Senescent human diploid fibroblasts are able to support DNA synthesis and to express markers associated with proliferation

IAN R. KILL* and SYDNEY SHALL

Laboratory of Cell and Molecular Biology, School of Biological Sciences, Biology Building, University of Sussex, Brighton BN1 9QG, England

*Author for correspondence
Present address: Department of Biological Sciences, The University, Dundee DD1 4HN, Scotland

Summary

The characteristic limited reproductive life-span of normal human fibroblasts in culture is due to a steadily decreasing fraction of cells able to proliferate in the standard rich growth media. We have observed that restricting the growth factor supply to old cells for variable lengths of time in culture increases the fraction of cells that can enter S-phase; although these cells do not go on to divide. Thus, it seems that there is a transient phase between the proliferating state and the irreversibly post-mitotic, senescent state. Perhaps a 'quiescent-G0' state, which can be maintained in the presence of growth factors, is a stage on the pathway to mortalization and senescence.

Key words: human fibroblasts, limited lifespan, Hayflick limit, mortalization, growth-regulation, cell senescence, DNA synthesis.

Introduction

The growth of normal human diploid fibroblasts is characterised by a finite and predictable lifespan (Hayflick and Moorhead, 1961; Shall, 1987). The limited lifespan is due to a steadily decreasing fraction of proliferating cells (Smith and Hayflick, 1974; Westermark, 1978; Stein et al. 1978; Stein and Shall, 1978; Shall and Stein, 1979; Ponten et al. 1985). This widely studied example of cell growth regulation is poorly understood at the molecular level. Aged cultures only a small proportion of cells incorporate nucleotides or their analogues into DNA (Cristofalo and Sharf, 1973). To determine whether senescent cells do sense growth factor signals, Paulsson et al. (1986) and Rittling et al. (1986), made senescent cells serum-starved by incubation in low serum for 48 and 40 h, respectively. Restimulation of these 'Go' cells with growth factors or with serum resulted in the expression of the immediate-early, early, mid-G1 and late-G1 genes in these cultures. Therefore, it was suggested that this inability to proliferate is due to a 'downstream' block, either very late in G1 (Paulsson et al. 1986), or early in S (Rittling et al. 1986), and is not due to an inability to sense and/or to respond to growth signals. However, their conclusion must be re-examined because we report here that quite unexpectedly, serum-starved, senescent cells that were not proliferating previously, can be induced to proceed through G2 and to enter S phase and G2.

Thus, it appears that there is a transient phase between the proliferating state and the irreversibly post-mitotic, senescent state. Perhaps, a 'quiescent-G0' state that can be maintained in the presence of growth factors, is a stage on the pathway to mortalization and senescence.

Materials and methods

Cell culture

Adult human dermal fibroblasts (strain 1BR.3) were obtained from a punch biopsy (Arlett et al. 1975). The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum (NCS) and antibiotics (10 units ml−1 of penicillin, 50 μg ml−1 of streptomycin) to the 27th passage, at which time the culture had undergone 65 accumulated population doublings. Senescent cells were plated onto 13 mm diameter glass coverslips at a density of 3 x 104 cells cm−2. Cell cultures were maintained in DMEM supplemented with 10% (v/v) NCS and antibiotics for 2 days, then washed twice in DMEM, and finally refed with DMEM supplemented with 0.5% (v/v) NCS and antibiotics. After 2, 7 or 14 days, cultures were refed with DMEM supplemented with 10% (v/v) NCS and antibiotics. Control cultures were maintained in 10% (v/v) NCS continuously.

Detection of DNA synthesis

To detect DNA synthesis, cells on coverslips were incubated with 25 μM 5-bromodeoxyuridine in the presence of 2 μM fluorodeoxyuridine to inhibit thymidylate synthetase (Gratzner, 1982), at various times and durations following serum restimulation. Cells were washed twice with PBS and then fixed with 3.7% formaldehyde in PBS for 10 min at room temperature to denature the DNA and washed extensively with PBS. Cells were covered with 10 μl of a rat monoclonal antibody raised against 5-bromodeoxyuridine (1:10 in PBS/FCS, Sera-Labs) and incubated for 1 h at room temperature or overnight at 4°C in a humidified atmosphere. In all the experiments described in this paper, the antibody solutions were made up in PBS containing 1% (v/v) foetal calf serum (FCS); this mixture is called PBS/FCS in all procedures. After washing, a fluorescein-conjugated rabbit anti-rat antibody (1:30 in PBS/
p-phenylenediamine, and sealed with nail polish. Stained cells were viewed with a Zeiss Photomicroscope III fitted with a 63× water-immersion objective using epifluorescence. For assessment of the proportion of stained cells, 1000 total or 200 positive nuclei were counted in randomly selected fields.

Detection of proliferation-associated antigens

At 24 and at 48 h following serum restimulation, cells on coverslips were washed twice with PBS and fixed with methanol/acetone (1:1, v/v) for 10 min at 4°C, then they were rehydrated, washed with PBS and covered with 10 μl of one of the following antibody solutions: Ki-67 (1:50, in PBS/FCS); anti-PCNA (1:1000 in PBS/FCS, a kind gift from Dr R. Bravo, EMBL, Heidelberg); anti-topoisomerase II (1:250 in PBS/FCS, a kind gift from Dr L. Liu, Johns Hopkins University School of Medicine, Baltimore). The cells were incubated for 1 h at room temperature or overnight at 4°C in a humidified atmosphere, and were then washed with PBS and covered with 10 μl of one of the following antibody solutions: Ki-67, fluorescein-conjugated rabbit anti-mouse IgG (1:20 in PBS/FCS); for PCNA, rhodamine-conjugated goat anti-human IgG (1:25 in PBS/FCS); for topoisomerase II, fluorescein-conjugated swine anti-rabbit IgG (1:40 in PBS/FCS). After 1 h at room temperature in a humidified atmosphere the cells were washed with PBS, mounted and scored, as described above.

Increase in cell number

Control cultures at passage 27 were plated onto 35 mm dishes at about 2.1 × 10⁴ cells/dish and maintained in medium containing 10% NCS (v/v) for 2 days. Two dishes were harvested for determining cell number (day 0), and the remaining dishes were kept for a further 7 days when they were harvested for cell counting (day 7). Treated cultures at passage 27 were plated onto 35 mm dishes at about 2.1 × 10⁴ cells/dish and maintained in medium containing 10% NCS (v/v) for 2 days, then washed twice in serum-free medium, re-fed with medium containing 0.5% NCS (v/v), and incubated at 37°C in 5% carbon dioxide:air for 7 days. The medium was replaced with fresh medium containing 10% NCS (v/v) and two dishes were harvested for cell counting (day 0). The remaining dishes were maintained in medium with 10% NCS (v/v) for a further 7 days, when they were harvested for cell counting (day 7).

For estimation of cell numbers, four independent estimations on each duplicate sample were made, using a Coulter counter.

Results

Synthesis of DNA by 'senescent' human diploid fibroblasts

We are interested in defining the molecular mechanism of the block to proliferation in senescent cultures. We asked whether the ability of senescent cells to respond to growth factors included traverse through S-phase and G2. Therefore we determined the fraction of senescent cells that were able to incorporate the thymidine analogue, 5-bromodeoxyuridine (BrdUrd), following serum stimulation of serum-starved cells induced by a 2-day, a 7-day or a 14-day period in low serum (Table 1, A). Human dermal fibroblasts were serially passaged in medium containing 10% serum for 65 accumulated population doublings. Fewer than 5% of these cells were then incorporating BrdUrd in a 48 h continuous exposure, indicating that the culture was moderately senescent. Following a 2-day period of low serum, after re-stimulation with serum, 12.2% of cells incorporated BrdUrd during the first 24 h; however, this increased to 14.1% over 48 h. Thus, rather unexpectedly, over 3.5 times more senescent cells can be induced to enter S-phase by this procedure than occurs in non-serum-starved cultures. A 30 min exposure to BrdUrd at 48 h indicated that synthesis had almost ceased by this time. More surprisingly, after a longer period (7 days) of serum starvation, there were over seven times more cells entering S-phase (28.5%) following restimulation with serum than were observed in the control cultures. Increasing the period spent under conditions of low serum to 14 days resulted in an 8-fold increase in the fraction of cells entering S-phase (32.2%) following restimulation with serum than were observed in the control cultures.

In the second experiment, we again determined the fraction of cells able to synthesise DNA following 2 and 7 days spent under conditions of low serum, followed by restimulation with serum (Table 1, B). Again, we found that by treating cultures with low serum followed by restimulation with high levels of serum, more cells were able to synthesise DNA than in untreated cultures. In cultures maintained in low serum for 2 days, and then restimulated, over 3.5 times more cells had entered S-phase by 48 h than in control cultures. The fraction of cells synthesising DNA had increased 8-fold after 48 h of restimulation in cultures that had been kept in low serum for 7 days, compared to the control cultures.

Expression of antigens commonly associated with proliferating cells by serum-starved, senescent, human diploid fibroblasts

To confirm this result, we determined the fraction of cells that expressed antigens commonly associated with proliferating cells in parallel cultures treated in an identical manner (Table 2).

### Table 1. Percentage of senescent, serum-starved, human diploid cells synthesising DNA following serum stimulation

<table>
<thead>
<tr>
<th>Time of exposure to BrdUrd (h following stimulation)</th>
<th>0-24</th>
<th>24-48</th>
<th>0-48</th>
<th>47.5-48</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum starvation:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Control culture</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>(b) 2 days</td>
<td>12.2</td>
<td>14.1</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>(c) 7 days</td>
<td>14.1</td>
<td>28.5</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>(d) 14 days</td>
<td>15.0</td>
<td>32.2</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>B. Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum starvation:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Control culture</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>(b) 2 days</td>
<td>2.2</td>
<td>13.3</td>
<td>12.6</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>(c) 7 days</td>
<td>21.2</td>
<td>20.4</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

Control cultures (a) were maintained in 10% NCS (v/v) continuously. After 2 days (b); 7 days (c) or 14 days (d), the cultures were refed with DMEM, supplemented with 10% (v/v) NCS and antibiotics. Less than 1.0% of cells in parallel cultures maintained in DMEM supplemented with 0.5% (v/v) NCS throughout the 48 h exposure to BrdUrd were labelled. n.d., not determined.
Table 2. Percentage of senescent, serum-starved, human diploid cells expressing antigens, usually associated with cell proliferation, following serum stimulation.

<table>
<thead>
<tr>
<th>Time after restimulation (h)</th>
<th>Topoisomerase II</th>
<th>PCNA</th>
<th>Ki-67</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Control culture</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>(b) 2-day serum starvation</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>0 h</td>
<td>4.6</td>
<td>3.0</td>
<td>4.5</td>
</tr>
<tr>
<td>24 h</td>
<td>2.0</td>
<td>2.3</td>
<td>10.8</td>
</tr>
<tr>
<td>48 h</td>
<td>2.8</td>
<td>2.9</td>
<td>22.0</td>
</tr>
<tr>
<td>(c) 7-day serum starvation</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>0 h</td>
<td>1.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>24 h</td>
<td>8.8</td>
<td>2.9</td>
<td>22.0</td>
</tr>
</tbody>
</table>

The cells were cultured, serum starved and restimulated as described in Materials and methods. At 0, 24 and 48 h following serum stimulation, the cells were fixed and stained. The fraction of cells expressing each antigen was determined by counting 1000 total or 200 positive nuclei in randomly selected fields.

Discussion

These data show that following a short (2 day) period in low serum, a much larger fraction of cells in senescent cultures were able to support DNA synthesis and to express markers associated with proliferation than is seen in control, untreated senescent cultures. Extending the period of serum starvation from 2 to 7 days, results in a 6- to 13-fold increase in the fraction of cells synthesising DNA and expressing these proliferation markers. Extending the period of serum starvation from 7 to 14 days only slightly increases the fraction of cells able to synthesise DNA (7-fold to 8-fold). Thus, while apparently senescent human cells can be induced to enter S-phase and G2, not all the senescent cells in the culture enter the cell cycle. The low-serum treatment apparently induces more cells to divide (about 13%, Table 3) than do so in a comparable untreated culture (about 6%, Table 3), but not to the extent predicted by the fraction of cells that were able to synthesise DNA following the same treatment (about 29%, Table 1A, experiment 1(c)). This result suggests that at least some cells that are able to synthesise DNA remain blocked with regard to proliferation at a point in or beyond S-phase; the antigen Ki-67 is positive and PCNA is negative, and this combination of antigens strongly suggests that the cells have passed through the S-phase and are now in G2. A note of caution, however, is required because the cell numbers involved were rather small.

The available data argue against the possibility that our results are due to a partial synchronization of the culture by the experimental procedure. For example, it has been shown that the average duration of the cell cycle increases only slightly during in vitro ageing (Westmark, 1978; Macieira-Coelho et al. 1966; Grove and Cristofalo, 1976; Ponten et al. 1983). Therefore, it is highly unlikely that our results are due to a partial synchronisation of the culture by the serum starvation treatment because the average duration of the cell cycle in aged cultures is only about double (approximately 2 days) that of young cultures (about 1 day). Almost all cells capable of synthesising DNA under control conditions should become labelled in a 48 h continuous exposure to BrdUrd. Therefore, it would require cell cycle times 6- to 13-fold longer to explain our results in this way, because we observe an increase of this magnitude in the fraction of cells that enter S-phase after serum starvation. Further-
more, we can exclude partial synchronization because only
20% of the cells that enter S-phase go on to divide,
whereas we would expect all the cells synthesising DNA to
divide within the 7-day period allowed for passage from
S-phase to cell division.

Cellular proliferation is regulated by a complex interaction
of positively and negatively acting factors (Vogel et al.
1978; Inglot et al. 1980). Recent reports (Brooks and
Riddle, 1988; Loo et al. 1987) have implicated the serum
supplement of normal growth media as a source of both
classes of factors. Aged cultures maintained in high levels
of serum are apparently unable to respond fully to the
mitogenic factors which are present, since they contain a
fraction of cells that are unable to synthesise DNA or to
express certain markers usually associated with prolifer-
ation. In contrast, young cultures grown under the same
conditions are able to respond more fully to the same
factors. Thus, it seems that these cells are changing their
ability to respond to mitogenic factors with time in
culture. However, a greater percentage of cells respond to
stimulating factors upon readdition of high levels of serum
to cultures treated with low serum, when compared to
replicate cultures maintained in high serum continuously.
This implies that the low serum treatment induces a
change in responsiveness in a demonstrable population of
cells. We suggest that this change may be due to an
increased responsiveness to growth-promoting factors
and/or a decreased responsiveness to growth-inhibiting
factors.

One possible explanation of these results may be
provided by the work of Collins and Rozengurt (1984a;
1984b), who have demonstrated down-regulation of
growth factor receptors by treatment with apparently
unrelated growth factors. Thus, removal of certain growth

Fig. 1. Proliferation-associated antigens in senescent human cells. The figure shows fluorescence patterns observed with antibodies
to: (B) PCNA, (D) topoisomerase II, (F) and Ki-67. The identical fields in each case stained with DAPI are shown in A, C and E.
All photomicrographs were taken at the same magnification. Bar=10 μm.

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Table 3. Cell number increase during 7 days following serum restimulation of senescent, serum-starved, human, diploid fibroblasts

<table>
<thead>
<tr>
<th>Mean cell number (±S.E.M.; n=8)</th>
<th>Mean increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>(a) Control culture</td>
<td>24 584±860</td>
</tr>
<tr>
<td>(b) 7-day serum-starved</td>
<td>19 689±143</td>
</tr>
</tbody>
</table>

Control cultures (a) and treated cultures (b) were derived from the cells described in the legend to Table 1. The treated cultures were derived from parallel cell stocks passaged on the same day as the treated cultures.

Factors may lead to the reappearance of other unrelated growth factor receptors. Perhaps culture of human, mortal cells continuously in high levels of serum leads to down-regulation of growth factor receptors by growth factors present in serum. This may result in a reduced responsiveness to the growth-promoting effects of growth factors. Eventually, aged cultures would contain a high proportion of cells with a reduced responsiveness to growth factors, due to the down-regulation of growth factor receptors. Those cells responding to the low serum treatment by synthesising growth factor receptors would show an increased response to the growth-promoting factors upon the re-addition of high levels of serum.

Alternatively, the phenomena that we have observed may be due to the behaviour of growth control genes. For example, we have observed that apparently senescent human cells may be blocked in either the G1 or the G2 phase of the cell cycle. This growth behaviour is strongly reminiscent of the properties of the gene coding for the protein kinase known as pp34-cdc2. Mutations in this gene in yeast are able to arrest cells in both these phases of the cell cycle; thus, we can imagine that this protein kinase may be responsible for initially blocking the senescent cells in G1, and then at a later time, this kinase may be associated with a subsequent block in late G1, and perhaps S.

Since the greater proportion of cells in aged cultures still do not respond to serum, even following prolonged treatment with low serum, then this may define the true, distinct, senescent state. We would therefore suggest that perhaps cells continue to cycle in response to growth-promoting factors until they enter a particular state in which they have ceased proliferating, but are not yet irreversibly mortalised, that is, reproductively sterile. Entry into this state may be due to some alteration in the responsiveness of those cells to growth factors. Furthermore, this state may only be demonstrated by the ability of cells to respond to growth factors after treatment with low serum. Eventually, under conditions of high (10% (v/v)) serum concentration, this fraction of cells will enter a true senescent state, defined by their inability to re-enter the cell cycle, even after a period spent at a low serum level. Thus, we may propose that cells may enter an intermediate senescent state, maintained in the presence of growth factors, which may be a stage on the pathway to mortalisation and senescence.

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References


Senescent human cells can enter S-phase


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