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All-*trans* retinoic acid induces cellular senescence via upregulation of p16, p21, and p27

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ABSTRACT

We here present a new anti-tumor mechanism of all-*trans* retinoic acid (ATRA). ATRA induced several biomarkers of cellular senescence including irreversible G_1 arrest, morphological changes, senescence-associated β-galactosidase, and heterochromatin foci in HepG2 cells. ATRA also upregulated levels of p16, p21, and p27 which lead to activation of Rb and subsequent inactivation of E2F1. These effects were abolished by the RNA interference-mediated silencing of p16, p21, and p27. Moreover, ATRA failed to induce cellular senescence in Huh7 and HCT116, in which p16, p21, and p27 were not upregulated by ATRA, confirming that ATRA induces cellular senescence via upregulation of p16, p21, and p27.

1. Introduction

Retinoids are natural and synthetic vitamin A derivatives and analogs that are involved in many important biological processes, including vision, morphogenesis, differentiation, growth, metabolism, and cellular homeostasis [1]. In addition, aberrant retinoid signaling mechanisms have been associated with cancer development in human and animals [1,2]. Therefore, retinoids are being increasingly included in both chemopreventive and therapeutical schemes for various tumoral diseases [2]. In general, they are believed to inhibit carcinogenesis by blocking the promotion of already initiated or transformed cells by three mechanisms: induction of apoptosis, arrest of further growth of abnormal cells, and induction of abnormal cells to differentiate back to normal [2–7]. However, the molecular mechanisms underlying the retinoid therapy are poorly understood.

Several genetic and biochemical data suggest that cellular senescence is an important tumor suppression process, preventing damaged cells from undergoing aberrant proliferation [8]. Cellular senescence is characterized by irreversible cell cycle arrest that can be triggered by numerous intrinsic and extrinsic stresses. Other senescence markers include a large flat morphology, induction of a senescence-associated β-galactosidase (SA β-gal) activity [9] and formation of several heterochromatin domains called senescence-associated heterochromatin foci (SAHF) [10]. Numerous studies have elucidated the molecular mechanisms that direct cellular senescence [8,11]. Both p16 and p21, whose expression is invariably elevated in senescent cells, act as two critical regulators of senescence [8]. As cyclin-dependent kinase inhibitors (CKIs), p16 and p21 block the activity of G_1- and G_1/S-Cdks, respectively, leading to up-regulation of Rb activity in the cells. Thus, continuous Rb activation induces cellular senescence by recruiting heterochromatin proteins such as histone deacetylase to the E2F-responsive promoters to form SAHF, resulting in stable repression of E2F target genes [8,11].

All-*trans* retinoic acid (ATRA), the most biologically active metabolite of vitamin A, has been extensively studied as an anti-cancer agent [2]. ATRA usually inhibits the growth of tumor cells by blocking cell cycle progression at the G_1 phase. This effect can be exerted directly or

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indirectly by modulating the expression of genes involved in the G_1-checkpoint regulation. For example, ATRA downregulates levels of positive G_1 regulators such as cyclin-dependent kinase 2 (Cdk2), Cdk4/6, and cyclin D1 [12,13]. In addition, it also upregulates levels of G_1 or G_1-S Cdk inhibitors (CKIs) such as p16, p21 and p27 [12,14]. All of these conditions have been shown to inhibit E2F transcription factors, resulting in cell cycle arrest at the G_1 phase. These observations prompted us to investigate whether ATRA can induce cellular senescence via upregulation of two critical regulators of senescence, p16 and p21. In addition, it was investigated whether p27 upregulation is also involved in the induction of cellular senescence by ATRA.

2. Materials and methods

2.1. Cell lines and transfection

Six human cell lines including HepG2 (KCLB No. 88065), Hep3B (KCLB No. 88064), Huh7 (KCLB No. 60104), HEK293 (KCLB No. 21573), MCF7 (KCLB No. 30022), and HCT116 (KCLB No. 10247) were obtained from the Korean Cell Line Bank. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For transient expression, 2×10^5 cells per 60-mm dish were transfected with 1 μg of appropriate plasmid(s) using WelFect-EX™PLUS (WelGENE) following the manufacturer's instructions. Cells were either mock-treated or treated with 10 μM ATRA (Sigma) for 72 h unless otherwise stated.

2.2. Luciferase reporter assay

2×10^5 cells per 60-mm diameter plate were transfected with 0.5 μg of E2F1-luc [15]. To control for transfection efficiency, 0.1 μg of pCH110 (Pharmacia) containing the *Escherichia coli lacZ* gene under the control of the SV40 promoter was cotransfected as an internal control. At 48 h after transfection, luciferase assay was performed and the value obtained was normalized to the β-galactosidase activity measured in the corresponding cell extracts.

2.3. RNA interference

pSUPER RNAi system (OligoEngine), a vector system for expression of short interfering RNA plasmid-based RNA interference system that uses H1 RNA-based polymerase III promoter, was employed to knock-down specific gene expression. Based on the target sequences of p16 (5′-CGC ACC GCC TAG TTA CGG T-3′) [16], p21 (5′-AAT GGC GGG CTG CAT CCA GGA-3′) [17], and p27 (5′-GTG GAA TTT CGA TTT TCA G-3′) [18], siRNA inserts composed of both sense and antisense sequences separated by a central hairpin sequence were designed. The siRNA inserts were ligated into pre-cut pSUPER vector and positive clones were selected.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR

Total cellular RNA was extracted using a total RNA isolation kit (Qiagen). RNA (3 μg) was reverse transcribed with the corresponding reverse primer. One-quarter of the reverse-transcribed RNA was amplified with *Taq* polymerase (95 °C for 5 min; 30 cycles at 95 °C for 1 min, 56 °C for 1 min; 72 °C for 30 s, 72 °C for 5 min) using forward primers, 5′-CGG GGT GAA A A GAT AAA G-3′, 5′-GAT TGC CTG TTC TGC TTC-3′, and 5′-TGG CGT GAT TCT GAG CAA-3′ and reverse primers, 5′-TGC GGT CAC CAT TCA TCC A-3′, 5′-TTG GGT GAC TTT CCT ACT-3′, and 5′-CTG CCA AGC TGC CCA AGG-3′ to detect levels of apolipoprotein J, fibronectin, and SM22, respectively [19]. The PCR condition for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was described before [20]. For quantitative RT-PCR, one twentieth of the RT reaction mixture was used for PCR amplification by the StepOne™ Real-Time PCR system (Applied Biosystems) and SYBR Premix Ex Taq TMII (Takara). GAPDH was used for cDNA normalization. All samples were quantified in triplicate. Relative expression was calculated using the comparative C_t method [21].

2.5. Western blot analysis

Cells were lysed in buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with antibodies against p16, p21, p27, E2F1 (Santa Cruz Biotechnology), phosphorylated Rb, γ-tubulin (Sigma), Rb (Calbiochem) for 2 h at room temperature and subsequently with the appropriate horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (H + L)-HRP (Bio-Rad) and anti-rabbit IgG (H + L)-HRP (Bio-Rad) for 1 h at room temperature. The chemiluminescent ECL kit (Amersham) was used to visualize protein bands on X-ray films.

2.6. Cell proliferation assay

For the determination of cell growth, 1×10^3 per well in 96-well plates were incubated for 20 h. Cells were either mock-treated or treated with ATRA (Sigma) under the indicated conditions. The MTT-derived formazan developed by cells was quantified by measuring absorbance at 550 nm as described before [22].

2.7. Cell cycle analysis

Cell cycle profile was analyzed using flow cytometry. Briefly, 2×10^6 cells were trypsinized, fixed in 80% ethanol, and resuspended in 50 μg/ml propidium iodide (Sigma) containing 125 U/ml RNase A (Sigma). For cell cycle profile, DNA content was analyzed by flow cytometry using the Cell-FIT software (Becton–Dickinson Instruments).

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2.8. BrdU incorporation assay

For the determination of DNA synthesis rate, the amounts of BrdU incorporated into DNA was measured by a colorimetric immunoassay (Roche). Briefly, 1×10^3 cells were cultured in the presence of various concentrations of ATRA for 48 h and treated with 10 μM BrdU for an additional 24 h. The fixed cells were reacted with anti-BrdU-peroxidase for 2 h and the color developed by adding trimethyl benzidine was measured at 490 nm and 405 nm.

2.9. SA β-gal assay

SA β-gal assay was performed as described by Dimri et al. [9]. Briefly, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min. SA β-gal activity was determined by incubation with SA-β gal staining solution (Cell Signaling) at 37 °C for 18 h. The cells were rinsed twice with PBS and washed with methanol followed by microscopic examination.

2.10. SAHF assay

Cells harvested in 300 μl of PBS were incubated with 700 μl ethanol for 1 h. After centrifugation, the cells were incubated in 500 μl PBS containing 1 μg/ml of 4′6-diamidino-2-phenylindole (DAPI; Sigma) at room temperature for 5 min. Cells placed on a slide glass were observed for SAHF using a fluorescence microscope at the excitation wavelength of 350 nm.

2.11. Statistical analysis

The values indicate means ± S.D. from at least three independent experiments. The difference between the means of the treatment group and its control was assessed with the paired two tailed *t* test; A *P* value of <0.05 (*) or <0.01 (**) was considered to be statistically significant.

3. Results

3.1. ATRA induces irreversible cell growth arrest

According to previous reports, RA inhibits growth of various cancer cells by inducing G_1 arrest [12], apoptosis [22], or differentiation [23]. Therefore, we first examined whether ATRA exhibits its anti-proliferative potential in a human hepatoma cell line, HepG2. Consistently, ATRA inhibited growth of HepG2 cells in a dose-dependent manner (Fig. 1A). In addition, data from both FACS analysis and BrdU incorporation assay showed that ATRA induces G_1 arrest in HepG2 cells (Fig. 1B and C). Moreover, the RA-treated cells barely recovered their growth after release from the ATRA-mediated growth arrest (Fig. 1D). These results suggest that ATRA induces irreversible G_1 arrest.

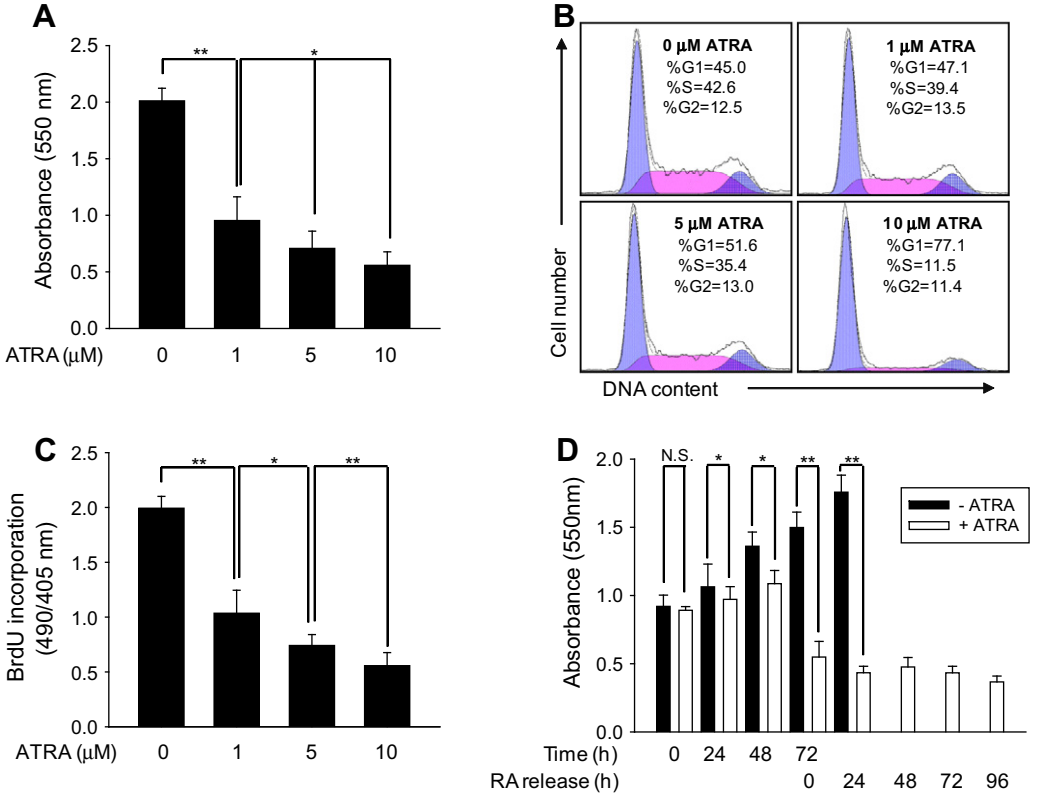

Fig. 1. ATRA inhibits cell growth by inducing irreversible G_1 arrest. HepG2 cells were treated with an increasing concentration of ATRA for 72 h. For (D), the treated cells were incubated for up to additional 96 h after transferring to a normal medium. MTT assay (A and D), FACS analysis (B), and BrdU incorporation assay (C) were performed to measure cell growth. Results are shown as means ± SD obtained from three different experiments (*$P < 0.05$; **$P < 0.01$; N.S., nonsignificant difference). The representative flow cytometry profile from three different experiments is shown in (B).

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3.2. ATRA induces cellular senescence

Irreversible G_1 arrest is one of the major biomarkers of cellular senescence [8]. Therefore, we investigated whether ATRA actually induces cellular senescence to inhibit cell growth. Indeed, several other biomarkers of cellular senescence including morphological changes (enlarged, flattened, and vacuolated), SA β-gal activity and SAHF were extensively induced by treatment with ATRA (Fig. 2A and B). In addition, ATRA markedly increased RNA levels of senescence-related genes such as apolipoprotein J, fibronectin, and SM22 [19] (Fig. 2C). Taken together, we conclude that ATRA induces cellular senescence of HepG2 cells.

3.3. ATRA induces cellular senescence via upregulation of p16 and p21

To investigate the molecular pathway by which ATRA induces cellular senescence, we first examined the effect of ATRA on the two key determinants of cellular senescence, p16 and p21. Treatment with ATRA markedly upregulated levels of p16 and p21 in the HepG2 cells (Fig. 3A, lane 2), as demonstrated before [14]. Under the same condition, ATRA downregulated levels of hyperphosphorylated Rb without affecting its total protein level. In addition, both transcriptional activity and protein levels of E2F1 were dramatically downregulated in the presence of ATRA (Fig. 3B, lane 2).

To examine whether upregulation of either p16 or p21 is responsible for the activation of Rb and subsequent inactivation of E2F1 in the presence of ATRA, we performed RNAi experiments using siRNA specific for either p16 or p21. As a result, knock-down of either p16 or p21 in the ATRA-treated cells resulted in upregulation of hyperphosphorylated Rb (Fig. 3A, lanes 4–7). Accordingly, both the transcriptional activity and protein levels of E2F1 were upregulated in the presence of ATRA (Fig. 3B, lanes 3–6). These results suggest that both p16 and p21 are involved in the ATRA-mediated activation of Rb and subsequent inactivation of E2F1.

To examine whether p16 and p21 are involved in the induction of cellular senescence by ATRA, we performed SA β-gal assay after knock-down of either p16 and/or p21. The potential of ATRA to induce cellular senescence was significantly impaired by silencing of either p16 or p21 (Fig. 3C). However, knock-down of both p16 and p21 was required for the complete abolishment of the potential of ATRA to induce cellular senescence. These results suggest that p16 and p21 act independently in the induction of cellular senescence by ATRA.

3.4. ATRA induces cellular senescence in a broad range of human cancer cells

Next, we investigated whether ATRA can induce cellular senescence in other human cancer cell lines. For this purpose, we first examined the potential of ATRA in two human hepatoma cell lines, Hep3B and Huh7 cells. ATRA effectively induced cellular senescence in Hep3B but not in Huh7 cells (Fig. 4). We also tested the effect of ATRA in three different types of human cell lines. ATRA could induce cellular senescence in HEK293 (a human embryonic kidney cell line) and MCF7 (a human breast cancer cell line) but not in HCT116 (a human colon cancer cell line). Therefore, it is likely that ATRA can induce cellular senescence in a broad range of cell types. Some cell lines such as Huh7 and HCT116 might have evolved resistance to ATRA-induced cellular senescence.

Interestingly, the potential of ATRA to induce cellular senescence was closely correlated to its ability to upregulate levels of p16 and/or p21 (Fig. 4A, lower panel). Levels of p16 were strongly upregulated in HepG2, Hep3B, and HEK293 cells. The p16 protein was not detectable in Huh7, MCF7, and HCT116 cells. On the other hands, levels of p21 were strongly upregulated in HepG2 and MCF cells but with much less extent in Hep3B and HEK293 cells. The p21 protein was not detected in Huh7 cells. The level of p21 in HCT116 cells was unaffected by ATRA. Therefore, neither p16 nor p21 was upregulated in Huh7 and HCT116, which exhibited resis-

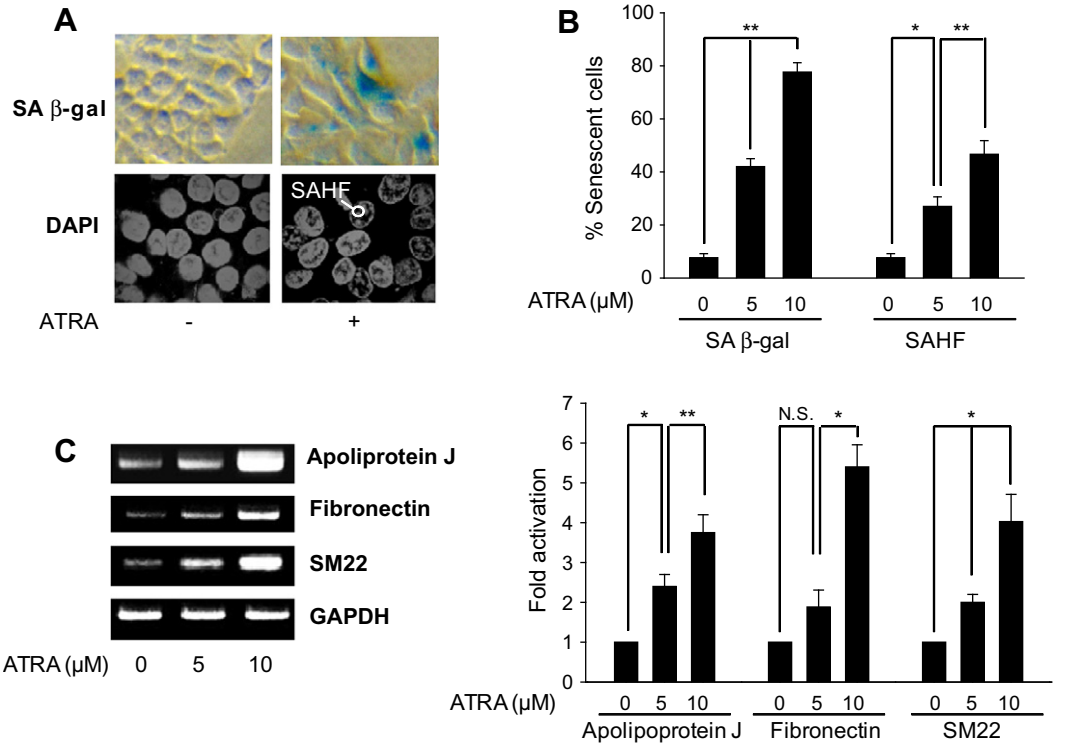

Fig. 2. ATRA induces cellular senescence of HepG2 cells. (A) HepG2 cells were either mock-treated or treated with 10 μM ATRA for 72 h. SA β-gal assay (upper panels) or SAHF assay (lower panels) were performed as described in Section 2. (B) HepG2 cells were treated with an increasing concentration of ATRA for 72 h. The percentage of senescent cells was measured by either SA β-gal assay or SAHF assay. Results are shown as means ± SD obtained from three different experiments. (C) RNA levels of apolipoprotein J, fibronectin, SM22, and GAPDH in HepG2 cells prepared as above were determined by ordinary RT-PCR analysis. The quantitative RT-PCR data from three different experiments are also shown on the right.

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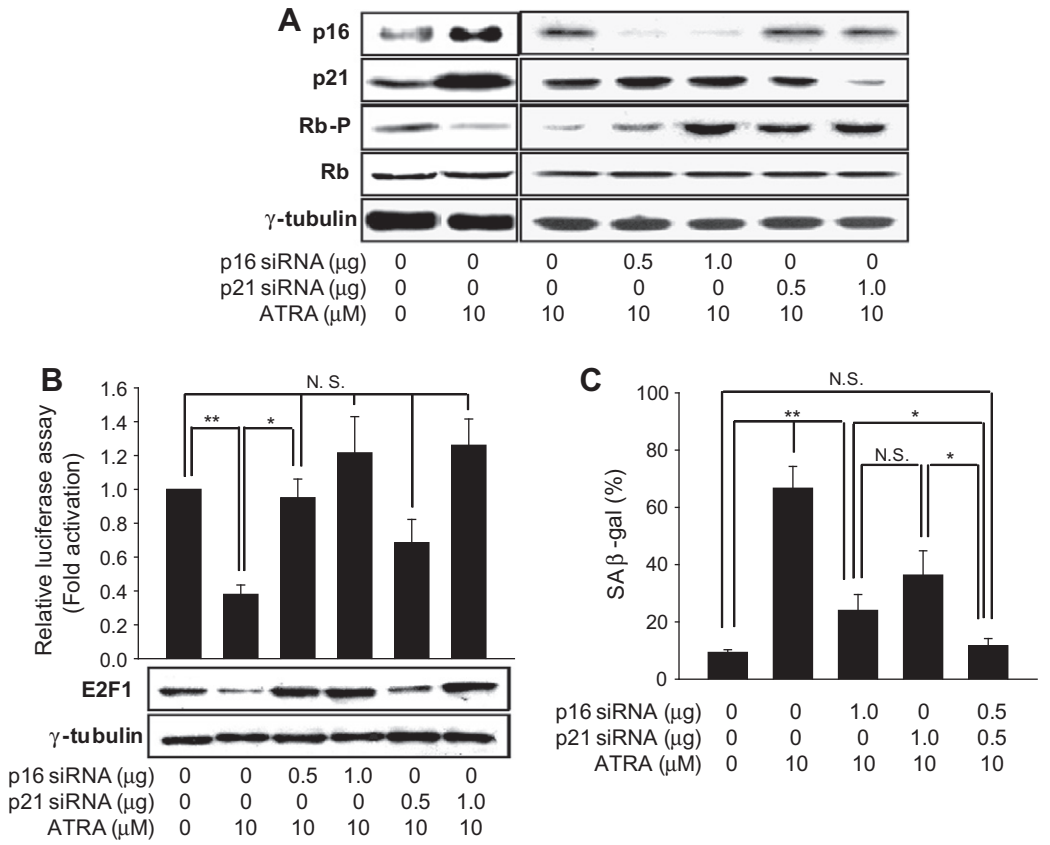

Fig. 3. ATRA induces cellular senescence by upregulating levels of p16 and p21. (A) HepG2 cells were transiently transfected with increasing amounts of either p16 RNAi (lanes 4 and 5) or p21 RNAi plasmid (lanes 6 and 7) and then treated with 10 μM ATRA for 48 h (lanes 2–7). Protein levels of p16, p21, Rb-P (phosphorylated Rb), total Rb, and γ-tubulin were determined by Western blots. (B) HepG2 cells were transiently cotransfected with E2F1-luc and an increasing amount of either p16 RNAi (lanes 4 and 5) or p21 RNAi plasmid (lanes 6 and 7) and then either mock-treated (lane 1) or treated with 10 μM ATRA for 48 h (lanes 2–6) followed by luciferase assay. Results are shown as means ± SD obtained from three different experiments prepared in triplicate. Protein levels of E2F1 and γ-tubulin were determined by Western blots (lower panels). (C) HepG2 cells were transfected with the indicated amount of either p16 RNAi or /and p21 RNAi plasmid and then treated with 10 μM ATRA for 72 h. SA β-gal assay was performed as described in Fig. 2B.

tance to ATRA-induced cellular senescence. Taken together with the data from RNAi experiments shown in Fig. 3C, we conclude that ATRA induces cellular senescence via upregulation of p16 and p21.

3.5. Upregulation of p27 is also involved in the induction of cellular senescence by ATRA

In addition to p16 and p21, p27 is also regarded as a key effector of cellular senescence [24]. Therefore, we investigated whether p27 is also involved in the induction of cellular senescence by ATRA. According to Fig. 5A, levels of p27 were upregulated by ATRA in HepG2 cells as demonstrated before [14] but not in Huh-7 and HCT116 cells. The potential of ATRA to upregulate levels of p27 is thus likely to be correlated to its ability to induce cellular senescence in these cell lines. In addition, knockdown of p27 in the ATRA-treated HepG2 cells resulted in upregulation of hyperphosphorylated Rb (Fig. 5B). Under the condition, the potential of ATRA to induce cellular senescence was severely impaired (Fig. 5C). These results suggest that p27 also mediates the potential of ATRA to induce cellular senescence.

4. Discussion

The first successful application of retinoids in human disease is given by acute promyelocytic leukemia (APL), which is caused by a reciprocal chromosomal translocation between retinoic acid receptor alpha (*RAR-α*) and promyelocyte leukemia protein (*PML*) genes, leading to the alteration of the signaling of both RAR and PML [25]. Thereafter, retinoids are being increasingly included in anti-tumor therapeutical schemes for the treatment of various tumoral diseases such as head and neck squamous cell carcinoma [7], hepatocellular carcinoma (HCC) [3,6], bladder cancer [26], ovarian carcinoma [4], thyroid carcinoma [27], and neuroblastoma [5]. However, the molecular mechanisms underlying the anti-cancer effects of retinoid therapy are poorly understood.

The use of supraphysical doses of ATRA in APL is supposed to overcome the negative effects of PML-RAR by inducing the dissociation of HDAC complexes from the PML-RARα and then the activation of differentiation processes, leading to remission in patients with APL [25]. In the case of HCC, a malfunction of retinoid X receptor alpha (RXRα due to phosphorylation by Ras-MAPK signaling pathway is profoundly associated with liver carcinogenesis. Therapeutic application of a synthetic retinoid can inhibit Ras-MAPK activation and RXRα phosphorylation, thereby inducing apoptosis of the HCC-derived cells [6].

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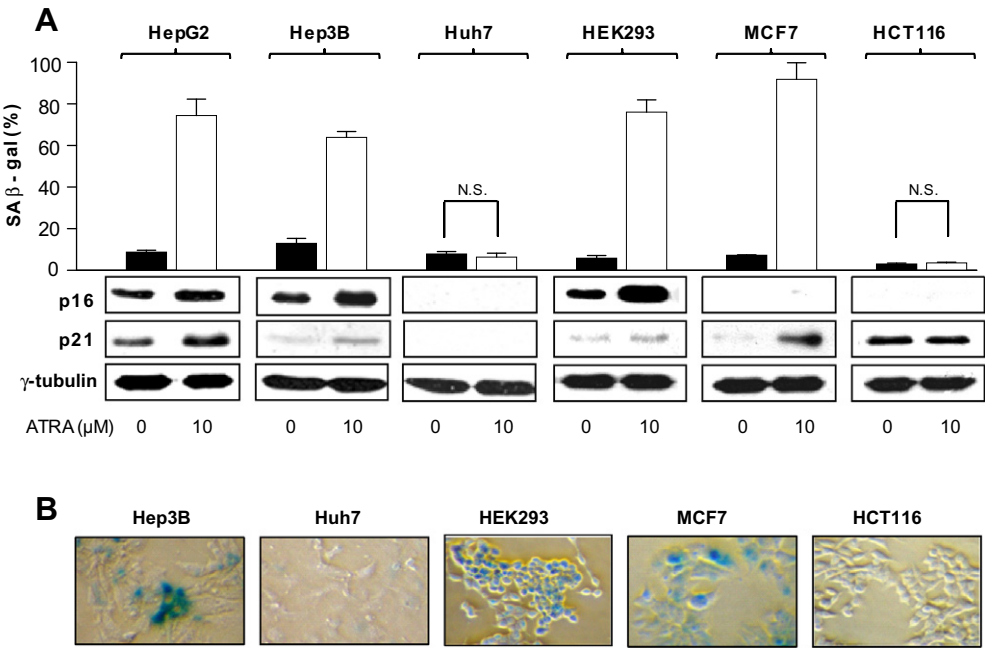

Fig. 4. ATRA induces cellular senescence in a broad range of human cell types. (A) Each cell line was treated with 10 μM ATRA for 72 h followed by SA β-gal assay. Protein levels of p16, p21, and γ-tubulin were determined by Western blots (lower panels). (B) The photographs show SA β-gal staining of six cell lines treated with 10 μM ATRA for 72 h.

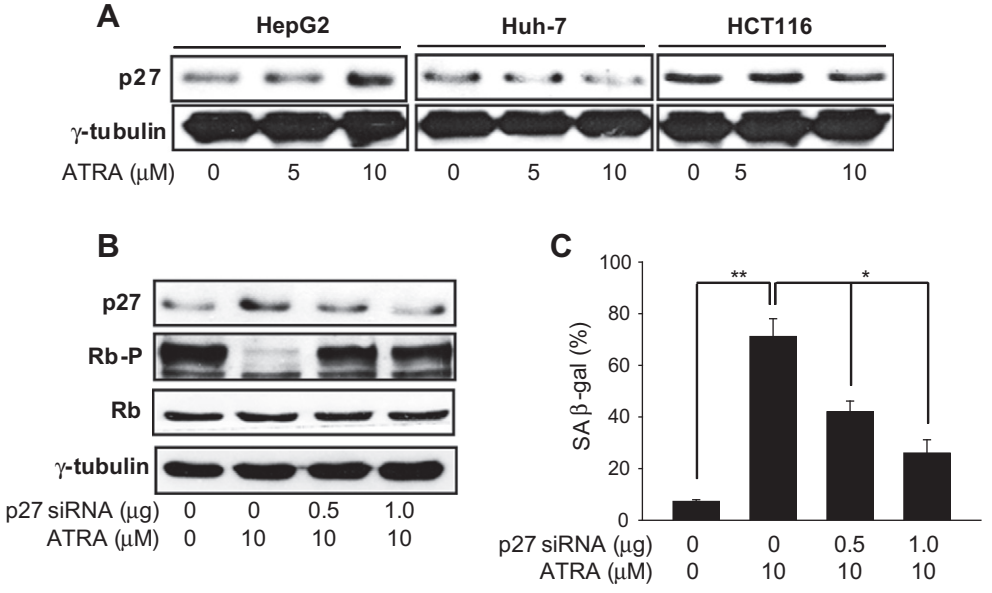

Fig. 5. p27 is also involved in the induction of cellular senescence by ATRA. (A) Each cell line was treated with an increasing concentration of ATRA for 72 h and levels of p27 and γ-tubulin were determined by Western blots. (B) HepG2 cells were transiently transfected with increasing amounts of p27 RNAi plasmid (lanes 3 and 4) and then treated with 10 μM ATRA for 48 h (lanes 2–7). Protein levels of p27, Rb-P (phosphorylated Rb), total Rb, and γ-tubulin were determined by Western blots. (C) HepG2 cells were transfected with the indicated amount of p27 RNAi plasmid and then treated with 10 μM ATRA for 72 h followed by SA β-gal assay as described in Fig. 2B.

The present study provides a new action mechanism of ATRA, i.e., induction of cellular senescence.

Cyclin-dependent kinase inhibitors such as p16, p21, and p27 are regarded as effectors of cellular senescence [8,24]. Consistently, induction of cellular senescence by ATRA required upregulation of at least one of p16, p21, and p27, as demonstrated in HepG2 cells by RNA interference experiments. It was possible to observe the roles of p16, p21, and p27 by comparing the potential of ATRA to induce cellular senescence in six different human cell lines

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in which levels of p16, p21, and p27 were differentially affected by ATRA. First, ATRA effectively induced cellular senescence in HepG2 cells, in which levels of p16, p21, and p27 were upregulated. However, upregulation of p16 does not seem to be absolutely required for induction of cellular senescence by ATRA, as demonstrated by MCF7 cells, in which p21 was upregulated by ATRA whilst p16 was not detected because of homozygous deletion of the gene [28]. Similarly, ATRA could induce cellular senescence in Hep3B and HEK293 cells in which p16 but not p21 was upregulated. Absence of p21 upregulation in these cells might be due to the impaired p53 activity resulted from either homozygous deletion of the gene in the Hep3B cells [29] or proteasomal degradation of the protein by the action of adenovirus E1B-55k in the HEK293 cells [30]. In contrast, ATRA could not induce cellular senescence in Huh-7 cells, in which neither p16 nor p21 was detectable because of homozygous deletion of their corresponding genes [31]. In addition, upregulation of p27 was not detected in Huh-7 cells. The same resistance to ATRA was also observed in HCT116 cells, in which levels of p16, p21, and p27 were unaffected by ATRA. Absence of p16 in this cell line could be explained by the frameshift mutation of the gene [32]. However, it is unknown why ATRA could not upregulate levels of p21, and p27 in HCT116 cells.

The mechanism by which ATRA upregulates levels of p16 and p21 is unknown. In general, retinoids act through RAR as either homodimer or heterodimer to activate target genes that signal retinoid biological effects [1]. Among several subtypes and isoforms of RAR, RAR-β_2 has attracted attention as a major executor of the anti-tumor potential of retinoids in a wide variety of cancers [33]. Consistently, knock-down of RAR-β_2 abolished the potential of ATRA to upregulate p16 and p21 (data not shown), suggesting that it mediates the potential of ATRA to induce cellular senescence. The target genes of retinoids usually contain retinoic acid responsive elements (RAREs) in their promoters, which serve as binding sites for the activated RAR. The RAR usually recruits a coactivator complex composed of proteins with histone acetylase activity that open the chromatin structure and then relieves transcriptional repression [1]. Interestingly, the p21 gene contains two consecutive retinoid X response elements in the promoter, which is responsible for RXR ligand-dependent p21 upregulation [34]. Indeed, ATRA enhances binding of RAR-β_2 and coactivators on the p21 promoter (data not shown). A number of RA responsive genes including p16 however do not contain a distinct RARE sequence and thus requires an alternative regulatory mechanism(s) [35]. In APL, ATRA triggers ubiquitin-mediated proteasomal degradation of PML-RARα, which depends on the binding of SUG-1 to AF-2 transactivation domain of RARα [36]. Proteasome-dependent degradation of G1 cyclins also results in G_1 cell cycle arrest and concomitant growth suppression in human bronchial epithelial cells [37]. Nakanishi et al. [38] also have suggested that ATRA regulates RA responsive genes via transcriptional regulatory cascades by activating a few transcriptional factors. However, none of these possibilities have been demonstrated for the upregulation of p16 and p21 by ATRA. According to our preliminary results, ATRA induced promoter hypomethylation of p16 and p21, resulting in upregulation of their expression (data not shown). However, the action mechanism of ATRA in this process is still unknown. More extensive studies are thus required to clarify the mechanism by which ATRA upregulates levels of p16, d p21, and p27. In conclusion, the present study demonstrates for the first time that ATRA induces cellular senescence. Considering that cellular senescence is characterized by irreversible cell growth arrest, its induction by ATRA might be important not only in the understanding of anti-cancer potentials of ATRA but also for the successful application of retinoid cancer therapy.

Conflict of interest

None declared.

Acknowledgements

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