Indole-3-carbinol activates the cyclin-dependent kinase inhibitor $p15^{INK4b}$ gene

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Abstract Indole-3-carbinol (I3C) is a naturally occurring compound found in vegetables such as broccoli and cauliflower, and has been shown to arrest human tumor cells in the G1 phase of the cell cycle. However, the molecular mechanism responsible for this effect has not been sufficiently elucidated. We report here that I3C activates the cyclin-dependent kinase (CDK) inhibitor $p15^{INK4b}$ gene through its promoter, accompanied by cell growth inhibition in HaCaT cells. Treatment with I3C almost did not affect the expressions of the other CDK inhibitors such as $p19^{INK4d}$, $p21^{WAF1}$ and $p27^{Kip1}$. These results suggest that $p15^{INK4b}$ is an important molecular target of I3C among CDK inhibitors.

Keywords: $p15^{INK4b}$; Indole-3-carbinol; G1 arrest; Promoter; RB; HaCaT

1. Introduction

Cell-cycle progression is regulated by proteins called cyclins and cyclin-dependent kinases (CDKs) that associate with each other [1]. The process of cyclin-dependent activation of CDKs is counterbalanced by CDK inhibitors [2]. Two families of CDK inhibitors were identified in mammalian cells and each has a different mode of action. One group, comprised of related proteins known as $p21^{WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$, inhibit cyclin E/A-CDK2 complexes [3]. The second family of CDK inhibitors is also called INK4 protein family, and its members, designated $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$, directly bind to CDK4/6 and are specific inhibitors of cyclin D-dependent kinases [4–6].

Indole-3-carbinol (I3C) is a naturally occurring compound found in vegetables of the *Brassica* genus such as broccoli, brussel sprouts and cauliflower [7,8]. When administered in the diet, I3C significantly decreases the incidence of spontaneous and chemically induced mammary tumors in mice [9,10]. I3C exerts antiproliferative activity in human cancer cells and inhibits cell adhesion, migration and invasion [11].

I3C induces cell-cycle arrest in the G1 phase [12–14]. However, the molecular mechanism responsible for this effect has not been sufficiently elucidated. We therefore examined the effect of I3C on the expressions of CDK inhibitors in human immortalized keratinocyte HaCaT cells. We show that I3C activates the CDK inhibitor $p15^{INK4b}$ gene through its promoter, accompanied by cell growth inhibition. Treatment with I3C almost did not affect the expression of other CDK inhibitors such as $p19^{INK4d}$, $p21^{WAF1}$ and $p27^{Kip1}$. These results suggest that $p15^{INK4b}$ is an important molecular target of I3C among CDK inhibitors.

2. Materials and methods

2.1. Cell culture and reagents

HaCaT cells (a gift from Dr. N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany) were maintained in DMEM containing 10% fetal bovine serum and 2 mM glutamine and antibiotics (penicillin/streptomycin), and were incubated at 37 °C in a humidified atmosphere with 5% CO_2. I3C (Wako, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO).

2.2. Analysis of cell-cycle progression

Unsynchronized HaCaT cells were exposed to I3C for 48 h. The cells were then treated with Triton X-100 and their nuclei were stained with propidium iodide before DNA content was measured using a Becton Dickinson FACS Calibur (Becton Dickinson, Mountain view, CA). At least 10 000 cells were counted and the ModFit LD V2.0 software package (Becton Dickinson, Franklin Lakes, NJ) was used to analyze the data.

2.3. Detection of apoptosis

Unsynchronized HaCaT cells were exposed to I3C for 48 or 72 h. The cells were then treated with Triton X-100 and their nuclei were stained with propidium iodide before DNA content was measured using a Becton Dickinson FACS Calibur (Becton Dickinson). At least 10 000 cells were counted and the ModFit LD V2.0 software package (Becton Dickinson) was used to analyze the data. DNA fragmentation was quantified by the percentage of cells with hypodiploid DNA (sub-G1).

2.4. Protein isolation and Western blot analysis

Cells were lyzed in lysis buffer (50 mM Tris–HCl (pH 7.5), 1% SDS). The protein extract was then boiled for 5 min and loaded onto a 12% (for $p15^{INK4b}$, $p19^{INK4d}$, $p21^{WAF1}$, $p27^{Kip1}$ and CDK6 detection) or 7% (for RB detection) polyacrylamide gel, electrophoresed and transferred to a nitrocellulose membrane. Rabbit anti-human $p15^{INK4b}$ polyclonal antibody (pAb) (C-20, Santa Cruz Biotechnology, CA), mouse anti-human $p19^{INK4d}$ mAb (DCS-100, NeoMarkers, Union City, CA), mouse anti-human $p21^{Cip1/Waf1}$ mAb (clone 6B6, Pharmingen, NJ),

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rabbit anti-human $p27^{Kip1}$ pAb (C-19, Santa Cruz Biotechnology, CA), mouse anti-human CDK6 mAb (DCS-83, MBL, Nagoya, Japan) and mouse anti-human pRB mAb (clone G3-245, Pharmingen) were used as the primary antibody. The signal was then developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK Limited).

2.5. RNA isolation and Northern blot analysis

Total RNA was isolated using a Sepasol RNA isolation kit (Nacalai Tesque Inc., Kyoto, Japan) and poly $(A)^+$ mRNA was separated from 100 μg of total RNA using an Oligotex™-dT30 ⟨Super⟩ mRNA purification kit (Takara Bio Inc., Shiga, Japan). Poly $(A)^+$ mRNA was fractionated on 1% agarose gels, transferred to nylon filters, and probed according to standard procedures [15]. Exon 1 of *p15* cDNA was used as a probe. Northern blot analysis was performed using standard methods [15] and mRNA levels were determined using a Fuji Image Analyzer Bas 2000 (Fujix, Tokyo, Japan).

2.6. Transient transfection and luciferase assay

The $p15^{INK4b}$-luciferase fusion plasmid [16] was co-transfected with pRL-TK (Promega, WI, USA) for standardization by Renilla luciferase activity. For each 24-well culture plate, HaCaT cells (5×10^4 cells) were transfected with 0.816 μg of DNA (0.8 μg of $p15^{INK4b}$-luciferase fusion plasmid and 0.016 μg of pRL-TK) using Lipofectin reagents (Invitrogen, Groningen, Netherlands). After 22 h, I3C was added, and 46 h after transfection the cells were harvested. The luciferase activity of each cell lysate was measured using a Dual-Luciferase Reporter Plasmid System (Promega, WI). The firefly luciferase activity of each sample was normalized by its Renilla luciferase activity and the fold activation was obtained by setting the control value to 1.0. All transfection assays were carried out in triplicate. The data are shown as the means ± S.D., and were analyzed using the Student's *t*-test. A *P*-value of less than 0.05 was considered to be statistically significant.

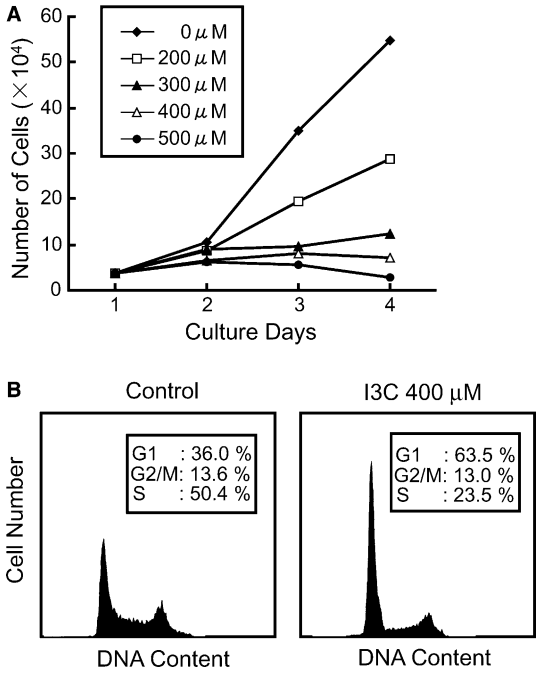

Fig. 1. Effect of I3C on the growth and cell cycle of HaCaT cells. (A) One day after inoculation of HaCaT cells, I3C at 200, 300, 400 or 500 μM was added, and cell growth was compared with a control cell culture with equivalent DMSO. The number of viable cells was counted by the Trypan-blue dye exclusion test. (B) Unsynchronized cells were incubated in the presence of DMSO or 400 μM I3C for 48 h, and the DNA content of the cells was determined by flow cytometry.

3. Results

3.1. I3C induces growth arrest in the G1 phase in HaCaT cells

We first examined the effect of I3C on the proliferation of human immortalized keratinocyte HaCaT cells. I3C inhibited the proliferation of HaCaT cells in a dose-dependent manner and 400 μM I3C had a cytostatic effect (Fig. 1A). To investigate the effect of I3C on cell-cycle progression, the DNA content of cell nuclei was measured by flow cytometric analysis. Treatment with I3C increased the percentage in the G1 phase, and decreased that in the S phase (Fig. 1B). This shows that I3C arrests the cell cycle of HaCaT cells at the G1 phase. Furthermore, 500 μM of I3C reduces the number of HaCaT cells within 3–4 days of the treatment. To examine whether I3C induces apotosis in these cells, we detected Sub-G1 populations (apoptotic cells) using flow cytometry analysis. Treatment with I3C (500 μM) for 3 days had no effect on Sub-G1 populations compared to vehicle only (data not shown). However, 500 μM of I3C for 4 days induced a significant increase of Sub-G1 populations (48%) compared to that with vehicle only (4%). This result shows that high concentrations of I3C induce apoptosis in HaCaT cells.

3.2. I3C increases $p15^{INK4b}$ mRNA and protein levels in HaCaT cells

We investigated whether I3C affects $p15^{INK4b}$ gene expression in HaCaT cells and found that I3C stimulates the expression of $p15^{INK4b}$ mRNA in a dose-dependent manner (Fig. 2A). The longer transcript is about 3.2 kb and the shorter one is about 2.2 kb. These two transcripts are detected in a previous report [4]. This is consistent with the result that I3C inhibited the growth of HaCaT cells in a dose-dependent manner (Fig. 1A). The time course study indicated that $p15^{INK4b}$ mRNA was up-regulated 12 h after treatment with I3C (Fig. 2B). Maximal induction was attained 36 h after treatment (Fig. 2B).

Next, we tried to elucidate whether the expression of $p15^{INK4b}$ protein could also be induced by treatment with I3C in HaCaT cells. We found that treatment stimulated $p15^{INK4b}$ protein expression in a dose-dependent manner (Fig. 2C). The time course study showed that expression was significantly increased 24 h after treatment (Fig. 2D). The effect of I3C on p15 protein reaches a peak 36 h after treatment, and declined at 48 h (Fig. 2D). This suggests that activation of p15 gene expression by I3C may decrease or p15 protein may degrade 48 h after treatment with I3C.

$p15^{INK4b}$ is a specific inhibitor of cyclin D-dependent kinases and the subsequent dephosphorylation of RB protein causes G1 arrest [4]. Therefore, we examined whether I3C can alter the phosphorylation status of RB protein in HaCaT cells by Western blotting. A hyperphosphorylated form of RB protein was converted into a hypophosphorylated form 24–36 h after treatment (Fig. 2E).

Taken together, these results indicate that I3C upregulates $p15^{INK4b}$ mRNA and protein levels and, subsequently, a hyperphosphorylated form of the RB protein is converted into a hypophosphorylated form in HaCaT cells.

3.3. I3C activates $p15^{INK4b}$ promoter activity in HaCaT cells

Since we found that $p15^{INK4b}$ mRNA expression is induced by I3C in HaCaT cells, we investigated whether I3C activates the promoter activity of the $p15^{INK4b}$ gene. Treatment with I3C activated the promoter activity in a dose-dependent

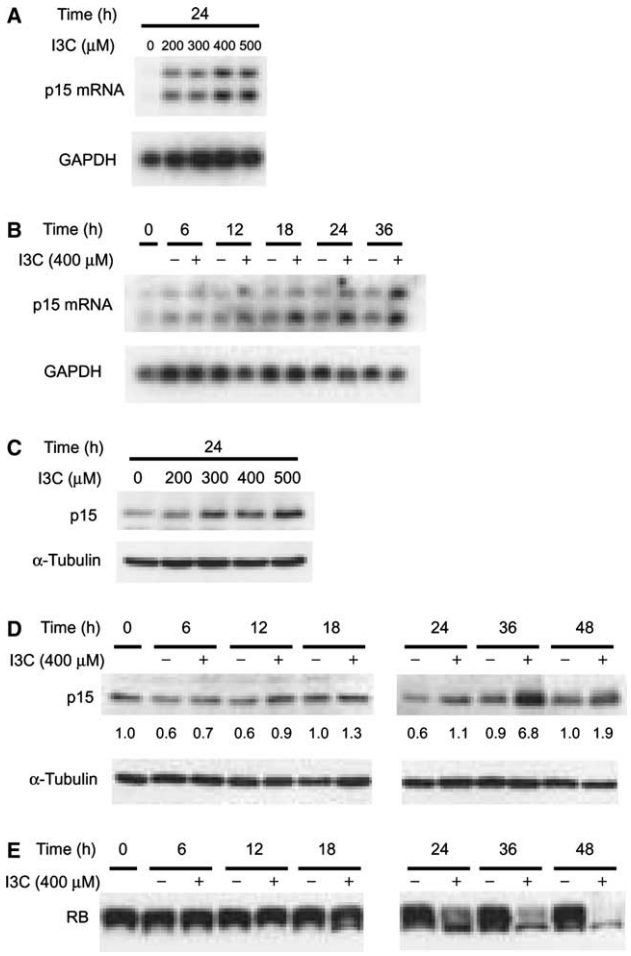

Fig. 2. Northern blot analysis of $p15^{INK4b}$ mRNA and Western blot analysis of $p15^{INK4b}$ or RB protein in HaCaT cells. (A,B) HaCaT cells were treated with various concentrations of I3C for 24 h (A) or 400 μM I3C for the indicated times (B), and the expression of $p15^{INK4b}$ mRNA was examined. The mRNA level of $p15^{INK4b}$ was standardized against that of glyceraldehyde-3-phosphate dehydrogenase. (C) HaCaT cells were treated with various concentrations of I3C, and the expression of $p15^{INK4b}$ protein was examined after 24 h exposure to I3C. α-Tubulin (Oncogene Research Product, CA) was chosen as a loading control for all Western blots. (D,E) HaCaT cells were exposed to DMSO alone (−) or 400 μM I3C (+) for the indicated times. The expressions of $p15^{INK4b}$ (D) and RB proteins (E) were detected. Data shown below the blot (D) represent fold induction in the $p15^{INK4b}$ protein expression quantified by densitometer analysis (Kodak 1D 2.0.2 Image Analysis Software, USA), and each value was compared with that of the control (0 h) which was estimated as 1.0.

manner (Fig. 3). These results indicate that I3C stimulates $p15^{INK4b}$ promoter activity in HaCaT cells.

3.4. Effect of I3C on the expressions of other CDK inhibitors in HaCaT cells

We examined the effect of I3C on the expressions of other CDK inhibitors in HaCaT cells by Western blotting. The abundance of $p19^{INK4d}$, $p21^{WAF1}$ and $p27^{Kip1}$ was almost unchanged by treatment with I3C (Fig. 4). We could not detect the expression of $p16^{INK4a}$ in HaCaT cells (data not shown). Furthermore, we could not evaluate the effect of I3C on $p18^{INK4c}$ protein expression due to weak band and high background in the Western blotting (data not shown). Therefore, these results suggest that $p15^{INK4b}$ is an important molecular target of I3C among CDK inhibitors.

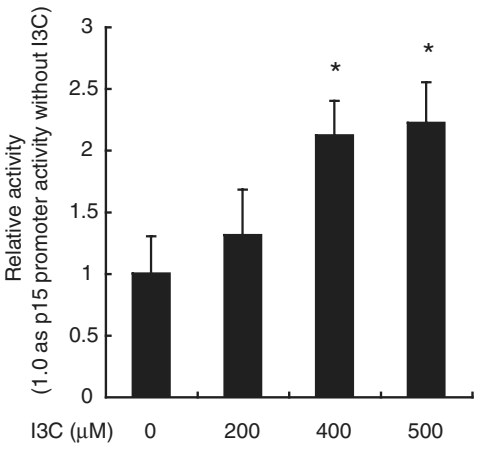

Fig. 3. Activation of $p15^{INK4b}$ promoter activity in HaCaT cells by treatment with I3C. HaCaT cells were transiently transfected with the $p15^{INK4b}$–luciferase fusion plasmid, and luciferase activities were measured after incubation in medium containing various concentrations of I3C for 24 h. The data are shown as the means ± S.D. ($n = 3$). *, $P < 0.05$.

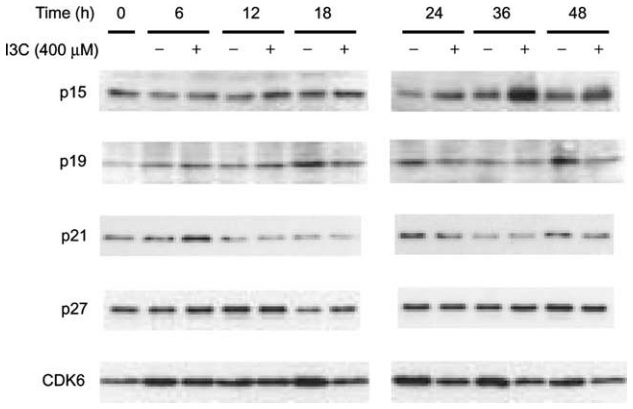

Fig. 4. Effect of I3C on the expressions of $p15^{INK4b}$, $p19^{INK4d}$, $p21^{WAF1}$, $p27^{Kip1}$ and CDK6 in HaCaT cells by Western blotting. HaCaT cells were exposed to DMSO (−) or 400 μM I3C (+) for the indicated times.

Finally, we examined the effect of I3C on the expression of CDK6 in HaCaT cells. It has been shown that the treatment with I3C decreases the expression of CDK6 in human breast cancer cells [12]. As shown in Fig. 4, the time course study indicated that CDK6 protein was down-regulated 18 h after treatment with I3C in HaCaT cells.

4. Discussion

Our results indicate that I3C arrests the cell cycle of HaCaT cells in the G1 phase and inhibits cell growth. Furthermore, I3C stimulates $p15^{INK4b}$ promoter activity and upregulates $p15^{INK4b}$ mRNA and protein levels, and that the hyperphosphorylated form of the RB protein is converted into a hypophosphorylated form in HaCaT cells. However, treatment with I3C almost did not affect the expressions of other CDK inhibitors such as $p19^{INK4d}$, $p21^{WAF1}$ and $p27^{Kip1}$. Taken

together, these results suggest that $p15^{INK4b}$ is an important molecular target of I3C among CDK inhibitors.

We recently examined the effect of ectopic $p15^{INK4b}$ expression on the growth of HaCaT cells by selecting for Geneticin resistant cells after transfection with $p15^{INK4b}$ expression vectors encoding the $p15^{INK4b}$ and neomycin resistance genes [16]. The recovery of antibiotic-resistant HaCaT cells was markedly inhibited by the ectopic expression of $p15^{INK4b}$, suggesting that this gene inhibits cellular growth in HaCaT cells [16]. Thus, activation of the $p15^{INK4b}$ gene may contribute to growth arrest induced by I3C.

It has been shown that the treatment with I3C decreases the expression of CDK6 in human breast cancer cells [12]. In this study, we observed that the expression of CDK6 is down-regulated by I3C in HaCaT cells. Taken together with our result that the expression of $p15^{INK4b}$, a specific inhibitor of CDK4/6, is up-regulated by I3C, it is suggested that cyclin D-CDK6 activity is inhibited by I3C through dual mechanisms in HaCaT cells.

We previously showed that butyrate inhibits cellular proliferation and induces the $p15^{INK4b}$ gene through its promoter in HaCaT cells [16]. Butyrate is one of the most abundant short chain fatty acids in the large intestine, and is generated by the bacterial fermentation of dietary fibers [17]. It also has anti-tumor properties in vivo and in vitro at physiological concentrations, suggesting that it may have preventive effects against large bowel cancer [18–20]. We examined whether combination treatment with I3C and butyrate is more efficacious for growth inhibition than either agent alone. Simultaneous treatment with I3C and butyrate enhanced growth inhibition in HaCaT cells (data not shown).

The inactivation of $p16^{INK4a}$ has been extensively reported for most human malignant tumors [21]. As a member of the INK4 family, $p15^{INK4b}$ has a function similar to that of $p16^{INK4a}$. This suggests that $p15^{INK4b}$ may function as a replacement for $p16^{INK4a}$ when $p16^{INK4a}$ is inactivated. Aytac et al. [22], recently showed that $p15^{INK4b}$ inhibits cellular proliferation independent of RB. The ectopic expression of $p16^{INK4a}$ inhibits the growth of cell lines depending on RB status [22]. However, in the case of $p15^{INK4b}$, cellular proliferation was inhibited in cells lacking RB as well as in cells exhibiting wild-type RB expression. Therefore, transcriptionally up-regulated agents of the $p15^{INK4b}$ gene may contribute to new strategies for the prevention or therapy of malignancies, which we term 'gene-regulating chemoprevention or chemotherapy' [23–25]. I3C, which activates $p15^{INK4b}$ gene expression, might be representative of gene-regulating chemopreventive or chemotherapeutical agents over a broad spectrum of tumors regardless of RB status.

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