Differential regulation of cyclin A, cyclin B and p21 concentrations in a growth-restricted human fibroblast cell line

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When the culture temperature was shifted from 35 °C to 39 °C, human fibroblasts immortalized by the temperature-sensitive simian virus 40 T antigen became larger and acquired the morphological characteristics of senescent fibroblasts. After culture at 39 °C for 48 h, most cells had ceased to proliferate. A rapid depletion of cells with S-phase DNA content was observed after the temperature shift. To elucidate the mechanism governing this rapid arrest of proliferation, we studied the expression of genes involved in the regulation of cell cycle progression. Cyclin A, cyclin B and p34^{cdc2} concentrations were not changed during growth restriction, whereas p21 was rapidly induced in these growth-restricted cells. Transient expression of exogenous p21 in

cells cultured at 35 °C led to growth restriction and morphological changes characteristic of senescence. Furthermore, we studied the reversibility of growth restriction induced by the temperature increase. The results showed that senescent morphology and growth arrest were not reversible. In these cells the p21 concentration remained high and p34^{cdc2} remained undetectable. This indicates that p21 accumulation might be responsible for the maintenance of senescence. Our findings provide information on the use of growth restriction of immortalized fibroblasts induced by a temperature shift as a model system to study senescence.

INTRODUCTION

Diploid human fibroblasts have a limited life span in cell culture. After a number of generations, these cells become senescent, show characteristic morphological changes and cease to proliferate [1–4]. Fibroblast senescence *in vitro* has been proposed as a model which can reflect what occurs in normal cells *in vivo* during cell senescence [5]. In cell culture, senescent fibroblasts become larger, less motile and exhibit decreased saturation density compared with early-passage fibroblasts [2,3,6–8]. Nuclear size and macromolecules such as RNA, protein and lipids are all increased. However, the amount of DNA reflects the G1, or 2N, complement in the majority of cells [6,9]. Continuous cell growth in the absence of DNA replication occurs in fibroblast senescence [10]. Only recently has some understanding been gained into the mechanisms governing senescence. A number of studies comparing young and senescent fibroblasts have revealed differences in gene expression. Some of the genes that are not expressed in senescence are cell-cycle-related genes, e.g. those encoding p34^{cdc2} and cyclins A and B [11].

Progression of mammalian cells through the cell cycle is regulated by the sequential activation and inactivation of a series of protein kinase complexes [12]. These complexes contain a catalytic unit, a cyclin-dependent kinase (CDK) and a regulatory subunit, known as a cyclin. An association with cyclins is required for the activation of CDKs. One of the CDKs, p34^{cdc2}, is associated with cyclin B and is responsible for cell cycle progression through the G2 ‘check point’ [13,14]. Cyclin A is associated with p34^{cdc2} and CDK2 in the S phase of the cell cycle, and these complexes may be involved in promoting DNA replication in G1 cells [14–19]. The failure of expression of these

genes has been proposed to be relevant to the lack of DNA synthesis and mitosis in senescent fibroblasts [11].

The protein p21 is encoded by a recently cloned gene, *Sdi1*, that is overexpressed in senescent fibroblasts [20]. It has been shown that Cip1, which is the same protein as p21, can associate with different CDK–cyclin complexes and inhibit the kinase activity that is required for cell cycle progression [21–23]. Independent research has shown that p53 causes induction of the *WAF1* gene, a gene which turned out to have identical coding to that of the p21 gene [24]. p21 was suggested to mediate the cell proliferation arrest induced by p53 [25].

In this study we have analysed the biochemical events that occur when cell lines established by a temperature-sensitive simian virus 40 (SV40) large T antigen become growth-restricted at the non-permissive temperature. The cell morphology, DNA content and cell-cycle-related proteins such as cyclin A, cyclin B and p21 were analysed. The results reveal that the DNA content was increased rapidly, the cyclin A and B concentrations were not decreased, and p21 gene expression was induced by growth restriction. The p21 protein concentration remained high even after growth-restricted cells were transferred to the permissive temperature. These findings provide a mechanism for the irreversibility of growth restriction induced by a rise in temperature.

EXPERIMENTAL

Cell culture

PA317 cells were obtained from A.T.C.C. and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) calf serum plus penicillin (100 units/ml) and streptomycin

Figure 1 Cell morphology of HFL 6-2 cells cultured at 35 °C and 39 °C

Cells were plated on 10 cm dishes at a density of 10^6 cells per dish and grown at 35 °C for 48 h. They were then transferred to incubation at 39 °C. Cells were observed with an Olympus IMT-2 phase contrast microscope 24, 72 and 120 h after the temperature shift. Magnification $\times 250$.

Figure 2 Cell growth of HFL 6-2 cells cultured at 35 °C and 39 °C

(a) Growth of HFL 6-2 cells cultured at 35 °C and 39 °C. Cells (5×10^4) were plated in 10 cm dishes and grown at 35 °C for 24 h. Then cells were transferred to incubation at 39 °C at zero time. Cells numbers were counted after trypsinization at the indicated time points. (b) Continuous culture of HFL 6-2 cells at 39 °C for 48 h leads to irreversible growth restriction. Cells (5×10^4) were plated in 10 cm dishes. After incubating at 35 °C for 24 h, cells were transferred to 39 °C for another 48 h. Cells were then transferred back to 35 °C. Cell numbers were determined after trypsinization at 24 h after the initial incubation at 35 °C (0 h on graph), at 24 and 48 h after the temperature shift to 39 °C, and at 24 h (time 72 h on graph) and 48 h (time 96 h on graph) after transfer back to 35 °C from 39 °C.

(100 $\mu\text{g/ml}$). Human fibroblasts were prepared by trypsinization from a biopsy specimen of condyloma accuminata, and were also maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin and streptomycin.

Synthesis of retrovirus

PA317 cell were transfected with 10 μg of pZipSVtsa58U19 (kindly provided by Dr. Parmjit Jat, Ludwig Institute, London, U.K.) by the calcium phosphate procedure [26]. G418-resistant

cells were selected at 400 $\mu\text{g/ml}$ and pooled. Virus production was confirmed by transferring G418 resistance to NIH 3T3 cells by infection as described [26].

Retrovirus infection and isolation of stably infected cells

Infections were performed as described by Jat and Sharp [26] with modifications. Briefly, retroviruses were prepared as a 12 h supernatant from a 90–100% confluent dish of an appropriate virus producer cell line. The viral supernatant was filtered through

a 0.45 μm -pore-size filter before use in infection. Infections were done on 10 cm-diameter dishes at 37 °C for 2 h in 2 ml of growth medium containing the virus and 8 $\mu\text{g}/\text{ml}$ Polybrene (Aldrich Chemical Co., Milwaukee, WI, U.S.A.), after which 8 ml of fresh medium was added. The cells were re-fed every 3–4 days and cultured at 35 °C. The media were changed every 4 days and colonies that grew were isolated on microdilution plates. Clones that grew were expanded into cell lines and passaged at 35 °C.

Flow cytometry analysis of DNA content

Cells were trypsinized and resuspended at 5×10^6 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 to 1.8 ml of solution A containing 30 $\mu\text{g}/\text{ml}$ trypsin, 3.4 mM sodium citrate, 0.1% Nonidet P-40 (NP-40), 1.5 mM spermine and 0.5 mM Tris, and incubated for 20 min at room temperature. A 1.5 ml portion of solution B [500 $\mu\text{g}/\text{ml}$ trypsin inhibitor (Sigma), 100 $\mu\text{g}/\text{ml}$ ribonuclease A (Sigma), 3.4 mM sodium citrate, 0.1% NP-40, 1.5 mM spermine, 0.5 mM Tris, pH 7.6] was added and the incubation continued for 20 min at room temperature. Next, 1.5 ml of ice-cold solution C (416 $\mu\text{g}/\text{ml}$ propidium iodide, 3.4 mM sodium citrate, 0.1% NP-40, 1.5 mM spermine, 0.5 mM Tris, pH 7.6) was added and incubated for 20 min at 4 °C. Flow cytometry analysis was performed using a FACScan (Becton & Dickinson).

Immunoblotting

Cellular proteins were extracted in SDS/PAGE loading buffer. After being boiled for 10 min, about 100 μg of crude protein lysate was separated by SDS/PAGE, transferred to a nitrocellulose filter, reacted with mouse monoclonal antibodies and visualized by rabbit anti-mouse IgG and Western Blue stabilized for alkaline phosphatase (Promega, Madison, WI, U.S.A.). Antibodies recognizing cyclin A, cyclin B and p34^{cdc2} were from Oncogene Science (Uniondale, NY, U.S.A.). Antibodies recognizing p21 were from Pharmingen (San Diego, CA, U.S.A.).

RNA isolation and Northern blot analysis

The single-step method of RNA isolation by acid guanidinium thiocyanate/phenol/chloroform extraction [27] was used to obtain total RNA from human fibroblasts. Total RNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, and then transferred on to a nitrocellulose filter. The filters were treated as follows for hybridization with probes at 42 °C. The hybridization buffer was 50% (v/v) formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSPE (1 $\times$ SSPE = 0.15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 10% (w/v) dextran sulphate, 0.1% SDS and 200 μg of denatured salmon sperm DNA/ml. The probe used was p21 cDNA (kindly provided by Dr. D. Beach, Cold Spring Harbour Laboratory, NY, U.S.A.) labelled with [α -³²P]dCTP by nick translation. After hybridization, the filter was washed in 0.1 $\times$ SSC/0.1% SDS at 65 °C, and then air-dried and autoradiographed at -70 °C.

RESULTS

Establishment of a fibroblast cell line from human condyloma accuminata by temperature-sensitive SV40 T antigen

We established a cell line, HFL 6-2, by infecting primary human fibroblasts with retroviruses encoding a temperature-sensitive mutant of the large T antigen using the procedure described in the Experimental section. HFL 6-2 cells were proliferative in culture for more than 6 months. The characteristics of HFL 6-2

Figure 3 Flow cytometric analysis of HFL 6-2 cells cultured at 35 °C and 39 °C

Nuclei were prepared from 10^6 cells grown at 35 °C or 39 °C for 24, 48 or 72 h as described in the Experimental section. DNA content was analysed with a Beckton and Dickinson flow cytometer. The arrow indicates the results with human peripheral blood mononuclear cells used as an internal marker.

cells were identified after 20 passages; they contained prolyl 4-hydroxylase, a specific fibroblast marker, and did not contain cytokeratin (results not shown). At 35 °C, cells assumed a transformed phenotype and proliferated indefinitely. When the culture containers were transferred to 39 °C, the cell morphology started to change within 24 h. The cell size increased and cell division ceased (Figures 1 and 2).

Flow cytometric analysis of DNA content

The DNA content of cells can reflect the mechanism of growth arrest. For instance, when normal human fibroblasts in culture reach senescence, the DNA content reflects the G1 phase (or 2N) in the majority of cells. This indicates that initiation of DNA synthesis may be inhibited. To identify the mechanism of growth restriction of HFL 6-2 cells, flow cytometric analyses of the DNA content of cells grown at 35 °C and at 39 °C for 24, 48 and 72 h were performed. The deviation of the 'two copies' (2C) peak from a lymphocyte standard indicated the aneuploid nature of HFL 6-2 cells. In fact, cytogenetic analysis revealed that there are on average 72 chromosomes per cell in HFL 6-2 cells (results not shown). The 2C and 'four copies' (4C) peaks were identified in

Figure 4 Analysis of p21, cyclin A, cyclin B and p34^{cdc2} concentrations in HFL 6-2 cells cultured at 39 °C

HFL 6-2 cells were plated at 35 °C and then transferred to 39 °C. At 2, 4, 8, 16 and 24 h after the temperature shift, cells were lysed and cellular protein was extracted. Equal amounts (100 mg) of cellular proteins were analysed by SDS/10% PAGE. Immunoblots using antibodies recognizing p21, cyclin A, cyclin B and p34^{cdc2} were performed as described in the Experimental section.

cells grown at 35 °C (Figure 3). When cells were transferred to 39 °C for 24 h, the S-phase population started to decrease. In cells maintained at 39 °C for 48 h, a DNA histogram revealed the accumulation of cells with 2C and 4C DNA content. Similar DNA contents were observed after cells had been cultured for 72 h at 39 °C. These findings were confirmed by repeating the experiment three times.

Analysis of cell-cycle-related proteins: p21, cyclin A, cyclin B and p34^{cdc2}

To analyse the mechanisms of growth restriction induced in HFL 6-2 cells by a temperature shift, we studied the concentrations of cyclin A, cyclin B, p34^{cdc2} and p21 at different times after a temperature shift from 35 °C to 39 °C (Figure 4). The results show that the cyclin A, cyclin B and p34^{cdc2} concentrations were unchanged 24 h after the temperature shift. The p21 protein concentration was increased 4 h after the cells were transferred to 39 °C, and p21 proteins were continuously accumulating when cells were cultured at 39 °C for 16 and 24 h. However, the majority of the HFL 6-2 cells had ceased to divide by 24 h after

Figure 5 Northern blot analysis of p21 mRNA in HFL 6-2 cells cultured at 39 °C

(a) At the indicated times of culture of the cells at 39 °C, total cellular RNA was extracted, separated on a 1.0% formaldehyde/agarose gel, blotted on to a nitrocellulose filter and hybridized to p21-specific cDNA probes as described in the Experimental section. Subsequently, the filter was hybridized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific cDNA probes to ensure that equal amounts of RNA were present in the different lanes. (b) Cells were cultured at 35 °C (lane A), and then transferred and cultured at 39 °C for 24 h (lane B). Cells were then transferred back to 35 °C and cultured for a further 24 h (lane C). p21 and GAPDH mRNA concentrations were determined as described in (a).

the temperature shift. This indicated that growth restriction may not require decreases in the concentrations of cyclin A and cyclin B. On the other hand, p21 gene induction may be responsible for growth restriction of T-antigen-transformed HFL 6-2 fibroblasts.

Northern blot analysis showed that, by 4 h after the temperature shift, the p21 mRNA concentration had started to increase (Figure 5a). This suggests that p21 accumulation is the result of induction of p21 gene expression. Transient exposure of HFL 6-2 cells to 39 °C revealed that p21 gene expression was tightly controlled by the temperature change. Before the temperature shift, the p21 mRNA concentration was low (Figure 5b, lane A). A 24 h exposure of the cells to 39 °C led to p21 induction (Figure 5b, lane B). By 24 h after the cells were transferred back to 35 °C from a 24 h exposure to 39 °C, the p21 mRNA concentration had decreased to the level present before the temperature shift (Figure 5b, lane C). p21 protein accumulation was also reversible on transfer back to 35 °C from a 24 h exposure to 39 °C (see Figure 7a).

p21 is stabilized in growth-restricted fibroblasts cultured at the permissive temperature

A 24 h exposure of HFL 6-2 cells to 39 °C led to a transient induction of p21 gene expression and the transient accumulation of p21 protein. However, 48 h of continuous exposure of HFL 6-2 cells to 39 °C led to irreversible growth restriction even after the temperature was shifted back to 35 °C (Figures 6 and 2b).

The irreversibility of growth restriction cannot be explained by our knowledge of the temperature-sensitive T antigen. We

Figure 6 Continuous culture of HFL 6-2 cells at 39 °C for 48 h lead to irreversible morphological changes

Cells were plated on 10 cm dishes at a density of 10^6 cells per dish. After incubating at 35 °C for 48 h, cells were transferred to 39 °C for another 48 h. The cells were then transferred back to 35 °C. Cells were observed with an Olympus IMT-2 phase contrast microscope and pictures were taken after 48 h of the initial incubation at 35 °C (a), at 48 h after the temperature shift to 39 °C (b), and at 24 h (c) and 48 h (d) after the transfer back to 35 °C from 39 °C. Magnification $\times 250$.

Figure 7 Continuous culture of HFL 6-2 cells at 39 °C for 48 h leads to the irreversible accumulation of p21 protein

(a) Cells were plated on 10 cm dishes at a density of 10^6 cells per dish and grown at 35 °C for 48 h (lane A), and then transferred and cultured at 39 °C for 24 h (lane B). Cells were then transferred back to 35 °C and cultured for a further 24 h (lane C). Total cellular proteins (100 μ g/lane) were analysed by immunoblot using p21-specific antibodies as described in the Experimental section. (b) Cells were plated on 10 cm dishes at a density of 10^6 cells per dish and grown at 35 °C for 48 h. Cells were then transferred to 39 °C and grown for 48 h, and subsequently transferred back to 35 °C. Cells were collected 48 h after being plated (lane A), at 24 h (lane B) and 48 h (lane C) after the temperature shift to 39 °C, and at 24 h (lane D) and 48 h (lane E) after restoration to 35 °C from 39 °C. The p21 protein concentration was determined as in (a).

measured the change in p21 concentration in cells experiencing temperature changes. At 35 °C, the p21 concentration was very low (Figure 7b, lane A). When the culture containers were transferred to 39 °C for 24 h (Figure 7b, lane B) or 48 h (lane C),

the p21 concentration was increased. Cells were also cultured at 39 °C for 48 h and then transferred back to 35 °C for 24 h (Figure 7b, lane D) or 48 h (lane E). Upon return to 35 °C from 39 °C, the p21 concentration remain high for at least 48 h. These observations suggest that the irreversible growth restriction is due to the continuous existence of a high concentration of p21 protein in growth-restricted cells.

DISCUSSION

We have established a human fibroblast cell line, HFL 6-2, by using the temperature-sensitive SV40 T antigen gene. These cells proliferated indefinitely at 35 °C. When transferred to 39 °C, however, the cells ceased to proliferate and underwent morphological changes characteristic of senescence. In this work we have analysed the biochemical events that occurred after growth restriction. The results revealed that the cyclin A, cyclin B and cdc2 gene products were not repressed within 24 h after a temperature shift. p21 (Sdi1) synthesis was induced by 4 h after a temperature shift, and there was an increase in p21 in culture with prolonged exposure to the restrictive temperature. Also, p21 induction was not reversible if cells were shifted back to 35 °C after 48 h of incubation at 39 °C. This is similar to senescent normal human fibroblasts and T-antigen-transformed fibroblasts in crisis. As in senescent normal human fibroblasts, p21 was possibly induced by p53 in HFL 6-2 cells. It had been shown that a consensus p53 binding site is present in the promoter of p21, and p53 is capable of driving the p21 promoter [24]. Upon a temperature shift to 39 °C for HFL 6-2 cells, the T antigen was denatured and no longer associated with p53. p53 might activate the gene expression of p21.

It has been reported that the amount of DNA in senescent normal fibroblasts reflects the G1, or 2N, complement in the

majority of cells. Some cells with a 4N content represent G1 tetraploidy cells or cells arrested in G2 [28]. However, flow cytometric analysis of SV40-transformed cells in crisis revealed 4C and 8C contents, rather than 2C and 4C contents which were observed in pre-crisis transformed fibroblasts [29]. In these cells, the chromosome number increases significantly. In our study of HFL 6-2 cells cultured at 39 °C, the temperature shift resulted in the disappearance of cells with an S-phase DNA content. This DNA content change occurred within 24 h of the temperature shift and remain stable. However, 2C and 4C DNA contents remained unchanged in growth-restricted cells. This is different from the situation in SV40-transformed cells in crisis, which showed increased DNA content. SV40 DNA or the T antigen of SV40, when introduced into pre-senetic human fibroblasts, is capable of stimulating cell proliferation [29–33]. It is possible that coupling between DNA replication and mitosis is inactivated in transformed cells in crisis but remains intact in HFL 6-2 cells cultured at 39 °C.

It is possible that the rapid induction of p21 at restrictive temperatures allows it to interact with CDKs and in turn leads to proliferation arrest. The ablation of cyclin A and cyclin B could be a consequence of senescence in normal fibroblasts, but is not required for the acquisition of the senescent phenotype by HFL 6-2 cells. The presence of cyclins seems to relate strongly to cell growth cycles: cyclin A is accumulated in the S phase of the cell cycle and degraded at the late S/G2 phase [14–19], and cyclin B is accumulated at the late S/G2 phase and degraded in the M phase [13,14]. The arrest of the cell cycle by p21 would prevent the degradation of the cyclin A and cyclin B proteins. Hence the presence of cyclins A and B could be a result of mitosis, not the cause of it.

Furthermore, the presence of cyclins A and B seems to be independent of mitosis. The decrease of cyclins A and B has been proposed to relate to the lack of both DNA synthesis and mitosis in senescent fibroblasts [11], because when normal human fibroblasts reach senescence, or when SV40-transformed fibroblasts reach crisis, cyclin A and cyclin B gene expression is decreased [11,29]. However, when HFL 6-2 cells were transferred to a restrictive temperature, the cyclin A and cyclin B protein concentrations remained constant on incubation for 24 h. This implies that cyclins A and B are not major role-players in causing mitosis.

When cells were returned to the permissive temperature after 48 h incubation at 39 °C, cell proliferation did not resume. This was unexpected. At such a temperature, newly synthesized T antigen should still be functional and should maintain cell proliferation. A possible explanation involves the function of p21, since there was a persistent accumulation of p21 after the culture temperature was shifted back to 35 °C for 48 h after incubation at 39 °C for 48 h (Figure 7). p21 has been shown to counteract the effect of T antigen [21]. It is possible that the continuous accumulation of p21 abolished the effect of T antigen and maintained the growth arrest. Continuous p21 accumulation can also arrest cell cycle progression by inhibiting CDKs through direct binding [22,23].

Overexpression of exogenous p21 has been shown to lead to growth arrest of immortalized cells [24]. Recently we observed growth restriction and morphological changes characteristic of senescence in HFL 6-2 cells when we introduced a p21-expressing plasmid into the cells (Y.-P. Tsao, unpublished work). The mechanism through which p21 causes growth restriction and senescence remains to be determined. We are currently studying

the association between p21 and CDKs in growth-restricted and proliferative HFL 6-2 cells. However, it has been reported recently that the association of p21 with the cyclin B-p34^{cdc2} complex is increased in irradiated normal human fibroblasts and is not required for the G2 arrest of irradiated cells [34]. An association between p21 and CDKs may not indicate the biological function of p21. Studying the cycle-dependent kinase activity in growth-restricted HFL 6-2 cells will provide information about the function of p21.

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