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Protective role of tretinoin and *N*-acetyl-L-cysteine from antiproliferative action of cigarette smoke extract on alveolar epithelial cells

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To investigate the mechanisms by which tretinoin and *N*-acetyl-L-cysteine (NAC) reverse the growth inhibition of alveolar epithelial cells induced by cigarette smoke extract (CSE), MTT assay was used to evaluate cell viability. It was observed that both tretinoin and NAC could restore the viability of CSE-inhibited A549 cells. By incubation with fluorescent indicator H_2DCFDA, it was documented that CSE-stimulated accumulation of intracellular reactive oxygen species (ROS) was obviously decreased by tretinoin or NAC. Furthermore, using semi-quantitative and real-time quantitative RT-PCR as well as western blot methods, high expression of insulin-like growth factor binding protein-2 (IGFBP-2) in A549 cells treated with CSE was found at both transcriptional and protein levels, and concomitant with the restoration of cell growth after treatment with tretinoin or NAC, down regulation of IGFBP-2 was observed. From the present study, it is concluded that both RA and NAC can antagonize CSE-induced growth arrest of alveolar epithelial cells and that down regulation of IGFBP-2 may play an important role in the process.

1. Introduction

Chronic obstructive pulmonary diseases (COPD) is a worldwide leading cause of morbidity and mortality and its prevalence is still rising (Van et al. 2004). It is therefore important to understand the development of this disease in order to develop strategies of prevention, treatment, and cure. Cigarette smoke extract (CSE) is a complex mixture of chemicals containing high levels of oxidants and is the major etiologic factor in the development of COPD, of which emphysema is a major component. Among the various cell types in the lung, epithelial cells of the alveolar structure appear to be a major target for oxidant injury (Tuder et al. 2000; Yuma et al. 2001; Kasahara et al. 2000). It is now well established that type II cells are the stem cells of the alveolar epithelium. After oxidant injury, the rapidity of initiation of type II cells proliferation is crucial for a proper healing. Therefore, characterization of the mechanisms involved in the block of type II cells replication by oxidants and in its reversibility appears to be critical for the understanding and management of many lung diseases associated with oxidative stress. In previous studies in a rat type II epithelial cell line, several components of the insulin-like growth factor (IGF) system have been reported involved in the control of proliferation, Particularly, accumulation of the IGF binding protein (IGFBP)-2 was associated with the block of type II cells proliferation caused by hyperoxia, serum deprivation and glucocorticoid and so on (Cazals et al. 1994; Mouhieddine et al. 1994, 1996). However, the mechanisms regulating human alveolar type II epithelial cell replication upon cigarette smoke exposure remain poorly understood.

Retinoids, including retinol and retinoic acid (RA) derivatives, have been shown to be involved in the processes of lung repair after injury. Nabeyrat et al. (1998) showed that treatment of serum-deprived type II cells with RA led to a stimulation of cell proliferation concomitant with a marked decrease in the expression of IGFBP-2. Interestingly, Besnard et al. (2002) observed that RA abrogated TNF-α-induced growth arrest and that this effect was also associated with a dramatic decrease in IGFBP-2 expression. These results suggest a possible protective role of RA through mechanisms involving modulation of IGFBP-2 production. However, whether RA participated in the modulation of the proliferation of CSE-treated alveolar type II epithelial cells has never been investigated. Besides, it has been documented that the antioxidant *N*-acetyl-L-cysteine (NAC), a glutathione precursor, can inhibit cigarette smoke-related apoptosis in the bronchial epithelium of cigarette smoke-exposed rats, and has been applied in patients with COPD in order to reduce symptoms, exacerbations and the accelerated lung function decline (Dekhuijzen 2004). In the present study, RA and NAC were used in parallel for the first time to observe their effect on the proliferation of CSE-induced alveolar epithelial cells as well as the expression of IGFBP-2.

To provide information on the influence of CSE on the repair capacity of the alveolar epithelium, we chose to analyze the effect of CSE on the proliferative response of alveolar type II epithelial cells. An alveolar type II epithelial cell line (A549) employed as surrogate to represent human

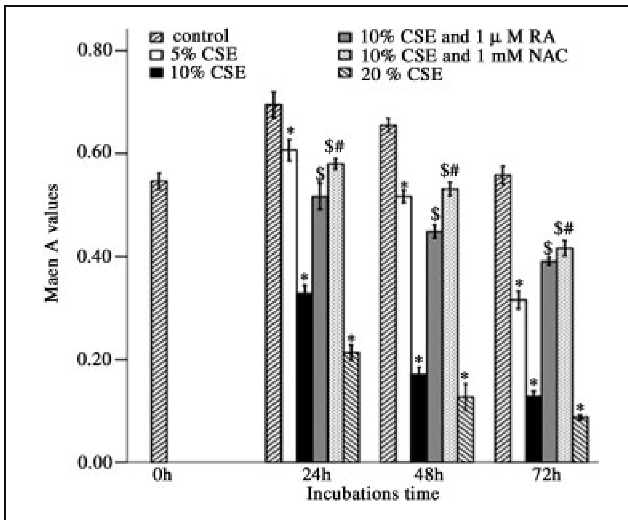

Fig. 1: The effect of CSE, and CSE with ATRA or NAC on A549 cells. After incubated with culture medium, 5% CSE, 10% CSE, 10% CSE + 1 μM ATRA, 10% CSE + 1 mM NAC and 20% CSE for 24 h, 48 h or 72 h respectively, cell viability was evaluated by MTT assay. OD represents optical density. * $P < 0.05$ versus control, $ $P < 0.01$ versus 10% CSE group; # $P < 0.05$ versus group treated with 10% CSE + 1 μM ATRA

alveolar type II epithelial cell. Our findings documented an antiproliferative action of CSE, and for the first time, we found the effect was associated with an increased expression of IGFBP-2. To discover any protective role of RA or NAC to reverse the CSE effect, we also examined the consequence of RA or NAC treatment on CSE-induced growth inhibition of type epithelial cells. We provided data indicating that both RA and NAC were able to restore the proliferative capabilities of the cells through possible mechanisms that involve a down regulation of IGFBP-2.

2. Investigations and results

2.1. Effects of CSE on cell viability

CSE reduced cell viability in a time- and concentration-dependent manner (Fig. 1). In the presence of 5% CSE, cell viability was reduced to 78.89% and 56.57% at 48 h and 72 h of incubation, respectively. After 48 h or more hours of incubation, CSE at concentrations of 10% or more reduced cell viability to 15.68%. After co-incubation with 1 μM RA or 1 mM NAC, the cell viability of 10% CSE group was greatly restored as presented in Fig. 1, and it was showed NAC was more effective than ATRA in reversing the growth of alveolar epithelial cells caused by CSE.

2.2. Levels of intracellular reactive oxygen species (ROS)

After incubation with CSE from 5% to 20%, 10% CSE +1 μM ATRA and 10% CSE + 1 mM NAC for 3 h, intracellular ROS levels were detected by flow cytometry. As shown in Fig. 2, CSE-treated cells had higher and higher ROS levels concomitant with the increase of CSE concentration. However, when NAC or ATRA was added together with 10% CSE, the intracellular ROS levels were dramatically decreased, and NAC displayed a greater potential in deleting intracellular ROS than ATRA.

2.3. Semi-quantitative and real-time quantitative RT-PCR

The mRNA levels of IGFBP-2 were examined after A549 cells were stimulated with CSE for 48 h. With 5%, 10% or 20% CSE treatment, the mRNA levels of IGFBP-2 in

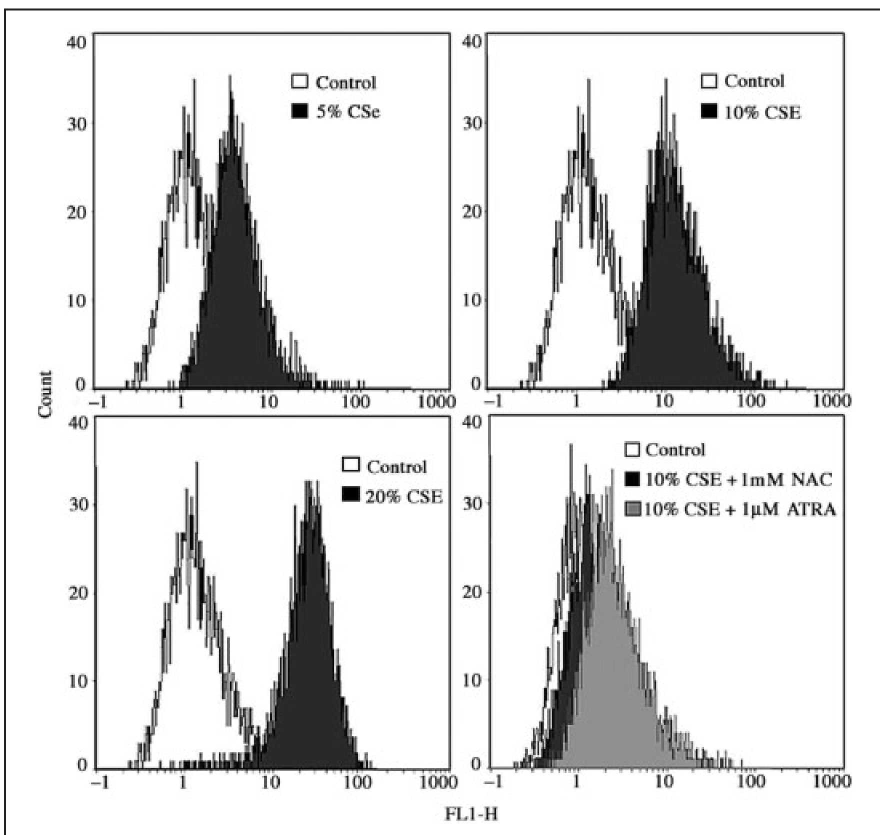

Fig. 2: Intracellular ROS levels of A549 cells after treated with CSE, and CSE plus NAC or ATRA. Intracellular ROS was detected by incubation with peroxide-sensitive fluorescent indicator H_2DCFDA and fluorescence was determined by flow cytometry analysis

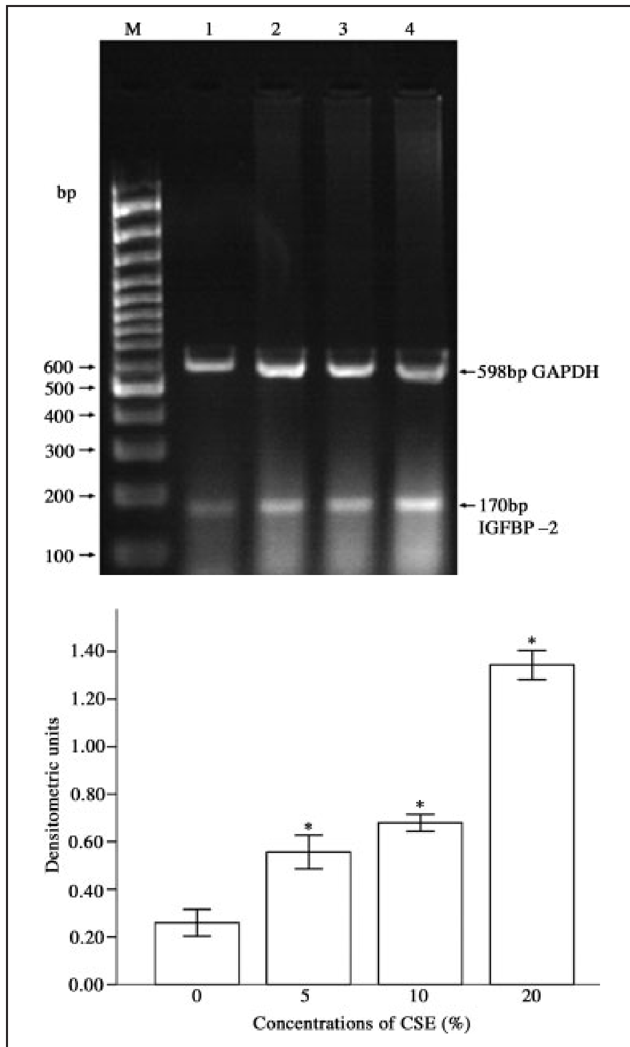

Fig. 3: The mRNA levels of IGFBP-2 in A549 cells treated with CSE. After incubated with different concentrations of CSE for 48 h, total RNA was extracted and RT-PCR was performed as described in the Experimental part. A: lane 1–4 are the bands of control, 5%, 10% and 20% CSE groups respectively, M: DNA marker, bp: base pair. This is a representative of three independent experiments. B: histograms are a quantitative representation of mRNA levels of IGFBP-2, data were obtained by densitometric measurement, then normalized to an endogenous control GAPDH and presented as means ± SD. The results are expressed in arbitrary densitometric units. * $P < 0.01$ compared with control group

alveolar epithelial cells reached, 2.15, 2.62 and 5.15-fold of control group, respectively (Fig. 3), which indicated that CSE treatment augmented the expression of IGFBP-2 at transcriptional level in a dose-dependent manner.

After co-incubation with 1 μM ATRA or 1 mM NAC for 48 h, using a real-time quantitative RT-PCR method, it was observed that both ATRA and NAC could reverse the CSE-induced high expression of IGFBP-2 at transcriptional level (Fig. 4).

2.4. *Western blot*

After A549 cells were stimulated with CSE for 48 h, the expression of IGFBP-2 was increased to 1.42, 2.56 and 2.98-fold of the untreated control group with 5%, 10% or 20% CSE treatment, respectively. After co-incubation with 1 μM ATRA or 1 mM NAC, the expression of IGFBP-2 was reduced by 33.6% and 47.0% respectively compared with 10% in the CSE treatment group (Fig. 5).

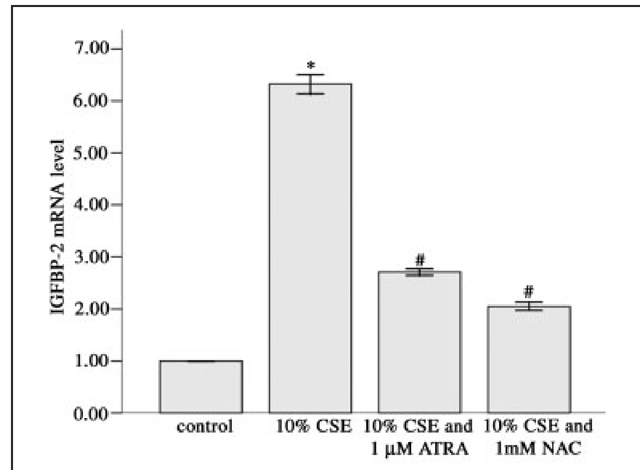

Fig. 4: The mRNA levels of IGFBP-2 in A549 cells treated with 10% CSE, 10% CSE combined with ATRA or NAC. After incubated with culture medium, 10% CSE, 10% CSE + μM ATRA and 10% CSE + 1 mM NAC for 48 h, the IGFBP-2 mRNA levels were detected using real-time quantitative RT-PCR method as described in the Experimental part, and presented as mean ± SD. The value of untreated cells was set at 1. * $P < 0.01$ versus control, # $P < 0.05$ versus 10% CSE group

3. Discussion

The chemical composition of cigarette smoke is complex, and therefore it is not easy to predict which compounds or combinations, may be involved in its toxicity. Recently, it has been reported that there are many ROS inducers among numerous chemical components of cigarette smoke (Onoue et al. 2004). In addition, cigarette smoke is a rich source of ROS. Oxidative stress is one of the classical signals for cell death, tightly linked with induction of several death genes and signaling pathways. It has been suggested that cigarette smoking increases apoptosis or necrosis through the activation of ROS and oxidative stress (Wang et al. 2000; Carnevali et al. 2003; Howard et al. 1998). In the present study, it was found that the inhibition of cell viability was accompanied with rapid and accelerating induction of intracellular ROS. Taken together, the inhibitory effect of CSE on alveolar epithelial cells might be initiated by oxidative stress.

In this experiment it was found for the first time that IGFBP-2 participated in the cellular response of human lung epithelial cells to CSE. Studies are now in progress to define precisely the mechanisms by which IGFBP-2 expression is induced during the process of cell growth inhibition caused by oxidative stress. Cazals et al. (1999) demonstrated that exposure of rat alveolar type II epithelial cells to hyperoxia was associated with accumulation of the active form of NF-kappa B, and that NF-kappa B could activate IGFBP-2 promoter in transient transfection assays. Considering the phenomenon was the result of oxidative stress caused by hyperoxia, which may also be applied to human alveolar epithelial cells induced by CSE. Another question is how IGFBP-2 blocks the progression of alveolar epithelial cells through the cell cycle. Purified human IGFBP-2 has been reported to inhibit the ability of IGF-II to stimulate DNA synthesis. In addition to acting as a modulator of IGF actions, recent reports strongly suggest that IGFBP may have direct cellular effects that are not dependent on the IGFs (Chadelat et al.1998). The presence of the arginine-glycine-aspartic acid (RGD) integrin recognition sequence in IGFBP-2 has led to the suggestion that this protein may bind to cell surfaces *via* one or more integrin receptors,

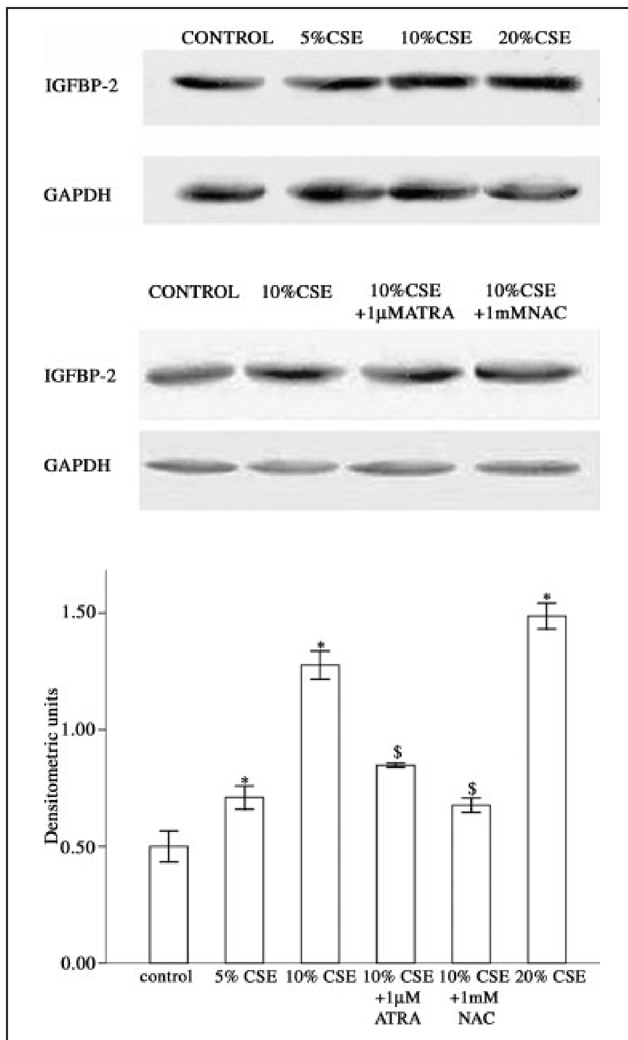

Fig. 5: The expression levels of IGFBP-2 in A549 cells. After incubated with different concentrations of CSE, 10% CSE + 1 μM ATRA and 10% CSE + 1 mM NAC for 48 h, total cellular protein was extracted and Western blot was performed as described in the Experimental part. A: The results of control, 5%, 10% and 20% CSE groups respectively; B: The results of control, 10% CSE, 10% CSE + 1 μM ATRA and 10% CSE + 1 mM NAC groups respectively, GAPDH was used as an endogenous control. C: Histograms are a quantitative representation of IGFBP-2 expression, data were obtained by densitometric measurement, then normalized to GAPDH and presented as means ± SD, the results are expressed in arbitrary densitometric units. * $P < 0.01$ versus control; $ $P < 0.05$ versus 10% CSE group

leading to the transmission of intracellular signals. Besides, Besnard et al. (2001) reported the presence of IGFBP-2 in the nucleus together with an increased intracellular expression of IGFBP-2 in cells exposed to O_2. Indeed, this cellular localization of IGFBP-2 has never been reported previously, concerning the mechanisms by which IGFBP-2 can enter the nucleus and can be linked to the key regulators of the cell cycle machinery in alveolar type II cells, experiments are in progress to provide answers to these questions. Therefore, it was strongly suggested that IGFBP-2 might play an important role in the control of various cellular functions through IGF-dependent and IGF-independent mechanisms, further studies of its properties and expression are warranted.
It was observed in the study that both RA and NAC could restore the growth of CSE-treated alveolar epithelial cells as well as the elevated expression of IGFBP-2 at both transcriptional and protein levels. The mechanisms by which they can be involved in lung growth and repair remain poorly understood, but it is likely that the pulmonary epithelium represents a major target. Manzano et al. (1999) found tretinoin-induced stimulation of antioxidant defenses including an increased gene transcription of catalase mRNA and γ-glutamyl-cysteine synthetase (γ-GCS) mRNA. Concerning the down regulation of intracellular ROS level by ATRA in the study, it could not be excluded that ATRA may share similar mechanisms in protecting CSE-induced alveolar epithelial cells, and the observed down regulation of IGFBP-2 may be the result of the antioxidation. NAC is a precursor of L-cysteine and the glutathione pathway, and a scavenger of free radicals because it interacts with ROS (Zafarullah et al. 2003), this was consistent with our experimental results. These support several conclusions.
First, CSE exposure causes growth inhibition of alveolar epithelial cells. Second, IGFBP-2 is negatively correlated with the proliferation of CSE-treated alveolar epithelial cells. Finally, both RA and NAC can antagonize CSE-induced growth arrest of alveolar epithelial cells and the down regulation of IGFBP-2 may play an important role in the process. What we found in the present study provides new proofs for the application of RA and NAC in ameliorating CSE-induced lung injuries. Nevertheless, the mechanisms by which CSE as well as RA and NAC regulate the growth of alveolar epithelial cells still need to be further clarified.

4. Experimental

4.1. Chemicals and reagents

Fetal bovine serum (FBS; Sijiqing, Hangzhou, China), Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT). Monoclonal anti-human IGFBP-2 antibody (R&D Systems, Inc., Minneapolis, MN), TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA). Cigarettes of a domestic brand (Lan Zhou) were purchased from Lanzhou Tobacco (Lanzhou, China). 3-(4,5-Dimethylthiazal-z-yl)-2,5-diphenylterazolium (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), all-trans retinoic acid (ATRA) and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma Chemical Company (St Louis, MO, USA). AMV one-step RT-PCR amplification kit, and primers for IGFBP-2 and glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) amplification were from Shanghai Sangon Biological Engineering & Technology and Service Co., Ltd. SYBR® RT-PCR kit and primers of IGFBP-2 and GAPDH for real time quantitative PCR were from TaKaRa Biotechnology Co., Ltd (Dalian, Liao Ning, China). Total protein extraction reagents, BCA quantification kit, horseradish peroxidase (HRP)-conjugated anti-mouse IgG and enhanced chemiluminescence system were provided by Shanghai Kangcheng Biological Engineering & Technology Co., Ltd. (Shanghai, China). All chemicals were of analytical reagent grade.

4.2. Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared by a modification of the method of Harvey and Aaron (1978). In brief, two cigarettes without filters were combusted with a modified syringe-driven apparatus. The smoke was bubbled through 50 ml of serum-free DMEM, and the resulting suspension was adjusted to pH 7.4 and then filtered through a 0.22 μm pore filter to remove bacteria and large particles. The resulting CSE was applied to epithelial cell cultures within 30 min of preparation.

4.3. Cell culture

The human type II alveolar epithelial cell line A549 was obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). A549 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-Glutamine in a humidified incubator with 5% CO_2 at 37 °C. The culture medium was renewed every 2 to 3 days.

4.4. Analysis of cell viability

Cell viability was determined by an MTT assay (Sladouski 1993). Cells were seeded in 96-well plates at 1×10^4 cells per well and cultured in DMEM containing 10% FBS until the cells attached. The medium was then changed to serum free for 16 h until the cells had grown to 70% ~ 80% confluence. The cells were then treated with different concentrations of CSE (5%, 10%, or 20% of CSE), 1 mM NAC + 10% CSE and 1 μM ATRA + 10% CSE for 24 h, 48 h and 72 h respectively in serum free medium, and the group with

only culture medium was considered as control. A total 10 μl of 5 g/L MTT was added to each well at the end of indicated times, and incubated for another 4 h at 37 °C. After the medium was removed, cells and dye crystal formazan were solubilized with 150 μl dimethyl sulphoxide (DMSO), and absorbance was measured at 570 nm by using a Bio-Tek Powerwave microplate reader (BioTek Instruments, Inc., USA).

4.5. Intracellular ROS measurement

Intracellular reactive oxygen species (ROS) levels were measured with the use of fluorescent dye H_2DCFDA staining method (Chan et al. 2005). H_2DCFDA is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS (Sauer et al. 2001). Cells of different groups were cultured in flasks, after treatment for 3 h, cells were washed once with PBS and then harvested with trypsinase, subsequently washed again with PBS and then resuspended in 1 ml of PBS. Cells were incubated with 20 μmol/L H_2DCFDA dissolved in PBS in the dark at 37° for 30 min and then washed twice with PBS and analyzed by flow cytometry.

4.6. Western Blot Analysis

After cells were treated with different concentrations of CSE, 10% CSE with or without RA or NAC for 48 h, then total protein was extracted using protein extraction reagent according to manufacturer's protocol. The concentrations of protein were determined using the BCA protein assay kit. Equal amounts of proteins (50 μg/well) were subjected to electrophoresis using 20% sodium dodecyl sulfate-polyacrylamide gels. Following electrophoretic transfer of proteins onto polyvinylidene difluoride membrane, then incubated with primary antibodies at 1/1000 (IGFBP-2) or 1/10000 (GAPDH), washed three times with TBS/T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The protein-antibody complexes were detected using the enhanced chemiluminescence according to the manufacturer's recommended protocol.

4.7. Semi-quantitative and real-time quantitative RT-PCR analysis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Semi-quantitative reverse transcription-polymerase chain reaction(RT-PCR) was performed with AMV one-step RT-PCR amplification kit according to the manufacturer's instructions. The primers for IGFBP-2 mRNA were 5′-ATGGCGATGACCACTCAGAAGG-3′ (forward), 5′-ACCCTTGCCCATCTGCCG-3′(reverse), and amplified fragment was 170 bp; The primers for GAPDH mRNA were 5′-CCACCCATGG-CAAATTCCATGGCA-3′ (forward), and 5′-TCTAGACGGCAGGT-CAGGTCCACC-3′ (reverse) and amplified fragment was 598 bp. Reactions were incubated at 40°, 30 min for reverse transcription and 94 °C, 2 min for pre-denaturation, then amplified using thermal cycler PTC-220 (MJ Research, Inc., MA, USA), and amplification conditions were as following respectively: 95°, 15 s; 55°, 40 s; 72°, 1 min for 30 cycles to IGFBP-2 and 95 °C, 15 s; 61.5°, 40 s; 72°, 1 min for 30 cycles to GAPDH, then elongated at 72 °C for 7 min, then the PCR products were analyzed in ethidium bromide-stained 1.8% agarose gels. Real-time RT-PCR was performed with SYBR® RT-PCR kit (TaKaRa Cat# DRR045S) and detected with real time analysis and amplification system RG-3000 (Corbett Research Pty Ltd, Mortlake, Australia). Reverse transcription was performed according to manufacturer's instructions. A 25 μl reaction mixture contained cDNA template which was equal to 100 ng of total RNA, 12.5 μl SYBR® Premix Ex Taq™ (2×), 0.2 μM forward and reverse primers (5′-AGCATCACCTTGGCCTGGAG-3′ forward, 5′-AGGCCATGCTTGTCA-CAGTTG-3′ reverse for IGFBP-2 mRNA and 5′-GCACCGTCAAGGCT-GAGAAC-3′ forward, 5′-ATGGTGGTGAAGACGCCAGT-3′ reverse for GAPDH mRNA). Reactions were incubated at 95° for 10 s, and then amplified for 45 cycles (95° for 5 s followed by 60° for 20 s in each cycle). GAPDH mRNA levels provided an endogenous control for PCR quantification studies. As the amplification efficiency of IGFBP-2 mRNA was approximately equal to that of GAPDH mRNA, the relative quantification of IGFBP-2 mRNA was expressed using the $2^{-\Delta\Delta Ct}$ (fold difference) method (Burkhardt et al. 2006) upon normalization to GAPDH mRNA and relative to a calibrator (untreated control). Ct is the cycle number at which the amount of amplified gene of interest reaches a fixed threshold, $\Delta Ct = Ct$ of target gene (IGFBP-2) − Ct of endogenous control (GAPDH), and $\Delta\Delta Ct = \Delta Ct$ of samples − ΔCt of calibrator.

4.8. Statistical analysis

All experiments were repeated three times with duplicate samples each time. Results were presented as means ± SD. Statistical analysis was performed using one-way ANOVA test and statistical significance is defined as $P < 0.05$.

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