Expression of p21 Is Not Required for Senescence of Human Fibroblasts

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Abstract

Senescence and immortalization have been studied in skin fibroblasts derived from two individuals with the Li-Fraumeni syndrome. These cells inherit one wild-type and one mutant p53 allele and lose the former during culture. Despite this loss, cultures of Li-Fraumeni syndrome cells progress normally from early passage to replicative senescence. Senescent cells also expressed barely detectable levels of p21 mRNA, and, in marked contrast to normal cultured cells, levels of p21 expression decreased during in vitro aging. Further maintenance for up to 10 months of post-mitotic cultures has led to the isolation of cells with an extended lifespan. Four potentially immortal cultures have continued to proliferate, and two have completed more than 110 population doublings. These results indicate that p53 and p21 are not required for replicative senescence in human fibroblasts. However, their inactivation may enhance the probability of spontaneous immortalization.

Introduction

Human fibroblasts in culture undergo a limited number of population doublings. Following an initial period of proliferation, cultures begin a period of proliferative decline which ends in a state defined as replicative senescence (1, 2). Evidence suggests that this process has a genetic component and two genes, among others, that have been implicated are p53 and p21 (3—5). The former is the most commonly mutated gene detected in human cancer cells and its loss in cultured rodent cells reproducibly enhances the frequency of spontaneous immortalization (the overcoming of senescence). The cdk3 inhibitor p21 (WAF1/Cip1/sld1) is transcriptionally activated by p53 and is highly expressed in senescent normal human fibroblasts. These observations have led to the hypothesis that p21 might be an important factor in inducing or maintaining the senescent phenotype (3. 5). The action of p21 and other cdk inhibitors can lead to hypophosphorylation and activation of the retinoblastoma gene product pRB. Cells then become arrested in late G1. Both DNA-damaged and senescent cells are inhibited, the latter irreversibly, at this stage of the cell cycle (6). An understanding of the mechanism of replicative senescence is of relevance because immortalization is thought to be a critical step in carcinogenesis (7). Therefore, identification of the genes that are expressed during senescence, or not expressed in immortal cells, will have a potential bearing on our understanding of tumorigenesis. At present these genes are unknown, although several human chromosomes, including chromosomes 1, 4, 6, 7, 9, and 11, have been reported to induce senescence upon introduction into immortal recipients (8).

Patients with the LFS suffer high incidences of cancer, often before the age of 30 years. The most common tumors are breast carcinomas, sarcomas of bone and soft tissue, and brain tumors, with some individuals suffering more than one primary tumor. LFS patients inherit one mutant and one wild-type p53 allele, with loss of the latter being associated with tumorigenesis (9). Cultured fibroblasts from LFS patients spontaneously lose the wild-type p53 allele and have been reported to undergo spontaneous immortalization, although some investigators have been unable to reproduce this latter observation (10—12). Our initial experiments demonstrated that LFS cells undergo replicative senescence in the absence of wild-type p53. This feature of these cultures provided us with an opportunity to examine the hypothesis that p21 expression is required for senescence because of transcriptional activation of p21 by p53. Since p21 can also be transcriptionally activated independently of p53 (13), these studies also provide information on whether p21 transcription is dependent on p53 function during senescence. We also wished to confirm that LFS cells undergo spontaneous immortalization because a reliable source of newly immortal human cells could provide suitable material for identifying senescence genes using differential analysis with presenescence immortal and/or senescent cells. This supposition is based on the hypothesis that immortalization involves the inactivation of a senescence-inducing gene (or genes), presumably via the deletion, mutation, or epigenetic disturbances. The availability of immortal cells that have undergone a minimal number of cell divisions after overcoming senescence (25 pdls produce about 2 X 10^12 cells, assuming clonal origin, which is enough to allow for cryopreservation and the initiation of RNA isolation) would reduce the probability of other, unassociated mutagenic events, assuming a spontaneous mutation frequency in cultured human cells of about 10^-5/gene/cell division (14). Verification of the tendency of LFS cells to immortalize would also provide some support for the contention that this change in cell behavior is an important event in carcinogenesis.

Materials and Methods

Cell Culture. Two strains of skin fibroblasts derived from two individuals with LFS were used. These were MDAH087 and MDAH172 and were a gift from M. Tainsky. The former has a CCG to TGG mutation of codon 248 in exon 7 of one allele of p53 and the latter a CGC to CAC mutation of codon 175 in exon 5 of one allele of p53 (15). The other respective p53 allele is wild type. Cells were grown in modified Eagle’s Medium (Life Technologies, Inc.) supplemented with fetal bovine serum (10%; Sigma) and gentamicin (10 μg/ml). Clones of LFS cells were isolated following seeding of cells at sparse mass culture density (about 4 X 10^5/cm²) or at clonal density (200 cells/75-cm² culture flask). Isolation of individual clones was achieved using Q-tips to scrape cells from the culture vessel surface and transfer them to a suitable recipient culture dish. Pooled clones (designated + + + + and #9 for those derived from strains MDAH087 and MDAH172, respectively) were derived by trypanoscopy and harvesting one culture vessel seeded at about 4 X 10^5 cells/cm² containing multiple colonies and transferring the total contents of each to a 75-cm² flask. Cultures were then expanded. Although the number of clones pooled was not counted, we estimate the number to be over 100. At the time of plating cells at sparse density for clone production, both MDAH087

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3 The abbreviations used are: cdk, cyclin-dependent kinase; LFS, Li-Fraumeni syndrome; pdl, population doubling.

4 M. Tainsky, personal communication.
and MDAH172 had undergone less than 20 pdls, at which point the majority of cells of both types were determined to have wild-type p53. Life span studies were performed by counting cells and calculating pdls until cultures failed to undergo one pdl in 1 month. Cells were seeded at a density that allowed for about three pdls before saturation density was reached. This was 10^3 and 2 x 10^3 cells/cm^2 for WI38 cells and LFS cells, respectively. Flasks were gassed with 5% CO₂ in air and incubated at 37°C in a culture room. Cultures were fed weekly or harvested when confluent, counted with a Coulter Counter, and reseeded as described above. The percentage of cells in a given culture that had undergone DNA synthesis was calculated according to the method of Cristofalo and Scharf (16). Transfection of immortal LFS cells with wild-type mouse p53 was performed as follows. Cells were seeded at 2 x 10^5/cm^2 dish in medium (5 ml). After an incubation period of 24 h, cells were cotransfected with pSV2M(2)6 (containing wild-type mouse p53 under metallothionein promoter control) and pMAMneo using Lipofectin (Life Technologies, Inc.). Transfected cells were then selected with G418 (400 μg/ml) after allowing cultures to recover from their treatment for 3 days. Individual clones were isolated as described above.

DNA Sequencing. Sequencing of p53 was performed to confirm the loss of wild-type p53 alleles in our LFS clones and pooled clones. This was achieved using standard methods on cell lysates as the source of genomic DNA to be amplified and sequenced using [32P]dCTP.

Northern Blotting. RNA was isolated according to the method of Chomczynski and Sacchi (19). Five μg of glyoxylated RNA were resolved in 1.5% agarose and electrophoresed on Nytran membranes. Hybridizations were performed at 65°C overnight in a solution comprised of 0.25 M sodium phosphate, 0.1% sodium polyphosphate, 7% SDS, 2 mM EDTA, and 200 μg/ml salmon sperm DNA. The cDNA probe for p21 consisted of the coding region of p21 and a portion of the untranslated 3' region and was made available by Dr. M. Tainsky) were sequenced using [32P]dCTP.

Results and Discussion

Skin fibroblasts derived from patients with LFS and WI38 fetal lung-derived fibroblasts when serially subcultivated progressed from early passage to senescence similarly. Fig. 1 shows representative growth kinetics of WI38 and LFS cells. Both demonstrated an initial period of steady cell division before they began a proliferative decline toward replicative senescence. During the later phase of in vitro aging, all cultures displayed classical signs of senescence characterized by increased debris in the growth medium and increased numbers of larger cells, many with altered morphologies. The percentage of cells undergoing DNA synthesis also declined with increasing in vitro age. After a 24-h labeling period with tritiated thymidine, the percentage of labeled nuclei in young WI38 cultures (pdl 20–30) was more than 90% but generally 20% or less in very old and senescent cells. The corresponding values in LFS cells were 50–60% (LFS cultures doubled more slowly than WI38 cultures) and less than 20%, respectively. These data support the view that LFS cells undergo normal in vitro aging and can be used as a valid model for studies of senescence.

The cells used in this study and some of their salient properties are listed in Table 1. The most relevant point is that all LFS cultures senesced without wild-type p53, although each strain maintained the mutant allele of p53 that was inherited (see above). It is also apparent from Table 1 that all three clones of MDAH172 that were studied (three others have been isolated but not used) have given rise to cultures with extended life spans which will be considered immortal when they have completed 150 pdls. This has also been the case with both parental cultures of MDAH172 and MDAH087, although clones of the latter have eventually failed to expand. Observation and subsequent isolation of these actively proliferating cells required long-term maintenance of senescent cultures with the longer lived cells appearing as rare clones against a senescent background. This period of postsenescence culture has ranged from 3 to >10 months before successful passage of the culture. Life-extended cultures grew slowly following initial passage with a doubling time of several days. However, growth rates have increased subsequently as these cultures have progressed beyond 100 pdls. Currently, clone 1 and 3 cells double in approximately 4 days at a life span of 120 pdls. Each of these longer lived cultures has a distinctive cellular morphology. MDAH172 clone 1 cells are relatively fibroblastic, whereas MDAH172 clone 3 cells are larger, less elongated, and their cytoplasms often appear to be granular. MDAH172 clone 2 cells are different altogether. When subconfluent, cells often display dendritic processes, have large multinucleated nuclei, and in these respects resemble senescent cells. The tumorigenicity of these potentially immortal cells is currently being investigated following s.c. injection of cells (10^7) into nude mice. It is expected, as others have already reported (12, 20), that they will be nontumorigenic.

Fig. 2A shows a representative Northern blot in which the expression of p21 was estimated in normal and LFS fibroblasts. We confirm that p21 expression is elevated severalfold in senescent normal cells compared to young dividing counterparts. A ratio of 8.3:1 was obtained using densitometry after correcting for levels of β-actin (Fig. 2A, Lanes 5 and 3, respectively). However, we were unable to find any evidence that a similar situation occurs in senescent LFS cells. Levels of p21 were barely detectable in all LFS samples analyzed whether from young, old, or senescent cultures (Fig. 2A). These data include several other samples from both MDAH087 and MDAH172.
Cultures of the parental cultures MDAH172 and MDAH087 and their derivative clones and pooled clones were grown until they failed to undergo one doubling in 1 month. All underwent senescence. The terminology used for MDAH087 pooled clones was based on their initial rate of growth following isolation. Thus, the prefix +++ indicated rapid growth and + only slow growth, and + + denoted cultures with an intermediate rate. For MDAH172, pooled clone cultures were numbered 1–9, with the prefix # to distinguish those from MDAH087 pooled clone cultures and MDAH172 single clone cultures. Only cultures + + + and #9 have been used in this study. Isolation of clonal cultures and pooled clone cultures was performed at the same time, but with the former being derived from more sparsely seeded cultures. The pdl for the various clones represents the point at which enough cells were available for DNA sequencing and preparation for cryopreservation. For single clones, 20 pdl would produce 10⁶ cells (assuming single-cell origin), whereas for pooled clones less pdl would be required. This is reflected in a difference of five pdl assigned to single clones and pooled clones at the time of DNA sequencing. Therefore, selection of clones at pdl 15 for MDAH172 would allow for single clonal growth to 10⁶ cells by pdl 35 and pooled clonal growth by pdl 30. Four of the five cultures of MDAH172 have given rise to cells with an extended life span. Currently, life-extended MDAH172 clones 1 and 3 have completed in excess of 110 pdls and may be immortal. A total of three proliferating colonies had been identified in two T75s of MDAH087 parental cells, but at present these have not expanded enough to be harvested or passaged. Sequencing of p53 demonstrated that the wild-type allele was undetectable by about pdl 35–40.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Life span (pdl)</th>
<th>Senescence</th>
<th>Extended life span</th>
<th>Young cultures</th>
<th>Old cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDAH087 parent</td>
<td>55</td>
<td>+</td>
<td>+</td>
<td>+ (pdl 22)</td>
<td>– (pdl 40)</td>
</tr>
<tr>
<td>MDAH087 clone 1</td>
<td>51</td>
<td>+</td>
<td>–</td>
<td>– (pdl 40)</td>
<td>– (pdl 35)</td>
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<tr>
<td>MDAH087 clone 2</td>
<td>51</td>
<td>+</td>
<td>–</td>
<td>– (pdl 40)</td>
<td>– (pdl 35)</td>
</tr>
<tr>
<td>MDAH087 + + + 2</td>
<td>61</td>
<td>+</td>
<td>+</td>
<td>+ (pdl 16)</td>
<td>– (pdl 35)</td>
</tr>
<tr>
<td>MDAH172 parent</td>
<td>44</td>
<td>+</td>
<td>–</td>
<td>– (pdl 35)</td>
<td>– (pdl 30)</td>
</tr>
<tr>
<td>MDAH172 clone 1</td>
<td>51</td>
<td>+</td>
<td>–</td>
<td>– (pdl 35)</td>
<td>– (pdl 30)</td>
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<tr>
<td>MDAH172 clone 2</td>
<td>54</td>
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<tr>
<td>MDAH172 clone 3</td>
<td>51</td>
<td>+</td>
<td>–</td>
<td>– (pdl 35)</td>
<td>– (pdl 30)</td>
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<tr>
<td>MDAH172 clone 4</td>
<td>67</td>
<td>+</td>
<td>–</td>
<td>– (pdl 35)</td>
<td>– (pdl 30)</td>
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</table>

Cultures that were within 10% of the end of their lifespan (data not shown). By exposing film to appropriate blots for several days, we were able to determine that p21 mRNA levels actually decreased with in vitro age of LFS cultures (Fig. 2B). For example, p21 mRNA levels were determined by densitometry to be about four times lower in senescent than in younger MDAH172 cells (Fig. 2B). That LFS cells have normally responsive p21 alleles was demonstrated after transfection of a wild-type mouse p53 gene under metallothionein promoter control into immortal MDAH087 cells (Fig. 3). Following zinc induction, we have shown by Northern blot analysis a dramatic induction of p21 mRNA. These results do not support the view that p21 expression is a requirement for senescence. Expression of p21 during senescence appears to be correlated positively to the presence of wild-type p53, since there is a reduction in p21 mRNA that corresponds to the loss of successive wild-type p53 alleles (WI38 > young LFS with one wild-type p53 allele > old LFS with only mutant p53 alleles). Levels of p21 mRNA were similar in senescent LFS cells and in life-extended MDAH172 clone 1 cells, which would be expected if the above relationship is valid (data not shown). It is possible that p21 expression in LFS cells can be elevated by p53-independent mechanisms, although we have not tested this.

The cdk inhibitor p21 was originally identified as a CDNA that was expressed 10–20 times higher in senescent human fibroblasts than in young cells (3). This led to the hypothesis that p21 was an important factor in inducing or maintaining the senescent phenotype. To add weight to this hypothesis, it was reported that although expression levels of p53 did not increase with in vitro culture age, DNA binding in vitro and transcriptional activity in vivo of the protein did (5). Therefore, the transcriptional activation of p21 by p53 could explain the onset of senescence. The involvement of p53 in this process is in agreement with the observation that human and rodent cells lacking p53 undergo spontaneous immortalization (or the overcoming of...
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Fig. 3. Northern blot analysis demonstrating that immortal LFS cells contain normally responsive p21 alleles. MDAHO87 immortal cells (pdl 101) were transfected with a wild-type mouse p53 gene under metallothionein promoter control in plasmid pSV2M(2)6 (provided by Dr. M. Sleigh). Upon induction with zinc sulfate (100 μM for 4 h at intervals of 24 h), levels of p21 mRNA increased dramatically to a maximum following two exposures. Cells were harvested at the end of each respective 4-h zinc sulfate exposure. Levels of p21 mRNA remained high even 24 h after a final zinc sulfate exposure (Lane 5). Lane 1, no zinc sulfate exposure; Lane 2, 1 × 100 μM zinc sulfate for 4 h; Lane 3, 2 × 100 μM zinc sulfate for 4 h at a interval of 24 h; Lane 4, 3 × 100 μM zinc sulfate for 4 h at intervals of 24 h; Lane 5, as for Lane 4 but 24 h after the final zinc sulfate exposure. Lanes 6-10, correspond to the respective zinc sulfate exposure of Lanes 1-5 but on immortal MDAHO87 cells that had not been transfected.

senescence) at elevated frequencies (4, 12). This is especially true of normal human cells, since there is no example of confirmed spontaneous immortalization of such cells. Our results and those of others demonstrate that wild-type p53 function is not a requirement for the onset and completion of replicative senescence in human fibroblasts (11, 12). In addition to this, we have shown that elevated expression of p21 is not a prerequisite for human cell senescence and, in fact, that p21 mRNA levels fall as LFS cultures age and become senescent.

References
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