PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Effects of retinoic acid on proliferation, phenotype and expression of cyclin- dependent kinase inhibitors in TGF-β1-stimulated rat hepatic stellate cells

Guang Cun Huang, Jin Sheng Zhang and Yue E Zhang

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Abstract

AIM To study the molecular mechanisms of retinoic acid (RA) on proliferation and expression of cyclin-dependent kinase inhibitors (CKI), i.e. p16, p21 and p27 in cultured rat hepatic stellate cells (HSC) stimulated with transforming growth factor beta 1 (TGF-β1).
METHODS HSC were isolated from healthy rat livers and cultured. After stimulated with 1mg/L TGF-β1, subcultured HSC were treated with or without 1nmol/L RA. MTT assay, immunocytochemistry (ICC) for p16, p21, p27 and β-smooth muscle actin (β-SMA) protein, *in situ* hybridization (ISH) for retinoic acid receptor beta 2 (RAR-β2) and p16, p21 and p27 mRNA and quantitative image analysis (partially) were performed.
RESULTS RA inhibited HSC proliferation (41.50%, *P*<0.05), decreased the protein level of β-SMA (55.09%, *P*<0.05), and induced HSC to express RAR-β2 mRNA. In addition, RA increased the protein level of p16 (218.75%, *P*<0.05) and induced p21 protein expression; meanwhile, p27 was undetectable by ICC in both control and RA-treated HSC. However, RA had no influence on the mRNA levels of p16, p21 or p27 as determined by ISH.
CONCLUSION Up-regulation of p16 and p21 on post-transcriptional level may contribute, in part, to RA inhibition of TGF-β1 initiated rat HSC activation *in vitro*.

Department of Pathology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China
Guang Cun Huang, graduated from Medical Center of Fudan University (former Shanghai Medical University) in 1996, majoring in forensic medicine, now assistant and master at the Department of Pathology, School of Basic Medical Sciences, Fudan University, specialized in the study of hepatic pathology, having 3 papers published.
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Correspondence to: Dr. Guang Cun Huang, Department of Pathology, Medical Center of Fudan University (former Shanghai Medical University), 138 Yixueyuan Road, Shanghai 200032, China
Tel. 0086-21-64041900 Ext.2537
Email. zdxu@shmu.edu.cn
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INTRODUCTION

Hepatic stellate cells (HSC) play crucial roles in the development of liver fibrosis[1-6]. Stimulated HSC transform from vitamin A-rich quiescent cells to myofibroblast-like cells characterized by the expression of α-smooth muscle actin (α-SMA), loss of retinoids and diminished retinoid signaling[4-15]. Exogenous retinoids such as retinoic acid (RA) may recover the contents of retinoids and nuclear retinoic acid receptors (RAR) in HSC and therefore suppress hepatic fibrogenesis, but the mech anisms of RA on HSC inhibition were not well understood[3,16-27]. Recent studies on other cell types have shown that modulation of cell cycle regulatory proteins might contribute to RA-induced inhibition of cell prolifer ation and differ-entiation[28-37]and Kawada *et al*[38] reported that expression of G1 cyclin was involved in cell cycle transition of HSC from G1 to S. The present study was designed to investigate the effects of RA on negative cell cycle regulators cyclin-dependent kinase inhibitors (CKI) in cultured rat HSC stimulated with transforming growth factor beta-1 (TGF-α1). The results showed that RA inhibited HSC activation may be in part due to post-transcriptional up-modulation of p16 and p21.

MATERIALS AND METHODS

Reagents

Collagenase IV, pronase E, Nycodenz, RA and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide or tetrazolium (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant human TGF-β1 was from Oncogene Science (Uniondale, NY, USA). Dulbecco's modified Eagle's medium (DMEM) was Gibco/BRL- product (Life Technologies, Inc. Grand Island, NY, USA). Newborn calf serum, plastic tissue culture flasks and multi-plates were from Corning Incorporated (Corning, NY, USA). Polyclone anti-α-SMA antibody was purchased from Dako A/S (Glostrup, Denmark). Antibodies to p16, p21 and p27 were from Santa Cruz

Biotechnology, Inc. (Santa Cruz, CA, USA). ABC kit and DAB were from R & D Systems (Minneapolis, MN, USA). DIG Nucleic Acid Label and Detect Kit and Taq DNA polymerase were from Roche Diagnostics GmbH (Mannheim, Germany).

Isolation and culture of HSC

Cells were isolated from healthy Sprague-Dawley male rats (weighing 400g-450g) as described by Weiner *et al*[26] with minor modifications by the laboratory[39], seeded onto 25cm^2 plastic tissue culture flasks and incubated at 37℃ in a humidified 5% CO^2/95% air. The medium was replaced 24h after seeding and every 48h thereafter. After they reached confluence (10d after planting), activated HSC were subcultured onto plastic tissue culture multi-plates with or without coverslips.

Experiments were performed on cells between serial passage 1 and 3 using three independent cell lines.

Cell treatments

Activated HSC were depleted of serum for 48h, followed by incubation with 1mg/L TGF-α1 for another 48h, and then the medium was removed and cells maintained in DMEM with or without 1nmol/L RA for 48h. Preliminary dose dependence experiments indicated that 1mg/ L TGF-β1 or 1nmol/L RA had significant influence on HSC proliferation.

Proliferation assay

Cell proliferation was measured by MTT assay as previously described[40] with minor modifications. Briefly, during the last 4h of incubation the cells were loaded with 10μL of freshly prepared and filtered MTT (5g/L in PBS) per well. The medium was then replaced with 100μL absolute ethanol and the cells were left for 30min for color development, followed by reading on Vmax®R Kinetic Microplate Reader (Molecular Devices Corporation, Sunnyvale, California, USA) at 570nm wavelength.

Immunocytochemistry (ICC)

Cells grew on coverslips were fixed, permeabilized, blocked with 1% serum in PBS, and then incubated with primary antibodies to either α-SMA, p16, p21 or p27. ABC assay and DAB system were used to detect the proteins[41] and photomicrographs were taken with an Olympus microphoto-microscope (Olympus Optical Co. LTD., Shinjuku-ku, Tokyo, Japan).

In situ hybridization (ISH)

cDNA probes for human RAR-β2 and p16 were gifts from the Depar tment of Biochemistry, School of Basic Medical Sciences, Fudan University; and cDNA fragments for rat p21 and p27 were presented as gifts by Dr. Chen Guang-Ping. Fragments were labeled with digoxigenin using random priming assay.

ISH was performed as previously described[39] with immunohistochemical detection using an alkaline phosphatase (AKP)conjugated anti-digoxigenin monoclonal antibody. Hybridization signal was visualized through the substrates of AKP (NBT and BCIP). Photomicrographs were taken with an Olympus microphoto-microscope again.

Image analysis

Quantitative analysis of protein and mRNA were performed by scanning using KS 400 Imaging System 3.0 (Carl Zeiss Vision GmbH, Germany) and means of density values were determined.

Statistical analysis

Data were presented as mean values ± S.D. and statistical significance w as assessed by Student's *t* test.

RESULTS

RA Inhibited HSC proliferation and α-SMA expression

As shown in Figure 1, there were fewer (41.50%, $P<0.05$) HSC in RA-treated cells compared with control cells. In addition, RA decreased expression of α-SMA (55.09%, $P<0.05$; Figure 2 and Table 1).

RA induced RAR-β2 mRNA

To evaluate retinoid signaling, ISH was performed to determine RAR-β2 gene expression. No mRNA was detected in control cells, but HSC treated with RA did express RAR-β2, indicate RA induced expression of RAR-β2 in HSC-(Figure 3), and therefore enhanced retinoid signaling.

Expression of CKI

To further clarify the mechanisms of RA on cell cycle regulation in HSC, prote in and mRNA levels of CKI were determined. As shown in Figure 4, p27 was undetectable by ICC in both control and RA-treated HSC. In addition, RA increased the protein levels of p16 (218.75%, $P<0.05$) and p21 protein was detected in HSC treated with RA (Figure 4 and Table 1).

ISH results showed that the mRNA level of p16, p21 or p27 was not influenced by RA (Figure 5 and Table 1).

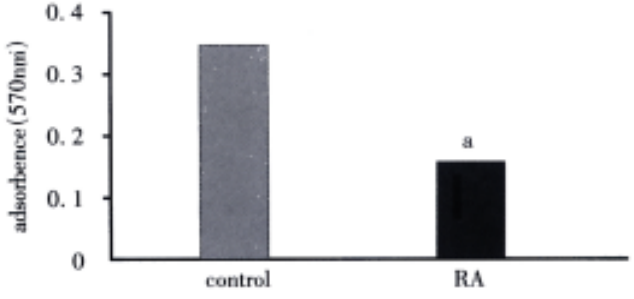

Figure 1 RA inhibited HSC proliferation. TGF -β1-stimulated rat HSC were cultured and treated with or without 1nmol/ L RA for 48h, followed by MTT assay as described in MATERIALS AND METHODS. [a]$P<0.05$ *vs* control.

Table 1 Effects of RA on α-SMA and CKI expression in HSC

Group	α-SMA (protein)	RAR-β2 (mRNA)	p16		P21		p27	
			Protein	mRNA	Protein	mRNA	Protein	mRNA
Control	0.285±0.050	ND	0.160±0.024	0.377±0.043	ND	0.285±0.043	ND	0.165±0.021
RA	0.157±0.042[a]	0.227±0.24	0.350±0.029[a]	0.353±0.023	0.0339±0.034	0.277±0.027	ND	0.179±0.023

ND: not determined; [a]$P<0.05$ *vs* control

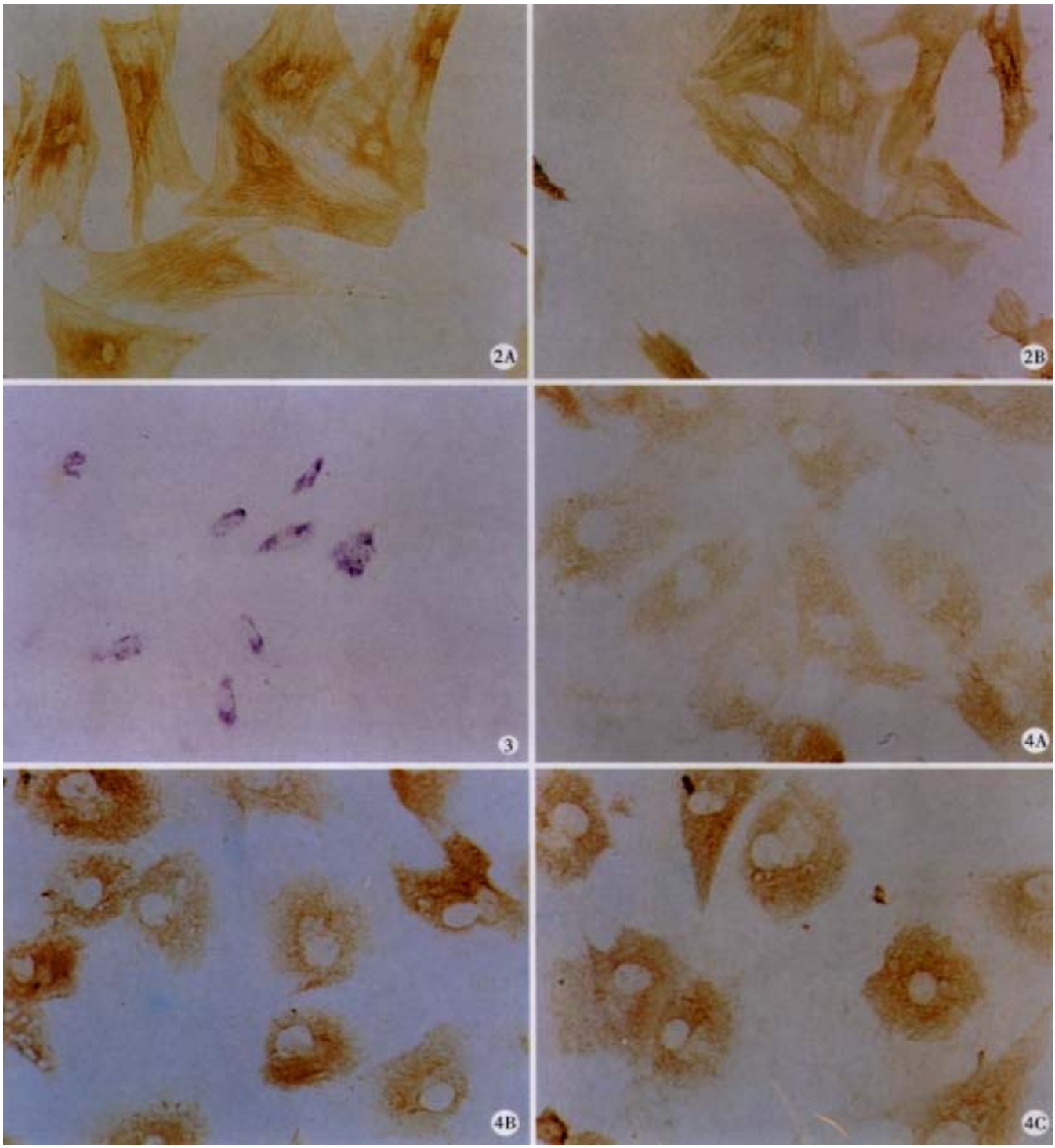

Figure 2 RA decreased the protein level of α-SMA. TGF-β1-stimulated HSC were treated with (A) as described in Figure 1, and then immunocytochemistry was performed to detect α-SMA protein. ABC×200 (B) or without RA

Figure 3 Expression of RAR-β2 in RA-treated HSC. *In situ* hybridization with DIG-labeled RAR-β2 cDNA probe was used to determine RAR-β2 mRNA expression in HSC. NBT/BCIP×200

Figure 4 Expression of CKI protein. Immunocytochemical study was performed to detect CKI, i.e. p16 (A,), p21 (B,). ABC×200 (C) expression in control (A) or RA-treated HSC

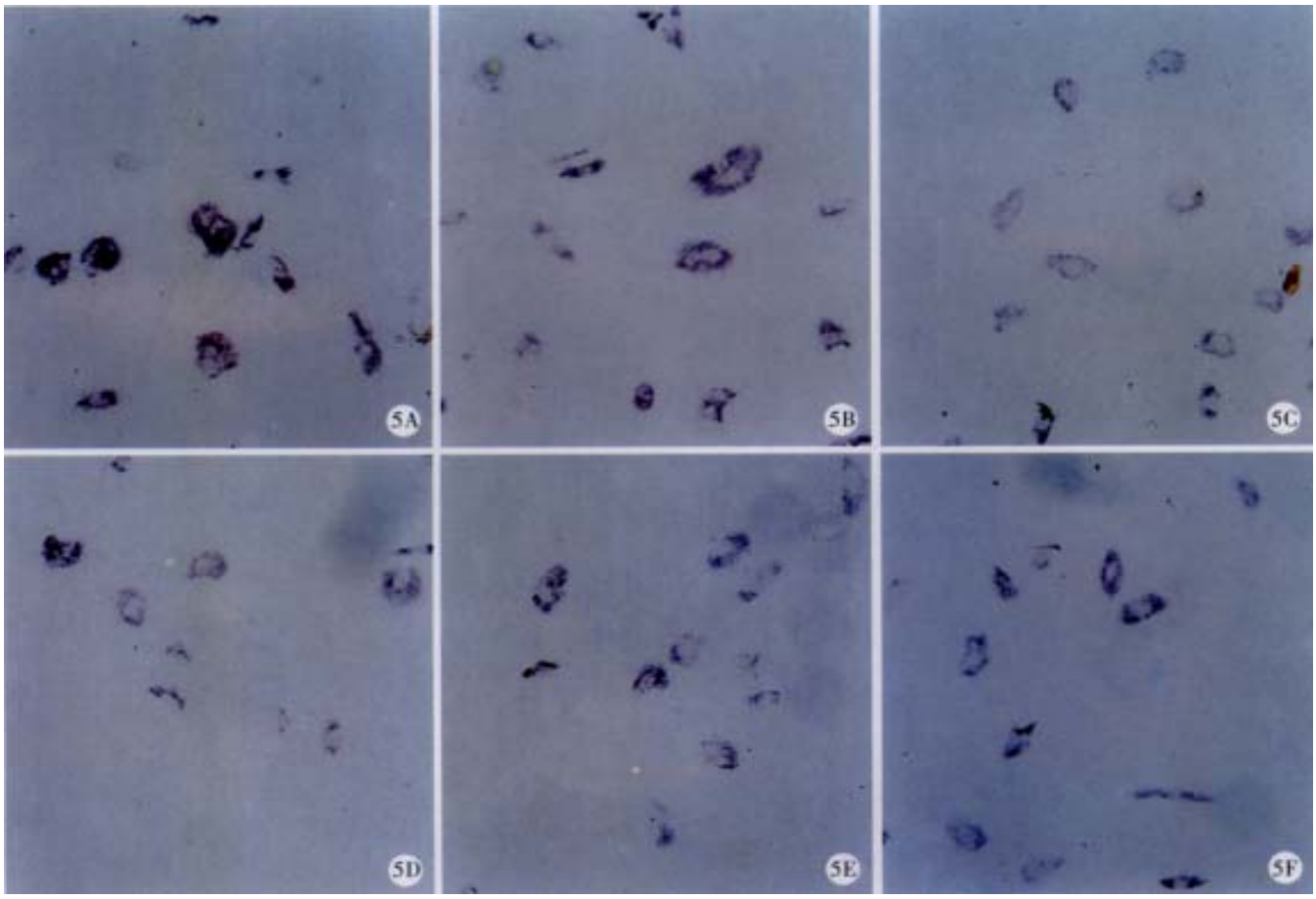

Figure 5 mRNA expression of CKI. mRNA of p16 (A,), p21 (B,) (C,) or p27 (D,) was determined with ISH in control (E,) or RA-treated HSC (F,). NBT/BCIP×200

DISCUSSION

TGF-β1 is one of the most fibrogenetic cytokines on HSC, which initiates HSC activation characterized by loss of retinoids, proliferation, and expression of α-SMA and extracellular matrix[2,6,42-45]. Our results showed that even 48h depletion of serum could not completely suppress the expression of α-SMA, implying that serum depletion can not reversibly suppress TGF-β1-initiated activation of rat HSC in culture.

RA may modulate cell growth and differentiation through retinoid signaling[30,46-49], mainly by nuclear retinoid X receptors and RAR. Present study showed that RA inhibited HSC proliferation and down-regulated α-SMA protein, demonstrating that RA may suppress HSC activation induced by TGF-β1. Our results showed that RA induced RAR-β2 mRNA, which may then modulate expression of some other genes including CKI[28-37,50-52]. In addition, cells in controls displayed no RAR-β2 mRNA, agreeing with its insufficient to completely suppress HSC activation again.

The protein level of p16 was increased in RA-treated HSC with detectable p21 protein, while RA had no influence on those mRNA levels, suggesting RA may up-regulate p16 and p21 gene expression on the post-transcriptional level. p16 or p21 can inhibit cyclin-CDK complexes and then prevent G1 transition[53-58]; therefore, our study indicates that RA induced inhibition of TGF-β1-initiated HSC activation may be in part due to up-modulation of p16 and p21 on the post-transcriptional level, and reveals a new mechanism of RA induced HSC inhibition.

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