

# The antioxidant (–)-epigallocatechin-3-gallate inhibits activated hepatic stellate cell growth and suppresses acetaldehyde-induced gene expression

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Activated hepatic stellate cells (HSC) are the primary source of excessive production of extracellular matrix during liver fibrogenesis. Although the underlying mechanisms remain incompletely understood, it is widely accepted that oxidative stress plays a critical role in liver fibrogenesis. Suppression of HSC growth and activation, as well as induction of apoptosis, have been proposed as therapeutic strategies for treatment and prevention of this disease. In the present report, we elucidated, for the first time, effects of the antioxidant (–)-epigallocatechin-3-gallate (EGCG), a major (and the most active) component of green tea extracts, on cultured HSC growth and activation. Our results revealed that EGCG significantly inhibited cultured HSC growth by inducing cell cycle arrest and apoptosis in a dose- and time-dependent manner. In addition, EGCG markedly suppressed the activation of cultured HSC as demonstrated by

blocking transforming growth factor- $\beta$  signal transduction and by inhibiting the expression of  $\alpha$ 1(I) collagen, fibronectin and  $\alpha$ -smooth muscle actin genes induced by acetaldehyde, the most active metabolite of ethanol. Furthermore, EGCG reacted differently in the inhibition of nuclear factor- $\kappa$ B activity between cultured HSC with or without acetaldehyde stimulation. Taken together, our results indicated that EGCG was a novel and effective inhibitor for activated HSC growth and activation *in vitro*. Further studies are necessary to evaluate the effect of this polyphenol in prevention of quiescent HSC activation *in vivo*, and to further elucidate the underlying mechanisms.

**Key words:** alcohol, apoptosis, fibrogenesis, gene regulation, green tea extract.

## INTRODUCTION

Hepatic fibrosis occurs as a wound-healing process after many forms of chronic liver injury, including virus infection, autoimmune liver diseases and sustained alcohol abuse [1]. Hepatic fibrogenesis is a process where production of extracellular matrix (ECM) surpasses degradation. Without effective treatments, reversible hepatic fibrosis at an early stage leads to irreversible cirrhosis. Suppression of hepatic fibrogenesis and prevention of cirrhosis has attracted the attention of researchers from the therapeutic perspective. Hepatic stellate cells (HSC), previously termed fat- or vitamin A-storing cells, or Ito cells, are the most relevant cell type for over-production of ECM and for the development of liver fibrosis. Upon stimulation, quiescent HSC become activated and trans-differentiate into myofibroblast-like cells characterized by several key phenotypic changes, including an increase in proliferation, accumulation of ECM, including  $\alpha$ 1(I) collagen, expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and loss of stored vitamin A droplets, as recently reviewed by Friedman [2]. Culturing quiescent HSC on plastic plates causes spontaneous activation leading to a myofibroblast-like phenotype, mimicking the process seen *in vivo*. This provides a simple and useful model for studying activation of these cells. Induction of apoptosis has recently been proposed as a strategy for terminating proliferation of activated HSC during liver fibrogenesis [3,4].

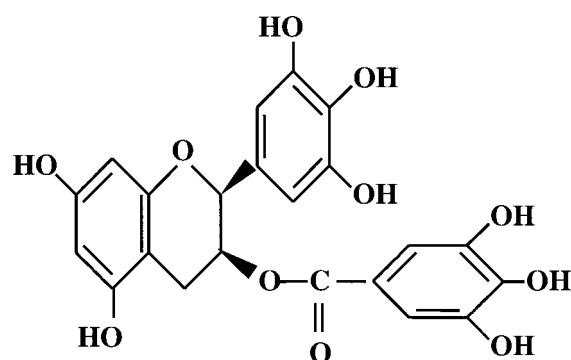
Although the underlying mechanisms remain incompletely understood, accumulating evidence has indicated that oxidative stress plays critical roles in activation of HSC [2,5,6]. Reducing

oxidative stress by antioxidants, such as D- $\alpha$ -tocopherol (vitamin E) and butylated hydroxytoluene, blocked HSC activation and suppressed the expression of collagen genes in HSC *in vitro* [5], as well as preventing fibrosis in iron-overloaded rat liver *in vivo* [7]. Experimental results suggested that reducing oxidative stress by antioxidants could be a potential and effective therapeutic strategy for treatment and prevention of hepatic fibrogenesis. However, the therapeutic efficacy of currently well-known antioxidants, such as superoxide dismutase, vitamin E and ascorbic acid, in treatment of human hepatic fibrosis is generally unimpressive [8].

Natural antioxidants, such as polyphenols from green tea extracts, have recently attracted considerable attention for preventing oxidative stress-related diseases including cancers, cardiovascular diseases and degenerative diseases [9]. Green tea is the most consumed beverage in the world [10]. (–)-Epigallocatechin-3-gallate (EGCG) is a major constituent and the most potent antioxidant of the polyphenols purified from green tea (Figure 1) [11]. The antioxidant potential of EGCG is far greater than that of vitamin E and/or C [12], which might allow it to succeed where other antioxidants have failed in preventing oxidative stress-related diseases. This polyphenol was previously shown not to harm the liver or kidney *in vivo* [13]. Its potent antioxidant capability and long history as beverage without adverse health effects make it a possible alternative to vitamin E for therapeutic treatment. Effects of the antioxidant EGCG on HSC growth and activation, however, have not been studied. The basis of this communication is to evaluate effects of this natural phyto-antioxidant on cultured HSC growth and acti-

Abbreviations used: BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGCG, (–)-epigallocatechin-3-gallate; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cells; LDH, lactate dehydrogenase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; RPA, RNase protection assay;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; T $\beta$ -RII, type II TGF- $\beta$  receptor; TGF- $\beta$ , transforming growth factor- $\beta$ ; TUNEL, terminal transferase deoxytidyl uridine end labelling.

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(-)-epigallocatechin-3-gallate (EGCG)

**Figure 1** The chemical structure of EGCG

vation, as well as to discuss the therapeutic potential of this antioxidant in treatment and prevention of hepatic fibrosis.

## MATERIALS AND METHODS

### HSC isolation and culture

HSC were isolated from male Sprague–Dawley rats (less than 300 g) as described previously [14,15]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HSC at passages 4–8 were used for experiments. EGCG (purity > 95%), purchased from Sigma (St. Louis, MO, U.S.A.), was used in experiments at the indicated concentrations. In some experiments, cells were serum-starved for 48 h prior to the addition of acetaldehyde (200  $\mu$ M). Serum-depleted media (0.4% FBS) were used to minimize the formation of adducts between acetaldehyde and serum proteins. Acetaldehyde at 200  $\mu$ M was widely used in previous HSC experiments [16]. The media with acetaldehyde was replaced every 12 h.

### Lactate dehydrogenase (LDH) release assays

Pre-confluent passaged HSC (1–2 days post-plating) in 6-well plates were incubated in 1 ml of DMEM containing 10% FBS treated with EGCG at the indicated concentrations for 24 h. After centrifugation, LDH in the supernatant of the conditioned media was determined as medium LDH. Attached cells were lysed completely by 1 ml of 10% FBS/DMEM with Triton X-100 (0.1%) [17]. After centrifugation, LDH in the supernatant was analysed as cellular LDH. LDH in DMEM with 10% FBS was defined as contamination arising from FBS and subtracted from medium and cellular LDH values. LDH activities were determined with an LDH assay kit (Sigma). Results were shown as the percentage of total LDH, i.e. [medium LDH/(medium LDH + cellular LDH)]  $\times$  100%.

### Determination of cell growth

Semi-confluent HSC ( $5.5 \times 10^4$ /well) were serum-starved for 24 h in DMEM containing 0.4% FBS. Cells were maintained in DMEM with 0.4% FBS or subsequently stimulated with 10% FBS in the presence or absence of EGCG at the indicated concentrations for the indicated times. After washing, cell growth was determined by attached cell numbers counted using a

computer-equipped cell counter (Coulter Corporation, Miami, FL, U.S.A.).

### [<sup>3</sup>H]Thymidine-incorporation assays

Passaged HSC ( $5.5 \times 10^4$ /well) in 6-well plates were serum-starved for 24 h prior to treatments. Cells were then either maintained in serum-depleted media (0.4% FBS) or incubated in serum-rich media (10% FBS) with or without EGCG at the indicated concentrations for an additional 24 h and subsequently pulsed for 4 h with methyl-[<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml; Amersham Bioscience, Arlington Heights, IL, U.S.A.). At the end of the incubation, cells were washed three times with cold PBS to remove unincorporated [<sup>3</sup>H]thymidine, followed by fixation with ice-cold methanol for 30 min at 4 °C. Cells were lysed in 750  $\mu$ l of 0.25 M NaCl/0.25% SDS. The reaction was stopped with 150  $\mu$ l of 1 M HCl. The whole lysate was removed from each well and mixed with 4 ml of the scintillation fluid Soluscint O (National Diagnostics, Highland Park, NJ, U.S.A.) and counted in a liquid scintillation analyser. Results were expressed as c.p.m. from triplicate experiments.

### Bromodeoxyuridine (BrdU) staining

Pre-confluent HSC in slideflasks were incubated in DMEM with 10% FBS with or without EGCG at the indicated concentrations for 24 h. Prior to harvesting cells (2 h), BrdU was added at a final concentration of 25  $\mu$ g/ml. Cells were fixed and stained using a BrdU staining kit from Zymed, following the protocol provided by the manufacturer.

### Flow-cytometric analyses of the cell cycle and apoptosis

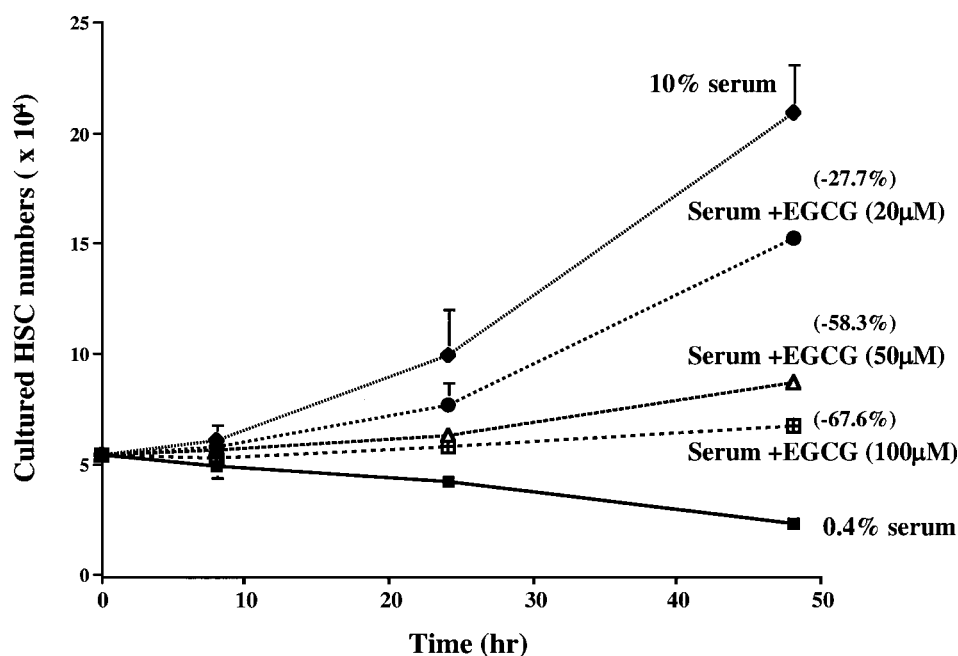
Pre-confluent HSC incubated in DMEM with 10% FBS were treated with EGCG at 50  $\mu$ M for the indicated times. Cells were harvested by brief trypsin/EDTA treatment and washed several times with cold PBS. HSC ( $\geq 1 \times 10^6$  cells/sample) were suspended in 2 ml of FACS buffer [1% FA buffer (Difco), 0.1% sodium azide and 1% FBS]. Cells were fixed with ethanol, and then labelled with propidium iodide (Sigma). Cells that were positively labelled with propidium iodide were detected with a Coulter® EPICS® XL-MCL flow cytometer and analysed using its System II™ software.

### Detection of apoptotic HSC by terminal transferase deoxynucleotidyl uridine end labelling (TUNEL)

Pre-confluent HSC cultured in DMEM with 10% FBS in slideflasks were treated with or without EGCG (50  $\mu$ M) for the indicated times. Cells were washed three times with cold PBS before fixation. Apoptotic HSC were detected using the DeadEnd™ Colorimetric TUNEL System (Promega), following the protocol provided by the manufacturer.

### Western blotting analyses

Whole-cell protein extracts were prepared from pre-confluent HSC treated with or without EGCG (50  $\mu$ M). SDS/PAGE with 10% resolving gel was used to separate proteins (25  $\mu$ g/lane). The separated proteins were detected by using primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Protein bands were visualized by utilizing chemiluminescence reagent (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.).



**Figure 2** EGCG inhibits activated HSC growth

Passaged HSC ( $5.5 \times 10^4$ /well) in 6-well plates were incubated in DMEM with 0.4% FBS for 24 h. Cells were then maintained in DMEM with 0.4% FBS, or stimulated with 10% FBS in the presence or absence of EGCG at the indicated concentrations for the indicated times. After washing, cell growth was determined by attached cell numbers. Values were expressed as means  $\pm$  S.D. ( $n \geq 6$ ). Error bars, if not indicated, were contained within the data points. Values in parentheses are the percentage inhibition caused by EGCG compared with cells treated with 10% FBS.

### Plasmids and transient transfection

The plasmid p3TP-Lux is a transforming growth factor- $\beta$  (TGF- $\beta$ )-inducible luciferase reporter, containing the plasminogen activator inhibitor-1 (PAI-1) gene promoter, kindly provided by Dr Joan Massague (Memorial Sloan-Kettering Cancer Center, NY, U.S.A.). The nuclear factor- $\kappa$ B (NF- $\kappa$ B) reporter plasmid pNF- $\kappa$ B-Luc was purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). The type II TGF- $\beta$  receptor (T $\beta$ -RII) reporter plasmid pT $\beta$ -RII (-219), containing 219 bp of the 5' promoter region of T $\beta$ -RII gene, was generously provided by Dr Seong-Jin Kim (Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.) [18]. Transfection and luciferase assays were described previously [15]. Transfection efficiency was determined by co-transfection of a  $\beta$ -galactosidase reporter, pSV- $\beta$  gal (0.5  $\mu$ g/well; Promega).

### RNA isolation and RNase protection assay (RPA)

Total RNA was isolated by the TRI Reagent (Sigma), following the protocol recommended by the manufacturer. To prepare RNA probes for T $\beta$ -RII, pT7-T $\beta$ RII was linearized with *Eco*RI [19]. The 115 bp of 28 S rRNA probe was used as an internal control (Ambion, Austin, TX, U.S.A.). The antisense probes were synthesized and  $^{32}$ P-labelled by MAXIScript<sup>TM</sup> (Ambion). RPA was carried out using RPA II<sup>TM</sup> kits (Ambion) following the protocol provided by the manufacturer. The radioactivity in each band was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.), as described previously [14,15].

### Real-time PCR

DNase I-treated total RNA (1  $\mu$ g) was used for synthesis of the first strand of cDNA. Reverse transcription conditions were as follows: 42  $^{\circ}$ C for 15 min, 95  $^{\circ}$ C for 5 min and 5  $^{\circ}$ C for 5 min (one cycle). Real-time PCR was carried out in 25  $\mu$ l of reaction solution [2.5  $\mu$ l of 10 $\times$  buffer, 5 mM dNTPs, 10 mM MgCl<sub>2</sub>, 200 nM primers and 0.75 units of platinum<sup>®</sup> *Taq* polymerase; all from Invitrogen] plus 1  $\mu$ l of SYBR Green (1:2000; BioWhittaker, Richland, ME, U.S.A.). No genomic DNA contamination or pseudogenes were detected by PCR without the reverse transcription step in the total RNA used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The reactions started at 95  $^{\circ}$ C for 7 min, followed by 40 cycles of 95  $^{\circ}$ C for 20 s, 54  $^{\circ}$ C for 30 s and 72  $^{\circ}$ C for 30 s. Melting peaks of PCR products were determined by heat-denaturing them over a 35  $^{\circ}$ C temperature gradient at 0.2  $^{\circ}$ C/s from 60 to 95  $^{\circ}$ C [20]. The cycle numbers crossing an arbitrary threshold ( $C_t$ ) were determined using Smart Cycler<sup>®</sup> software, version 1.2b (Cepheid, Sunnyvale, CA, U.S.A.). Fold change in target mRNA relative to GAPDH was calculated as suggested by Schmittgen et al. [20]:

$$\text{Fold change} = 2^{-\Delta\Delta C_t}$$

where

$$\Delta\Delta C_t = (C_{t \text{ target}} - C_{t \text{ GAPDH}})_{\text{time } X} - (C_{t \text{ target}} - C_{t \text{ GAPDH}})_{\text{time } 0}$$

Time  $X$  is any time point when the drug was given. Time 0 represents the experiment starting time (no drug added).

Primers used in the real-time PCR were:  $\alpha$ 1(I) collagen, forward, 5'- CCT CAA GGG CTC CAA CGA G-3'; reverse, 5'-TCA ATC ACT GTC TTG CCC CA-3';  $\alpha$ -SMA, forward, 5'- CCG ACC GAA TGC AGA AGG A-3'; reverse, 5'-ACA GAG

**Table 1** LDH release in cultured HSC treated with EGCG

The percentage of total LDH was calculated as [medium LDH/(medium LDH + cellular LDH)] × 100%. Values are expressed as means ± S.D. ( $n \geq 3$ ).

EGCG ( $\mu\text{M}$ ) ...	Total LDH (%)				
	0	20	50	100	1000
	8.7 ± 0.8	8.5 ± 0.3	7.4 ± 0.7	8.8 ± 0.7	38 ± 0.6*

\*  $P < 0.01$ , compared with cells with no EGCG treatment.

TAT TTG CGC TCC GGA-3'; fibronectin, forward, 5'-TGT CAC CCA CCA CCT TGA-3'; reverse, 5'-CTG ATT GTT CTT CAG TGC GA-3'; GAPDH, forward, 5'-GGC AAA TTC AAC GGC ACA GT-3'; reverse, 5'-AGA TGG TGA TGG GCT TCC C-3'.

#### Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [14]. The integrity of nuclear extracts was tested by EMSA with a  $^{32}\text{P}$ -labelled SP-1 consensus probe, resulting in distinct SP-1 shifts from all

extracts (results not shown). The NF- $\kappa\text{B}$  gel-shift probe was purchased from Santa Cruz Biotechnology.

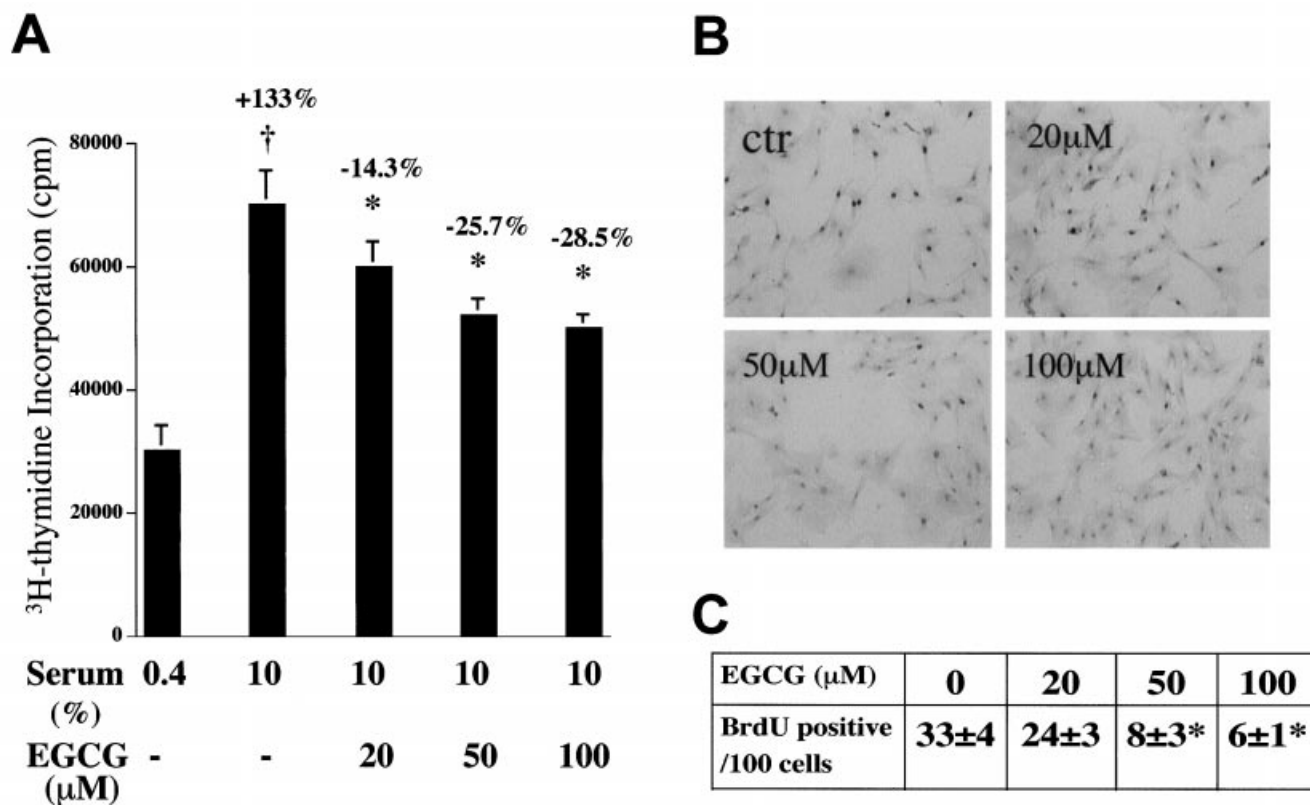
#### Statistical analysis

Differences between means were evaluated using an unpaired two-sided Student's  $t$  test.  $P < 0.05$  was considered significant. Where appropriate, comparisons of multiple treatment conditions with controls were analysed by ANOVA with the Dunnett's test for *post hoc* analysis.

## RESULTS

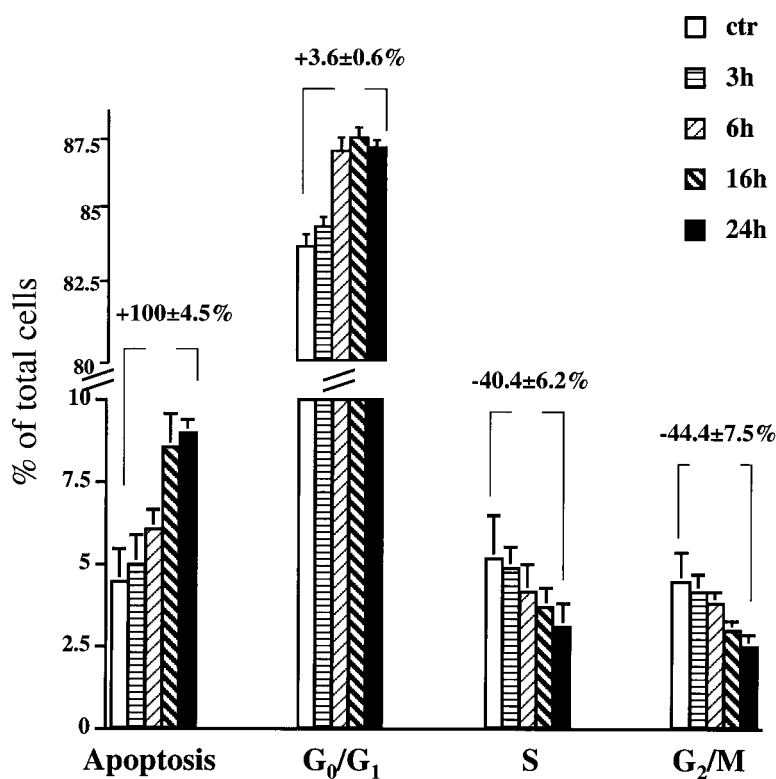
### EGCG inhibits cultured HSC growth

To begin to explore the effect of EGCG on activated HSC growth, serum-starved cells were maintained in DMEM with 0.4% FBS or stimulated with 10% FBS in the presence or absence of EGCG at the indicated concentrations for 8, 24 or 48 h. After washing, cell growth was determined by attached cell numbers. As shown in Figure 2, compared with cells maintained in DMEM with 0.4% FBS, 10% FBS significantly increased cell numbers, which was markedly reduced by EGCG. After 48 h incubation, compared with 10% FBS stimulation, EGCG at 20,



**Figure 3** EGCG significantly reduces incorporation of  $^3\text{H}$ thymidine and BrdU into cultured HSC chromosomal DNA

Passaged HSC were cultured in DMEM with 0.4% or 10% FBS containing EGCG at the indicated concentrations for 24 h. Methyl- $^3\text{H}$ thymidine (1  $\mu\text{Ci}/\text{ml}$ ) or BrdU (25  $\mu\text{g}/\text{ml}$ ) was added to the media several hours before harvesting cells (see the Materials and methods section for details). (A) Determination of  $^3\text{H}$ thymidine incorporation into chromosomal DNA of cultured HSC. Results (means ± S.D.) were expressed as c.p.m. from triplicate experiments. † $P < 0.05$  versus cells incubated in DMEM with 0.4% FBS; the value above the bar is the percentage increase in  $^3\text{H}$ thymidine incorporation stimulated by 10% FBS compared with cells grown in DMEM with 0.4% FBS. \* $P < 0.05$  versus cells incubated in DMEM with 10% FBS without EGCG treatment; values are percentage inhibition caused by EGCG compared with cells treated with 10% FBS only. (B) Representative of activated HSC stained by BrdU in random view fields (original magnification, × 100). (C) Percentage of BrdU-positively stained HSC treated with EGCG at the indicated concentrations. Values were expressed as means ± S.D., each of which was from 10 random views. \* $P < 0.05$  versus cells with no EGCG treatment.



**Figure 4** EGCG results in re-distribution of activated HSC cell cycle

Pre-confluent activated HSC were treated with EGCG (50  $\mu$ M) for the indicated times. After fixation and labelling with propidium iodide, cells positively labelled with propidium iodide were detected by flow cytometry. The percentage of cell numbers at each stage was from three independent experiments. The percentages shown are the difference in cell number caused by EGCG treatment for 24 h compared with cells with no EGCG treatment (control, ctr).

50 or 100  $\mu$ M in the media significantly reduced cell numbers by  $27.7 \pm 2.1$ ,  $58.3 \pm 1.4$  and  $67.6 \pm 1.5\%$ , respectively (Figure 2). EGCG toxicity to HSC was carefully studied in cultured HSC by examining LDH release. EGCG, compared with the control, made no significant difference in LDH release at concentrations up to 100  $\mu$ M (Table 1). Based on no detectable LDH release, and a rapid recovery of cell proliferation after withdrawal of EGCG (results not shown), it was concluded that EGCG up to 100  $\mu$ M was not toxic to cultured HSC. EGCG at 50  $\mu$ M was chosen for the following experiments. To elucidate mechanisms underlying EGCG inhibition of cultured HSC growth, we hypothesized that this antioxidant might reduce HSC proliferation and/or induce HSC apoptosis.

#### EGCG inhibits DNA synthesis in activated HSC

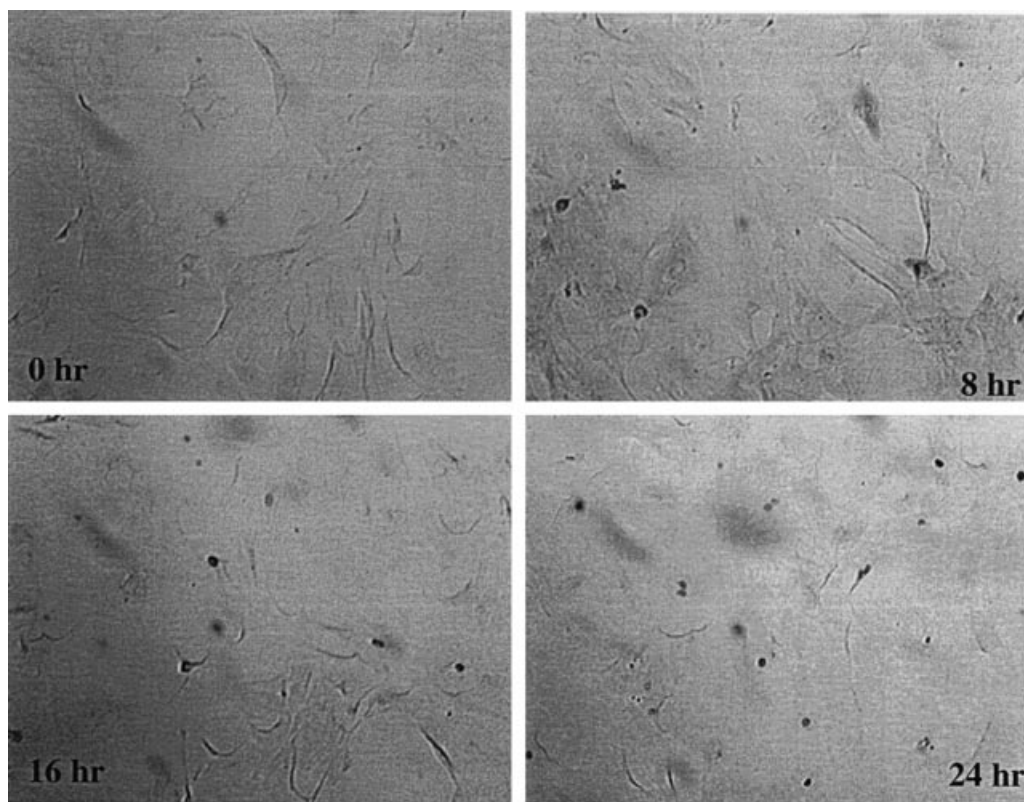
To evaluate the effect of EGCG on DNA synthesis and cell proliferation of cultured HSC, incorporation of methyl- $^3$ H]thymidine and BrdU into chromosomal DNA was examined. Serum-starved HSC were either maintained in serum-depleted media (0.4% FBS), or incubated in serum-rich media (10% FBS), with or without EGCG at the indicated concentrations for 24 h and pulsed with methyl- $^3$ H]thymidine. As shown in Figure 3(A), compared with 0.4% FBS, 10% FBS markedly stimulated incorporation of  $^3$ H]thymidine into chromosomal DNA of cultured HSC by 133%. EGCG at 20, 50 or 100  $\mu$ M significantly reduced the  $^3$ H]thymidine incorporation by  $14.3 \pm 1.4$ ,  $25.7 \pm 3.6$  and  $28.5 \pm 3.3\%$ , respectively, suggesting that EGCG inhibited

DNA synthesis and cell proliferation in a dose-dependent manner.

BrdU staining of cultured HSC confirmed that this antioxidant caused an apparent dose-dependent reduction in BrdU incorporation into chromosomal DNA (Figures 3B and 3C). Taken together, these results indicated that EGCG inhibited HSC proliferation in a dose-dependent manner.

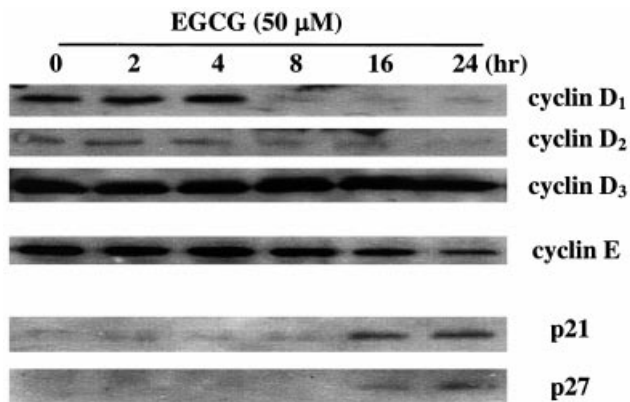
#### EGCG inhibits activated HSC growth by inducing cell cycle arrest and apoptosis

Flow-cytometric assays were carried out to further evaluate the effect of EGCG on the cell cycle of activated HSC. As shown in Figure 4, compared with no EGCG controls, this antioxidant, in a time-dependent pattern, altered the percentage of cells in the G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phases by  $+3.6 \pm 0.6$ ,  $-40.4 \pm 6.2$  and  $-44.4 \pm 7.5\%$ , respectively, after 24 h treatment. These results indicated that EGCG caused a cell cycle re-distribution of activated HSC by reducing cells staged at DNA synthesis and cell division, while increasing arresting cells. In addition, EGCG induced apoptosis of these activated HSC by 2-fold after 24 h treatment (Figure 4). TUNEL staining confirmed the effect of EGCG on induction of activated HSC apoptosis (Figure 5). Western blotting analyses further demonstrated that EGCG apparently inhibited the expression of cell cycle-stimulating proteins, including cyclin D<sub>1</sub> and D<sub>2</sub> and cyclin E, in cultured HSC, while causing no notable change in cyclin D<sub>3</sub> (Figure 6). In addition, EGCG induced a marked increase in cell cycle in-



**Figure 5 EGCG induces time-dependent HSC apoptosis**

Pre-confluent HSC cultured in DMEM with 10% FBS in slide flasks were treated with or without EGCG (50  $\mu$ M) for the indicated times. After fixation, apoptotic HSC were detected by the DeadEnd™ Colorimetric TUNEL System. Representatives of activated HSC stained by TUNEL are presented (original magnification,  $\times 100$ ).



**Figure 6 EGCG alters the expression of cell cycle proteins in activated HSC**

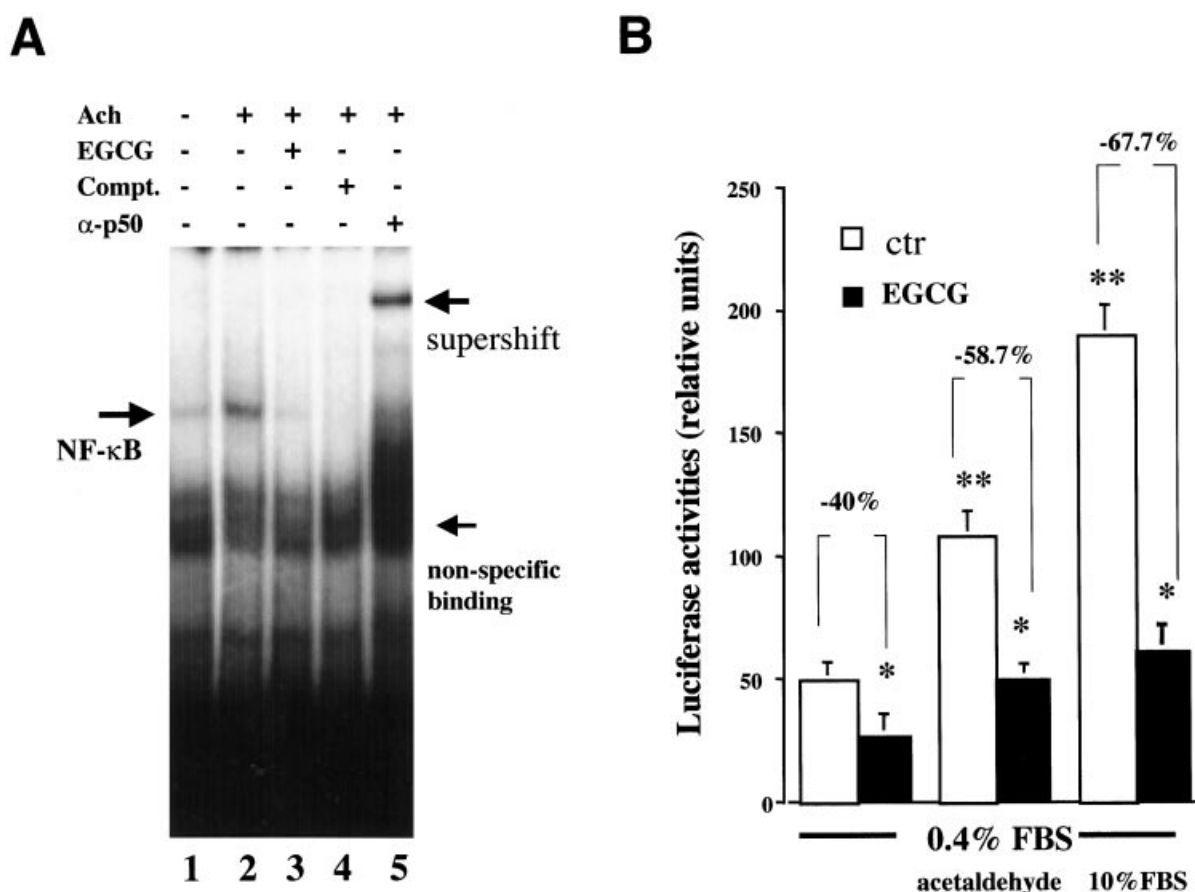
Whole-cell protein extracts were prepared from pre-confluent HSC treated with or without EGCG (50  $\mu$ M) for the indicated times. SDS/PAGE with 10% resolving gel was used to separate proteins (25  $\mu$ g/lane). Cell cycle proteins were detected by using primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Each result was representative of three independent Western blotting analyses. EGCG had no effect on cyclin D<sub>3</sub> protein expression, which was used as an internal control for equal protein loading.

inhibitory proteins, including p21<sup>(WAF1/Cip1)</sup> and p27<sup>(Kip1)</sup> (Figure 6). Taken together, these results revealed that EGCG inhibited HSC growth, at least, by inducing cell cycle arrest and apoptosis.

#### EGCG significantly inhibits NF- $\kappa$ B activity in activated HSC

Activation of NF- $\kappa$ B is closely associated with activation and survival of HSC [5,21,22]. Acetaldehyde, the major active metabolite of ethanol, directly induces HSC activation during alcohol-caused liver injury. Acetaldehyde induces oxidative stress and NF- $\kappa$ B activation in various cell types [23–25]. It was therefore of interest to elucidate the effect of the antioxidant EGCG on NF- $\kappa$ B activation induced by acetaldehyde in cultured HSC. As shown in EMSAs, compared with the control (Figure 7A, lane 1), acetaldehyde caused an apparent NF- $\kappa$ B-binding band (Figure 7A, lane 2). A 50-fold excess of the unlabelled probe markedly, if not completely, diminished the binding band in a competition assay (Figure 7A, lane 4), suggesting the specificity of the NF- $\kappa$ B binding to the probe. Anti-p50 antibodies ( $\alpha$ -p50), but not normal rabbit IgG (results not shown), caused a significant supershift and abolished the NF- $\kappa$ B-binding band (Figure 7A, lane 5). Treatment of these cells with EGCG (50  $\mu$ M) resulted in an apparent reduction in the density of the NF- $\kappa$ B-binding band (Figure 7A, lane 3), suggesting that EGCG reduced the NF- $\kappa$ B DNA-binding activity in cultured HSC.

To evaluate effects of EGCG on the NF- $\kappa$ B *trans*-activating ability, HSC were transfected with the NF- $\kappa$ B reporter plasmid pNF- $\kappa$ B-Luc. After recovery, cells were incubated in DMEM with 0.4 or 10% FBS, or 0.4% FBS plus acetaldehyde (200  $\mu$ M), with or without EGCG (50  $\mu$ M) for an additional 24 h (Figure 7B). Compared with 0.4% FBS, acetaldehyde or 10% FBS remarkably increased luciferase activities by 2.3- or 3.9-fold, respectively, in these cells. Pretreatment of cells with EGCG 3 h



**Figure 7** EGCG reduces NF- $\kappa$ B activity in activated HSC

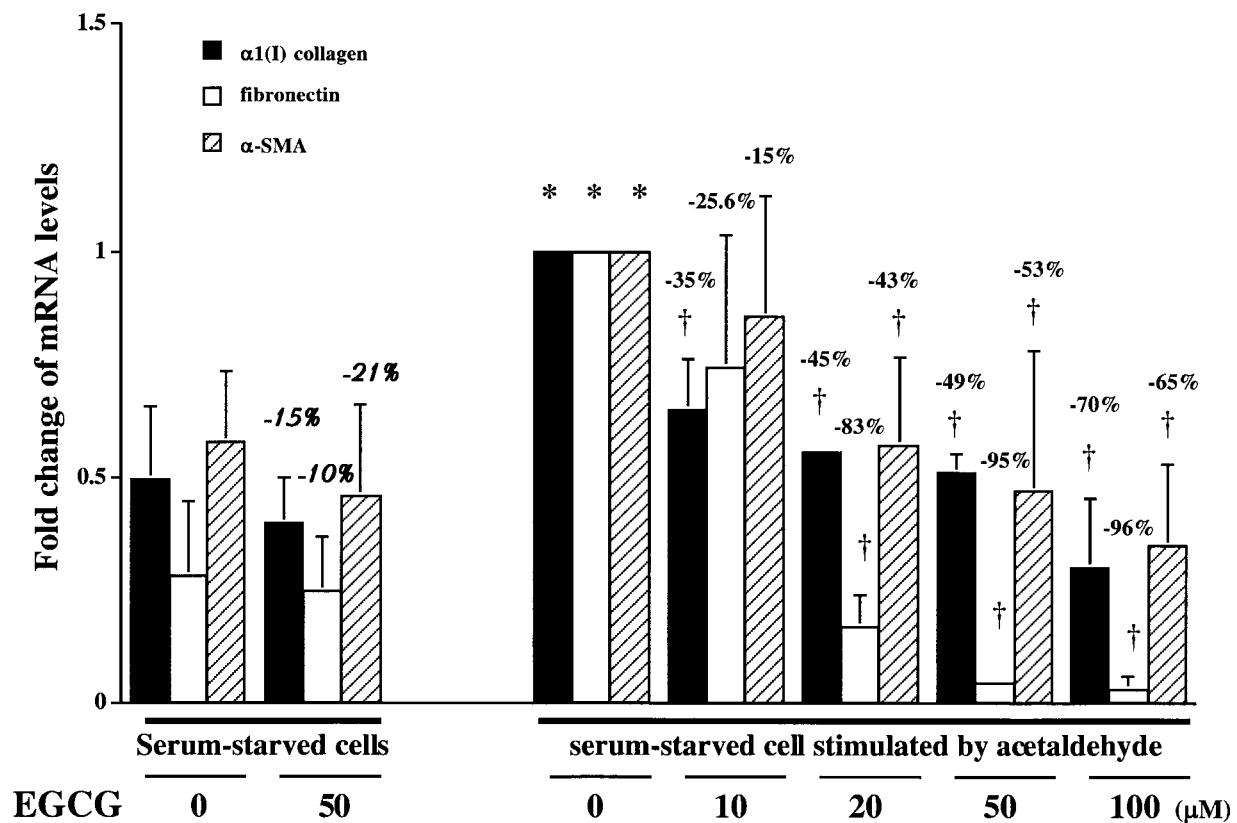
(A) The EGCG effect on NF- $\kappa$ B DNA-binding activity analysed by EMSA, using a  $^{32}$ P-labelled NF- $\kappa$ B-binding probe. Nuclear proteins were from cultured HSC treated with (lane 3) or without 50  $\mu$ M EGCG (lanes 1, 2, 4 and 5) in the presence (lanes 2-5) or absence (lane 1) of acetaldehyde (Ach; 200  $\mu$ M). A 50-fold excess of the unlabelled probe was used for the competition assay (Compt.; lane 4). Anti-p50 antibodies ( $\alpha$ -p50) were used for the supershift assay (lane 5). (B) The EGCG effect on the NF- $\kappa$ B *trans*-activating ability. After overnight recovery from transfection with pNF- $\kappa$ B-Luc, HSC were incubated in DMEM with 0.4% FBS with (black columns) or without (white columns) EGCG (50  $\mu$ M) 3 h prior to the addition of 10% FBS, or acetaldehyde (200  $\mu$ M), or nothing as a control, for an additional 24 h. Luciferase activities were expressed as relative units after  $\beta$ -galactosidase normalization ( $n \geq 6$ ). \* $P < 0.05$  versus corresponding cells without EGCG; \*\* $P < 0.05$  versus cells with no treatment. Values above bars are the percentage inhibition caused by EGCG compared with corresponding cells without EGCG treatment (control, ctr).

prior to the addition of acetaldehyde (200  $\mu$ M), or 10% FBS, significantly reduced luciferase activity by  $58.7 \pm 4.5$  or  $67.7 \pm 4.9\%$ , respectively, indicating that EGCG at 50  $\mu$ M inhibited the NF- $\kappa$ B *trans*-activating activity in cultured HSC. Taken together, these results demonstrated that EGCG inhibited NF- $\kappa$ B activation in activated HSC. In addition, compared with the inhibitory efficiency in cells stimulated by acetaldehyde ( $-58.7 \pm 4.5\%$ ) or 10% FBS ( $-67.7 \pm 4.9\%$ ), the inhibitory effect of EGCG on reducing luciferase activity was significantly diminished in serum-starved HSC ( $-40 \pm 2.5\%$ ). These results suggested that EGCG, with respect to inhibition of NF- $\kappa$ B activity, allowed a distinction to be made between cells with different activation status.

#### EGCG suppresses the expression of $\alpha$ 1(I) collagen, fibronectin and $\alpha$ -SMA induced by acetaldehyde

We and others have previously demonstrated that acetaldehyde induced the expression of ECM, including  $\alpha$ 1(I) collagen, fibronectin and  $\alpha$ -SMA in HSC [15,16,26]. To assess the effect of EGCG on the expression of genes induced by acetaldehyde, passaged HSC were serum-starved for 48 h prior to induction

with or without acetaldehyde (200  $\mu$ M) in the presence or absence of EGCG at the indicated concentrations for an additional 24 h. Real-time PCR revealed that, compared with the untreated serum-starved control, acetaldehyde increased the mRNA abundance of  $\alpha$ 1(I) collagen, fibronectin, and  $\alpha$ -SMA in these cells (Figure 8). EGCG treatment resulted in a marked dose-dependent reduction in the mRNA abundance increased by acetaldehyde (Figure 8), suggesting that EGCG inhibited the expression of these genes induced by acetaldehyde. Interestingly, the EGCG effect on inhibition of the expression of these genes was significantly diminished in serum-starved HSC without acetaldehyde stimulation (Figure 8). For example, compared with levels of mRNA in cells treated with acetaldehyde only, EGCG (50  $\mu$ M) caused a significant reduction in the mRNA levels of  $\alpha$ 1(I) collagen, fibronectin and  $\alpha$ -SMA by 49, 95 and 53%, respectively. In great contrast, compared with mRNA levels in cells without acetaldehyde treatment, EGCG at 50  $\mu$ M only resulted in a slight reduction in the mRNA levels of  $\alpha$ 1(I) collagen, fibronectin and  $\alpha$ -SMA by 15, 10 and 21%, respectively. This observation suggested that EGCG, regarding the inhibition of gene expression, might have an ability to distinguish cells based on their activation status. It bears mention that the expression of these



**Figure 8** EGCG suppresses the expression of genes induced by acetaldehyde

Passaged HSC were serum-starved for 48 h prior to the treatment with or without acetaldehyde (200 μM) in the presence or absence of EGCG at the indicated concentrations for an additional 24 h. The mRNA abundance of α1(I) collagen, fibronectin and α-SMA in the cells was determined by real-time PCR. Acetaldehyde or EGCG had no effect on GAPDH mRNA. The mRNA fold change was calculated by using GAPDH as an internal control (see the Materials and methods section for details). Values were expressed as means ± S.D. from three independent experiments. †*P* < 0.05 versus cells treated with acetaldehyde only, without EGCG; \**P* < 0.05 versus cells with no treatment. Values shown above the bars on the left-hand panel are the percentage inhibition caused by EGCG compared with cells grown in DMEM with 0.4% FBS, without EGCG; those on the right-hand panel are the percentage inhibition caused by EGCG compared with cells stimulated with acetaldehyde, without EGCG.

genes was at a relatively low level in these serum-starved cells. Without acetaldehyde induction, the EGCG effect on gene expression was hardly detectable by Northern blots (results not shown).

#### EGCG inhibits TGF-β signalling and reduces Tβ-RII mRNA induced by acetaldehyde in HSC

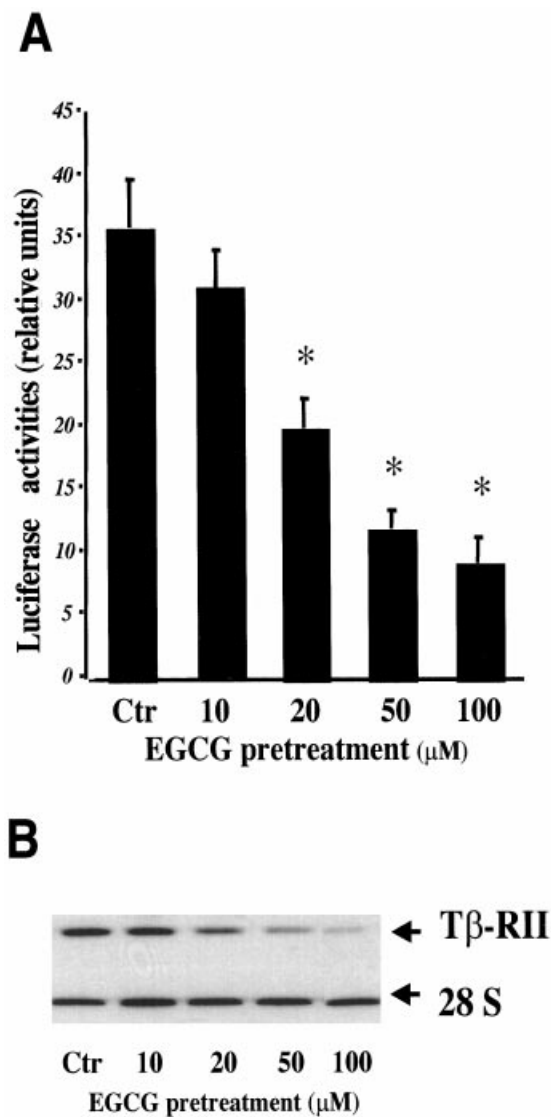
Our recent studies showed that acetaldehyde activated TGF-β signal transduction and induced the expression of the Tβ-RII gene in cultured HSC [26a], which made a significant contribution to α1(I) collagen gene expression in activated HSC. To elucidate mechanisms by which EGCG inhibited α1(I) collagen gene expression in cultured HSC, it was hypothesized that EGCG suppressed TGF-β signal transduction induced by acetaldehyde. To study this hypothesis, passaged HSC were transfected with p3TP-Lux, a TGF-β-inducible luciferase reporter plasmid. Cells were then pre-treated with EGCG at the indicated concentrations 3 h prior to the addition of acetaldehyde (200 μM). Compared with the control, which received no EGCG pre-treatment, EGCG caused a dose-dependent reduction in luciferase activity in these cells (Figure 9A), suggesting that EGCG inhibited acetaldehyde-activated TGF-β signal transduction. RPA further demonstrated that EGCG, in a dose-dependent fashion, apparently reduced

the level of Tβ-RII mRNA (Figure 9B), indicating that EGCG suppressed Tβ-RII gene expression induced by acetaldehyde.

#### DISCUSSION

To explore mechanisms of re-distribution of activated HSC cell cycle by EGCG, in the present studies we observed that this antioxidant reduced the expression of cyclins D<sub>1</sub>, D<sub>2</sub> and E, as well as induced the expression of p21<sup>(WAF1/Cip1)</sup> and p27<sup>(Kip1)</sup>. Similar EGCG effects on the expression of cell cycle proteins were observed in human epidermoid carcinoma cells (A431) [27]. Previous studies have indicated that the expression of particular cyclin Ds and activities of certain cyclin-dependent kinases (Cdk), as well as Cdk-inhibitory proteins (CKI), probably decided the fate of cells [28]. It was suggested that D-type cyclins might play critical roles in cell cycle progression, especially at early G<sub>0</sub>/G<sub>1</sub> phase [29,30]. Over-expression of cyclin D<sub>1</sub> in fibroblasts caused a short G<sub>1</sub> phase and dysregulation of gene expression, as well as enhanced tumorigenesis [31]. In contrast, microinjection of anti-cyclin D<sub>1</sub> antibodies or antisense cyclin D<sub>1</sub> cDNA prevented cells from entering the S phase [32]. These results collectively demonstrated that cyclin D<sub>1</sub> was a critical target of proliferative signals in G<sub>1</sub>. Another recent study suggested that cyclins D<sub>1</sub>, D<sub>2</sub>





**Figure 9** EGCG inhibits TGF- $\beta$  signal transduction and reduces the T $\beta$ -RII mRNA abundance increased by acetaldehyde in cultured HSC

Prior to (3 h) stimulation with 200  $\mu\text{M}$  acetaldehyde, EGCG at the indicated concentrations was added to HSC transfected with (A) or without (B) p3TP-Lux, a TGF- $\beta$ -inducible luciferase reporter plasmid (see the Materials and methods section for details). (A) Luciferase assays of HSC transfected with p3TP-Lux. Luciferase activities were analysed and expressed as relative units after  $\beta$ -galactosidase normalization ( $n \geq 6$ ). \* $P < 0.05$  versus cells treated with acetaldehyde, without EGCG. (B) RPA of endogenous T $\beta$ -RII mRNA of cultured HSC. 28 S rRNA was used as an internal control. Ctrl, control.

and E played a key role in transition of HSC cell cycle from the G<sub>1</sub> to the S phase [33].

Acute liver injury *in vivo* is first accompanied by activation and proliferation of HSC with no sign of apoptosis [34]. In the recovery phase, apoptotic HSC are detectable in parallel to a reduction in the total number of activated HSC present in the liver tissue [34]. Induction of apoptosis was recently proposed as a strategy to terminate proliferation of activated HSC for prevention of hepatic fibrogenesis [3,4]. The present study suggests that the reduction of cell numbers by EGCG might reflect a combined result of both inhibition of cell proliferation and induction of cell apoptosis, which could explain the apparent

difference in the percentage between cell numbers (Figure 2) and [<sup>3</sup>H]thymidine/BrdU incorporation (Figure 3). Further experiments in this report confirmed that EGCG not only inhibited activated HSC proliferation, but also induced apoptosis of these cells *in vitro*. Additional studies are necessary to elucidate effects of this antioxidant on regulating pro-apoptotic and anti-apoptotic proteins at levels of ligand bioavailability, receptor expression and signal generation, transduction and execution in activated HSC.

NF- $\kappa$ B has been described as a primary regulator and mediator of oxidative stress. It has been implicated in cell proliferation, cell cycle regulation and apoptosis [35]. Although the causal relationship remains unknown, previous studies demonstrated that activation and survival of HSC was closely associated with activation of NF- $\kappa$ B [5,21,22]. It was suggested that inhibition of NF- $\kappa$ B activation was a potential strategy for prevention and/or therapy of cirrhosis [21]. Previous studies indicated that oxidative stress, through inducing NF- $\kappa$ B activity, played an essential role in HSC activation [5]. Our present results demonstrated that EGCG significantly inhibited NF- $\kappa$ B activity induced by acetaldehyde in cultured HSC. In addition, as shown in Figure 7(B), EGCG allowed a distinction in the inhibition of NF- $\kappa$ B activity between serum-starved HSC with or without a stimulation to be made. Many previous studies believed that serum-starvation of activated HSC for 48 h rendered them quiescent [36,37]. The EGCG effect on differential inhibition of NF- $\kappa$ B activity has also been reported previously. For example, EGCG resulted in a dose-based differential inhibition of tumour necrosis factor- $\alpha$  and lipopolysaccharide-mediated activation of NF- $\kappa$ B in cancer cells, but not in normal cells [38,39]. Similarly, in this communication we observed that EGCG differentially inhibited the expression of ECM genes between serum-starved HSC, with or without acetaldehyde stimulation (Figure 9). The underlying mechanisms of the differential inhibition remain poorly understood.

Accumulating evidence has indicated a close link between alcohol and TGF- $\beta$ 1 in the process of hepatic fibrogenesis during liver injury [40,41]. Our recent studies observed that acetaldehyde activated TGF- $\beta$  signal transduction and induced the expression of T $\beta$ -RII, which played a marked role in acetaldehyde-increased expression of  $\alpha$ 1(I) collagen gene in HSC [26a]. The present results revealed that EGCG inhibited TGF- $\beta$  signalling and reduced the abundance of T $\beta$ -RII mRNA induced by acetaldehyde in cultured HSC, which, at least partially, explained the observation that EGCG inhibited the expression of the  $\alpha$ 1(I) collagen gene in HSC. Further experiments are necessary to elucidate the involved mediators and signal transduction pathways.

In summary, in this communication we have demonstrated for the first time that EGCG, a major component of green tea extracts, inhibits activated HSC growth, at least, by inducing cell cycle arrest and apoptosis. This antioxidant reduced the expression of cyclins D<sub>1</sub>, D<sub>2</sub> and E, while inducing the expression of p21<sup>(WAF1/Cip1)</sup> and p27<sup>(Kip1)</sup>. In addition, EGCG suppressed acetaldehyde-induced HSC activation, as demonstrated by inhibiting NF- $\kappa$ B activity, blocking TGF- $\beta$  signal transduction and reducing the expression of  $\alpha$ -SMA and ECM genes. Taken together, our results suggest that EGCG is a novel and effective inhibitor for activated HSC growth and activation *in vitro*. It should be emphasized that the results in this report were generated from cultured HSC, and that they might not necessarily and comprehensively reflect the situation in quiescent HSC *in vivo*. Additional experiments are necessary to determine effects of EGCG on cell growth and activation of quiescent HSC stimulated by alcohol/acetaldehyde *in vitro* and *in vivo*. It remains poorly

understood how EGCG could make a differential inhibition of NF- $\kappa$ B activation, or gene expression among cultured HSC with different activation status. Additional experiments, beyond the scope of this report, are necessary to investigate the underlying mechanism. Answers to this puzzle might provide a novel insight into the long history of green tea consumption without adverse health effects, while potentially preventing oxidative stress-related diseases. The characteristics of EGCG, including antioxidant potential, reduction of activated HSC growth, inhibition of NF- $\kappa$ B activation, induction of apoptosis and no adverse health effects, make it a potential anti-fibrotic candidate for treatment and prevention of hepatic fibrogenesis.

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