Reversal of the temperature-shift-induced growth restriction of a temperature-sensitive simian virus 40 T-antigen-transformed human fibroblast cell line by treatment with retinoic acid

Yeou-Ping TSAO*,†, Shu-Fang LI*, Shu-Wen KUO*, Jiang-Chuan LIU† and Show-Li CHEN*

*Department of Microbiology and Immunology, and †Department of Biology and Anatomy, National Defense Medical Center, Taipei, Taiwan, Republic of China

We previously established a human fibroblast cell line, HFL 6-2, that contains a temperature sensitive simian virus 40 (SV40) T antigen, permitting cell growth at 35 °C but restricting growth at 39 °C. p21 (Waf1/Cip1) was significantly induced by temperature shifts in HFL 6-2 cells. Here we show that all-trans-retinoic acid (RA) treatment prevented the growth restriction of HFL 6-2 cells at 39 °C. In the presence of RA, HFL 6-2 cells proliferated into sizeable colonies even at 39 °C. [3H]Thymidine incorporation and flow cytometry analysis revealed that cells exposed to RA maintained DNA synthesis at 39 °C. Prevention of growth restriction by RA was correlated with a lack of induction of p21 at the transcription level. These observations suggest that RA may prevent the senescence process by repressing p21 gene expression, and perturb the growth regulation of somatic cells.

INTRODUCTION

Cultured diploid human fibroblasts have a limited life span. After a number of cell generations these cells become senescent, exhibit characteristic morphological changes and cease to proliferate [1–7]. Growth arrest of normal cells after a given number of divisions has been proposed to be mediated through the function of the tumour suppressor genes coding for p53 and p21 (Waf1/Cip1) [8–10]. The progressive shortening of chromosome telomeres during the life span of cultured fibroblasts, resulting from the loss of terminal repeat elements during each round of chromosomal replication, is proposed to be responsible for the activation of p53 [9,10]. Simian virus 40 (SV40) large T antigen can extend the limited life span of primary cells and establish a continuous cell line in culture [11–15]. The mechanism of cell immortalization by the T antigen may be through its binding to and inactivation of the tumour suppressors p53 and the retinoblastoma protein [16].

We previously established a human fibroblast cell line, HFL 6-2, by transducing primary cells with a gene encoding a temperature-sensitive mutant of the SV40 T antigen [17]. HFL 6-2 cells grow at 35 °C but cease to proliferate at 39 °C. Furthermore, p21 gene expression was found to be activated in the growth-arrested cells [17], similar to the phenomenon observed for senescent fibroblasts [8].

p21 is encoded by the recently cloned SDII gene that is overexpressed in senescent fibroblasts [8]. CIP1, a gene identical with the SDII gene, encodes a product that can associate with different cdk–cyclin complexes and inhibit their kinase activities, which are required for cell cycle progression [18–20]. Independent research has shown that p53 induces the WAF1 gene, yet another gene identical with the SDII gene [21]. In addition, other studies have suggested that p21 mediates the arrest of cell proliferation induced by p53 [22]. The increased expression of the p21 gene in growth-restricted HFL 6-2 cells suggests that p21 might mediate the growth restriction in HFL 6-2 cells [17].

All-trans-Retinoic acid (RA) and its derivatives are known to exert significant effects on cell proliferation and differentiation [23–27]. The molecular mechanism of the action of RA on cell growth and differentiation has not been elucidated. However, treatment with RA has been shown to result in the activation and inhibition of transcription [28,29]. We previously found that treatment with RA suppressed the expression of nuclear oncogenes such as c-jun and c-fos [30]. In the present study we examined the possibility that p21 gene expression is suppressed by RA. After we had determined that RA can suppress p21 gene expression, we studied the effects of RA on the temperature-shift-induced growth restriction of HFL 6-2 cells.

EXPERIMENTAL

Cell culture and drug treatment

HFL 6-2 cells were established by infecting human fibroblasts with a retrovirus that encodes a temperature-sensitive (ts) SV40 T antigen as described [17]. HFL 6-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (Gibco) and 100 i.u./ml penicillin and streptomycin. Cells were maintained in logarithmic phase in a humidified atmosphere with 5% CO2. RA (Sigma) was dissolved in DMSO to a concentration of 1 mM and stored at −70 °C. Addition of RA to cell cultures was done in the absence of light.

Immunoblots

Cellular proteins were extracted in SDS/PAGE loading buffer. After being boiled for 10 min, approx. 100 μg of each crude protein lysate was separated by SDS/PAGE, transferred to nitrocellulose filters, reacted with mouse monoclonal antibodies and revealed by the enhanced chemiluminescence system (Amer sham) with procedures recommended by the manufacturer. Antibodies recognizing p21 were purchased from Pharmingen (San Diego, CA, U.S.A.).

Abbreviations used: CDK, cyclin-dependent kinase; DMEM, Dulbecco’s modified Eagle’s medium; RA, all-trans-retinoic acid; SV40, simian virus 40; NP40, Nonidet P40.

† To whom correspondence should be addressed.
RNA isolation and Northern blot analysis

A single-step method of RNA isolation by acid guanidinium thiocyanate/phenol/chloroform extraction [31] was used to obtain total RNA from cells. Total RNA was electrophoresed on a 1% (w/v) denaturing formaldehyde/agarose gel, and then transferred to a nitrocellulose filter. The filters were treated as follows for hybridization with probes at 42 °C. The hybridization buffer contained 50% (v/v) formamide, 5 × Denhart’s solution, 5 × SSPE (where SSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 10% (v/v) dextran sulphate, 0.1% SDS and 200 µg/ml denatured salmon sperm DNA. The probes were p21 cDNA (kindly provided by Dr. David Beach, Cold Spring Harbor Laboratory) and β-actin cDNA labelled with [α-³²P]dCTP by nick translation. After hybridization, the filter was washed in 0.1 × SSC (SSC is 0.15 M NaCl/0.15 M sodium citrate)/0.1% SDS at 65 °C, and then air-dried and autoradiographed at −70 °C.

p21 promoter activity assay

Transient transfection was performed by the calcium phosphate precipitation method. WWP-Luc or DM-Luc DNA (10 µg of each) were dissolved in 500 µl of 0.25 M CaCl₂ in a 15 ml centrifuge tube, 2 × Heps buffered solution (500 µl) consisting of 274 mM NaCl, 10 mM KCl, 1.4 mM Na₂HPO₄, 12 mM glucose and 42 mM Heps, pH 7.05 was added dropwise into the centrifuge tube with constant agitation. Precipitates were allowed to form at room temperature for 20 min and were then evenly added into a 100 mm-diam. dish that contained 10 ml of 1% fetal calf serum. After cells had been treated as described below, cells were stained with 0.05% Crystal Violet in methanol and colonies containing 30 or more cells were counted.

[³H]Thymidine incorporation

Cells were incubated with 5 µCi of [³H]thymidine for 6 h and harvested on glass fibre filters GFC (Whatman). After washing with 5% (w/v) trichloroacetic acid, the radioactivity of the trichloroacetic acid-insoluble fraction was measured with a scintillation counter (Beckman).

Flow cytometry analysis of DNA content

Cells were trypsinized and resuspended at 5 × 10⁶ cells/ml in citrate buffer containing 250 mM sucrose, 40 mM sodium citrate and 5%, DMSO, pH 7.6. A 200 µl sample was added into 1.8 ml of solution A (30 µg/ml trypsin, 3.4 mM sodium citrate, 0.1% Nonidet P40 (NP40), 1.5 mM sperin and 0.5 mM Tris, pH 7.6) and incubated for 20 min at room temperature. Then 1.5 ml of solution B [500 µg/ml trypsin inhibitor (Sigma), 100 µg/ml ribonuclease A (Sigma), 3.4 mM sodium citrate, 0.1% NP40, 1.5 mM sperin and 0.5 mM Tris, pH 7.6] was added and cells were incubated for 20 min at room temperature; 1.5 ml of ice-cold solution C (416 µg/ml propidium iodide, 3.4 mM sodium citrate, 0.1% NP40, 1.5 mM sperin and 0.5 mM Tris, pH 7.6) was then added and incubated at 4 °C for 20 min. Flow cytometry analysis was performed with a FACScan (Becton and Dickinson). Analysis of the percentage of cells in the S phase was performed with CellFIT cell cycle analysis software (Beckton and Dickinson).

RESULTS

Induction of p21 at the non-permissive temperature is suppressed by RA

We previously established the HFL 6-2 cell line by infecting primary human fibroblasts with a retrovirus that encoded a temperature-sensitive SV40 T antigen [17]. HFL 6-2 cells were found to proliferate actively at permissive temperatures (35 °C) whereas they ceased to proliferate at non-permissive temperatures (39 °C). We also found that treatment with RA can suppress the expression of genes such as c-jun and c-fos in Hep3B cells [30]. In the present study we investigated the effects of RA on p21 gene expression in HFL 6-2 cells at 35 or 39 °C. Cells were treated with 0.2 or 1 µM RA at 35 °C before shifting the temperature to 39 °C. The concentration of p21 started to increase after 8 h at 39 °C in the absence of RA treatment and further increased after 24 h at 39 °C. However, the level of p21 did not increase after 8 or 24 h at 39 °C in the presence of 0.2 or 1 µM RA (Figure 1A). Similar experiments revealed that p21 did not increase even after 48 h at 39 °C in the presence of 0.2 or 1 µM RA (Figure 1B). These results were confirmed in three independent experiments. We also analysed changes in p21 mRNA in the presence or absence of RA at 35 and 39 °C. Figure 2 shows that the p21 mRNA decreased significantly in the presence of 0.2 or 1 mM of RA after 24 or 48 h at 39 °C. Taken together, these results indicate that RA can prevent the induction of p21 gene expression in HFL 6-2 cells at the non-permissive temperature.

p21 promoter activity is repressed by RA treatment

To confirm the suppressive effect of RA on p21 gene expression, we studied the activity of the p21 promoter in HFL 6-2 cells

![Figure 1](image-url)
HFL 6-2 cells were exposed to RA (0, 0.2 or 1 µM) at 35 °C and then cultured at 39 °C in the presence of RA. At the indicated times (0, 24 and 48 h) after the temperature shift, total cellular RNA was extracted and analysed by Northern blot analysis with p21 cDNA as a probe. The blotting and hybridization conditions are described in the Experimental section. A β-actin cDNA probe was used as a control for RNA loading.

Table 1 The p21 promoter activity is repressed by RA treatment

<table>
<thead>
<tr>
<th>Culture temperature (°C)</th>
<th>WWP-Luc (µM)</th>
<th>WWP-Luc (1 µM)</th>
<th>DM-Luc (µM)</th>
<th>DM-Luc (1 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>7807 ± 3150</td>
<td>5870 ± 1325</td>
<td>6872 ± 4852</td>
<td>3806 ± 1516</td>
</tr>
<tr>
<td>39</td>
<td>1904 ± 1787</td>
<td>2153 ± 612</td>
<td>1927 ± 2254</td>
<td>1905 ± 788</td>
</tr>
</tbody>
</table>

Exposed to 1 µM RA. The p21 promoter-luciferase construct WWP-Luc contains 2.4 kb p21 promoter sequence downstream of the luciferase reporter gene [21]. DM-Luc is a mutant reporter construct in which the p53 binding site on the p21 promoter has been deleted [21]. WWP-Luc and DM-Luc plasmid DNA were transfected into HFL 6-2 cells at 35 °C. After 24 h, transfectants were cultured at either 39 or 35 °C for 24 h in the presence of 1 µM RA. Assay of luciferase activity showed that the p21 promoter activity was significantly lower in the cells treated with RA (Table 1). Repression of wild-type p21 promoter activity by RA was observed regardless of whether the cells were cultured at 35 or 39 °C. In addition, RA repressed mutant p21 promoter activity (DM-Luc) at both 35 and 39 °C, suggesting that RA repressed p21 promoter activity through a p53-independent pathway.

RA can prevent inhibition of DNA synthesis at non-permissive temperatures

We have previously shown that DNA synthesis was inhibited during growth restriction at 39 °C in HFL 6-2 cells [17]. The concomitant induction of p21 gene expression during the inhibition of DNA synthesis suggested that p21 inhibited the progression into the S phase of the cell cycle [17]. In the present study the finding that treatment with RA abolished p21 accumulation at 39 °C suggested that RA might also prevent the inhibition of DNA synthesis. This was tested by culturing HFL 6-2 cells in the presence of 0.2 or 1 µM RA at 35 or 39 °C for 48 h. [3H]Thymidine incorporation into cellular DNA was then determined. Table 2 shows that, without RA treatment, DNA synthesis in cells cultured at 39 °C for 48 h was decreased compared with that in cells cultured at 35 °C. The decrease in DNA synthesis at 39 °C was much less when the cells were cultured in RA. This result indicates that treatment with RA can prevent the inhibition of DNA synthesis induced by growing HFL 6-2 cells at 39 °C.

Prevention of the inhibition of DNA synthesis at 39 °C by RA was also demonstrated by flow cytometry analysis (Figure 3). HFL 6-2 cells were grown at 35 °C and were then exposed to 1 µM RA at either 35 or 39 °C. After 48 h, nuclei were prepared from these cells and the DNA content was analysed on a flow cytometer. The S-phase cell population decreased at 39 °C was much less when the cells were cultured in RA. This result indicates that treatment with RA can prevent the inhibition of DNA synthesis induced by growing HFL 6-2 cells at 39 °C.

Although the prevention of the inhibition of DNA synthesis by RA could be interpreted as being due to the stimulation of DNA synthesis by RA, treatment with RA did not stimulate DNA synthesis or increase the S-phase cell percentage at 35 °C (Table 2 and Figure 3). Therefore the possibility that RA stimulated DNA synthesis in HFL 6-2 cells can be ruled out.
RA partly preserves the proliferation capability of HFL 6-2 cells

We have shown that HFL 6-2 cells actively proliferate at 35 °C but cease to proliferate after culture at 39 °C for 72 h [17]. In the present study, Table 2 and Figure 3 show that treatment with RA prevented the reduction of DNA synthesis at 39 °C. The ability of HFL 6-2 cells to proliferate at 39 °C in the presence of RA was determined by examining foci formation: 4000 cells were plated in 100 mm-diam. dishes in the presence of 0, 0.2 or 1 μM RA at 39 °C and after 10 days the cells were stained with Crystal Violet and the numbers of foci that contained more than 30 cells were counted. All experiments were repeated three times. Results showed that only a few foci formed in the absence of RA, whereas many foci formed at 39 °C in the presence of RA. This result indicates that RA can at least partly preserve the growth potential of HFL 6-2 cells at 39 °C.

The preservation of the proliferation capability of HFL 6-2 cells by RA could also be due to an increase in the plating efficiency of the cells by RA. Treatment with RA, however, did not increase the number of foci at 35 °C (Table 3), eliminating this possibility.

DISCUSSION

We have previously established HFL 6-2 cells in which cell growth is permissive at 35 °C but restricted at 39 °C. The temperature shift to 39 °C was also found to induce the expression of p21 gene significantly. In the current study we found that growth restriction at 39 °C could be prevented by treating the cells with RA. The induction of p21 synthesis at the transcription level could also be prevented by treatment with RA. These observations imply that RA can inhibit the senescence process by repressing p21 gene expression.

RA seems to have differential effects on cell growth. Inhibitory effects of RA on cancer cell proliferation have been documented [32,33]. In contrast, RA has been shown to stimulate the DNA synthesis of primary renal cells, rat epidermal cells and normal peripheral blood cells [34–36]. It is possible that the effects of RA on cell growth are cell-type specific. The differential effects of RA on cell growth may also be dose dependent. At 10 μM, RA inhibited the growth of HFL 6-2 cells at both 35 and 39 °C (results not shown). However, at 1 μM, RA prevented growth arrest at 39 °C (Table 3) without affecting the growth rate of HFL 6-2 cells at 35 °C. A more advanced study of the effect of RA on the growth of HFL 6-2 cells might help to elucidate the mechanism of the growth regulation.

p21 gene expression is induced in senescent fibroblasts and in DNA-damaged cells experiencing G1 arrest [8,17,37]. Overexpression of p21 leads to growth arrest in colon cancer cells [21]. These observations, combined with the finding that p21 gene expression is induced by p53, suggest that p21 mediates the growth restriction signalling pathway of p53 in senescent and DNA-damaged cells [21]. In HFL 6-2 cells, T antigen degradation at 39 °C is believed to release bound p53, which then induces p21 gene expression and leads to growth restriction [17]. Inhibition of p21 gene expression by RA may prevent growth restriction at 39 °C. We observed similar phenomena in a human keratinocyte cell line that we established by using the same temperature-sensitive SV40 T antigen [38]. In these cells, p21 gene expression could be partly suppressed by the human papillomavirus 11 E5a oncoprotein, and the growth restriction that occurred at the restrictive temperature was delayed [38]. These observations strongly correlate the suppression of p21 gene expression with the prevention of growth restriction. However, recent studies suggest that a p21-independent pathway that requires p53 activity can result in G1 growth arrest [39,40]. One report showed that the introduction of mutant p53 prevented G1 arrest in senescent fibroblasts, but that the induction of the p21 gene still occurred [39]. A second report showed that a different p53 mutant gene arrested cell growth without inducing p21 gene expression [40].

The involvement of such p21-independent pathways in the growth restriction of HFL 6-2 cells at 39 °C is not clear. It is possible that these pathways are also inhibited by treatment with RA.

We have clearly shown in the present study that treatment with RA can reduce cellular p21 concentrations by repressing p21 gene expression. The mechanism of RA inhibition of p21 gene expression is still not clear. It is unknown whether retinoic acid receptor or retinoid ‘X’ receptor binding sites exist in the 5’ regulatory region of the p21 promoter. However, the RA responsiveness of the mutant p21 promoter, in which the p53 binding site is deleted (Figure 3), suggests that RA can repress p21 gene expression through a pathway independent of p53.

As well as in HFL 6-2 fibroblasts, we have found that treatment with RA can repress p21 gene expression in hepatoma cells (Hep3B) and lung cancer cells (H1299) (Y.-P. Tsao, unpublished work). The common response of these cell lines to RA suggests that p21 gene repression by RA is a general phenomenon. However, in RA-treated HL-60 leukaemia cells, differentiation is accompanied not only by growth restriction but also by the induction of p21 gene expression [41,42]. The differential regulation of p21 by RA in different cell types cannot yet be explained. One possibility is that RA might associate with different RA receptor isoforms in different cells and thus bind to different receptor binding sites in the p21 promoters. Another possibility is that the induction or suppression of the p21 gene is merely a downstream effect of a growth regulatory system and that the response of this system to RA treatment differs between cell types.

RA has been widely employed for various clinical purposes including cancer prevention and therapy [43,44]. It has diverse and complicated effects on cells that are only beginning to be understood. Understanding the cellular effect of RA will be important for improving the therapeutic effects and preventing the adverse effects of RA. This is the first report describing the repression of the p21 gene by RA. p21 is known to play important roles in senescence and in G1-phase arrest induced by DNA damage. p21 may also be important for gene therapy involving p53. Repression of p21 gene expression by RA may thus influence cellular responses to DNA damage, senescence, and the effects of p53-mediated gene therapy.
REFERENCES


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