decreased soluble guanylate cyclase activity in regenerating liver, fetal liver, and hepatoma. Proc. Natl. Acad. Sci. U. S. A. 24 (72), 1965–1969.

Kitagawa, M., Higashi, H., Jung, H.K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., Taya, Y., 1996. The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. EMBO J. 15, 7060–7069.

Ko, F.N., Wu, C.C., Kuo, S.C., Lee, F.Y., Teng, C.M., 1994. YC-1, a novel activator of platelet guanylate cyclase. Blood 84, 4226–4233.

Lang, S., Hartner, A., Sterzel, R.B., Schocklmann, H.O., 2000. Requirement of cyclin D1 in mesangial cell mitogenesis. J. Am. Soc. Nephrol. 11, 1398–1408.

Lavoie, J.N., L'Allemain, G., Brunet, A., Muller, R., Pouyssegur, J., 1996. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J. Biol. Chem. 271, 20608–20616.

Lehrke, I., Schaier, M., Schade, K., Morath, C., Waldherr, R., Ritz, E., Wagner, J., 2002. Retinoid receptor-specific agonists alleviate experimental glomerulonephritis. Am. J. Physiol., Renal Fluid Electrolyte Physiol. 282, F741–F751.

Lin, S.L., Chen, R.H., Chen, Y.M., Chiang, W.C., Tsai, T.J., Hsieh, B.S., 2003. Pentoxifylline inhibits platelet-derived growth factor-stimulated cyclin D1 expression in mesangial cells by blocking Akt membrane translocation. Mol. Pharmacol. 64, 811–822.

Matsushime, H., Roussel, M.F., Ashmun, R.A., Sherr, C.J., 1991. Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65, 701–713.

Mulsch, A., Bauersachs, J., Schafer, A., Stasch, J.P., Kast, R., Busse, R., 1997. Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. Br. J. Pharmacol. 120, 681–689.

Page, K., Li, J., Hershenson, M.B., 2001. p38 MAP kinase negatively regulates cyclin D1 expression in airway smooth muscle cells. Am. J. Physiol., Lung Cell. Mol. Physiol. 280, L955–L964.

Pan, S.L., Guh, J.H., Chang, Y.L., Kuo, S.C., Lee, F.Y., Teng, C.M., 2004. YC-1 prevents sodium nitroprusside-mediated apoptosis in vascular smooth muscle cells. Cardiovasc. Res. 61, 152–158.

Pandey, K.N., Nguyen, H.T., Li, M., Boyle, J.Y., 2000. Natriuretic peptide receptor-A negatively regulates mitogen-activated protein kinase and proliferation of mesangial cells: role of cGMP-dependent protein kinase. Biochem. Biophys. Res. Commun. 271, 374–379.

Peeper, D.S., Upton, T.M., Ladha, M.H., Neuman, E., Zalvide, J., Bernards, R., DeCaprio, J.A., Ewen, M.E., 1997. Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature 386, 177–181.

Pesce, C.M., Striker, L.J., Peten, E., Elliot, S.J., Striker, G.E., 1991. Glomerulosclerosis at both early and late stages is associated with increased cell turnover in mice transgenic for growth hormone. Lab. Invest. 65, 601–605.

Quelle, D.E., Ashmu, R.A., Shurtleff, S.A., Kato, J.Y., Bar-Sagi, D., Roussel, M.F., Sherr, C.J., 1993. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev. 7, 1559–1571.

Sherr, C.J., 1993. Mammalian G1 cyclin. Cell 73, 1059–1065.

Tamura, K., Kanaoka, Y., Jinno, S., Nagata, A., Ogiso, Y., Shimizu, K., Hayakawa, T., Nojima, H., Okayama, H., 1993. Cyclin G: a new mammalian cyclin with homology to fission yeast Cig1. Oncogene 8, 2113–2118.

Teng, C.M., Wu, C.C., Ko, F.N., Lee, F.Y., Kuo, S.C., 1997. YC-1, a nitric oxide-independent activator of soluble guanylate cyclase, inhibits platelet-rich thrombosis in mice. Eur. J. Pharmacol. 320, 161–166.

Terada, Y., Inoshita, S., Nakashima, O., Yamada, T., Tamamori, M., Ito, H., Sasaki, S., Marumo, F., 1998. Cyclin D1, p16 and retinoblastoma gene regulate mitogenic signaling of endothelin in rat mesangial cells. Kidney Int. 53, 76–83.

Tsai, T.J., Lin, R.H., Chang, C.C., Chen, Y.M., Chen, C.F., Ko, F.N., Teng, C.M., 1995. Vasodilator agents modulate rat glomerular mesangial cell growth and collagen synthesis. Nephron 70, 91–100.

Tulis, D.A., Bohl Masters, K.S., Lipke, E.A., Schiesser, R.L., Evans, A.J., Peyton, K.J., Durante, W., West, J.L., Schafer, A.I., 2002. YC-1-mediated vascular protection through inhibition of smooth muscle cell proliferation and platelet function. Biochem. Biophys. Res. Commun. 291, 101410–101421.

Wang, J.P., Chang, L.C., Raung, S.L., Hsu, M.F., Huang, L.J., Kuo, S.C., 2002. Inhibition of superoxide anion generation by YC-1 in rat neutrophils through cyclic GMP-dependent and -independent mechanisms. Biochem. Pharmacol. 63, 577–585.

Wang, S.W., Pan, S.L., Guh, J.H., Chen, H.L., Huang, D.M., Chang, Y.L., Kuo, S.C., Lee, F.Y., Teng, C.M., 2005. YC-1 exhibits a novel antiproliferative effect and arrests the cell cycle in G0–G1 in human hepatocellular carcinoma cells. J. Pharmacol. Exp. Ther. 312, 917–925.

Wohlfart, P., Malinski, T., Ruetten, H., Schindler, U., Linz, W., Karl Hartmut, S.S., Wiemer, G., 1999. Release of nitric oxide from endothelial cells stimulated by YC-1, an activator of soluble guanylyl cyclase. Br. J. Pharmacol. 128, 1316–1322.

Wu, C.C., Ko, F.N., Kuo, S.C., Lee, F.Y., Teng, C.M., 1995. YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase. Br. J. Pharmacol. 116, 1973–1978.

Wu, C.H., Chang, W.C., Chang, G.Y., Kuo, S.C., Teng, T.M., 2004. The inhibitory mechanism of YC-1, a benzyl indazole, on smooth muscle cell proliferation: an in vitro and in vivo study. J. Pharm. Sci. 94, 252–260.