J. Biochem. 126, 1052-1059 (1999)

5-Bromodeoxyuridine Induces Senescence-Like Phenomena in Mammalian Cells Regardless of Cell Type or Species[1]

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Received August 25, 1999; accepted September 21, 1999

5-Bromodeoxyuridine was found to induce flat and enlarged cell shape, characteristics of senescent cells, and senescence-associated β-galactosidase in mammalian cells regardless of cell type or species. In immortal human cells, fibronectin, collagenase I, and $p21^{waf1/sdi-1}$ mRNAs were immediately and very strongly induced, and the mortality marker mortalin changed to the mortal type from the immortal type. Human cell lines lacking functional $p21^{waf1/sdi-1}$, $p16^{ink4a}$, or p53 behaved similarly. The protein levels of $p16^{ink4a}$ and p53 did not change uniformly, while the level of $p21^{waf1/sdi-1}$ was increased by varying degrees in positive cell lines. Telomerase activity was suppressed in positive cell lines, but accelerated telomere shortening was not observed in tumor cell lines. These results suggest that 5-bromodeoxyuridine activates a common senescence pathway present in both mortal and immortal mammalian cells.

Key words: 5-bromodeoxyuridine, immortal, senescence, senescence marker, stress.

Normal human cells stop dividing in culture after a limited number of cell divisions, a phenomenon termed cellular senescence (*1*). Senescence is characterized most typically by specific morphological alterations, to a flat and enlarged cell shape (*2*), and the induction of senescence markers such as fibronectin (*3*), collagenase I (*4*), and senescence-associated β-galactosidase (*5*). It has also been shown that in normally senesced human fibroblasts, the cyclin-dependent protein kinase inhibitors $p21^{waf1/sdi-1}$ (*6*) and $p16^{ink4a}$ (*7*) are up-regulated, and the tumor suppressor p53 (*8*) is activated. Therefore, $p21^{waf1/sdi-1}$ is thought to play a role in arresting senescent cells at G_1 and G_2 of the cell cycle (*9*). $p16^{ink4a}$ is also thought to be involved in senescence as it is frequently mutated in human tumors (*10, 11*). The inactivation of p53 has been shown to lead to an extension of lifespan in normal human fibroblasts (*12*).

As cell division proceeds, telomere shortening occurs in normal somatic cells (*13*), one of the typical changes in senescence. This telomere erosion is thought to be achieved by p53 as a form of DNA damage and to result in cell cycle arrest (*14*). In fact, enforced expression of the telomerase catalytic subunit has been shown to extend the lifespan of normal human cells (*15*).

On the other hand, accumulating data show that normal human cells can enter senescence prematurely. For instance, treatment with hydrogen peroxide (*16*) and the introduction of an activated *c-ras* gene (*17*) have been shown to induce premature senescence in normal human fibroblasts with accelerated telomere shortening. Inhibitors of histone deacetylase (*18*) and DNA topoisomerases (*19*) have been shown to do so without affecting telomeres. In sharp contrast, the above treatments have different effects on immortal cells, namely, no effect or else the induction of apoptosis or cell lysis (*16-18*).

Immortal human cells can be established easily from tumors and occasionally from cells transfected with a DNA tumor virus, but very rarely from mutagenized normal cells. During the process of immortalization, it has been shown that several known and unknown genes need to be inactivated or mutated (*20*). Some of these genes should be responsible for the response to genotoxic and environmental stress as immortal cells can not enter senescence in response to such stresses. Other genes are thought to be involved in the negative regulation of telomeres as immortal cells have acquired a certain type of telomere-maintenance mechanisms.

During a screening of agents that induce premature senescence in normal human fibroblasts, we found that 5-bromodeoxyuridine (BrdUrd) clearly does so. Unexpectedly, however, immortal cell lines also responded to BrdUrd quite similarly. In the present study, we report the phenomena induced by BrdUrd in various normal and immortal types of mammalian cells.

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: BrdUrd, 5-bromodeoxyuridine; MEFs, mouse embryonic fibroblasts; PDLs, population doubling levels.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—Human embryonic lung fibroblasts TIG-7 and immortal cell lines were obtained from the Japanese Cancer Research Resources Cell Bank. Human colon carcinoma cell line HCT116 and its mutant HCT116/80S14 with targeted homozygous deletion of the *p21* gene (*21*) were kindly provided by B. Vogelstein (Johns Hopkins University School of Medicine). Mouse embryonic fibroblasts (MEFs) were prepared according to the standard protocol as described (*22*). Cells were cultured at 37°C in plastic Petri dishes containing Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum under 5% CO_2 and 95% humidity (*22*).

β-Galactosidase Assay—The assay was performed as described previously (*22*). Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde at room temperature for 5 min, and incubated at 37°C with a fresh staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$].

Immunostaining—Cells were mounted on glass coverslips placed in 35-mm dishes. After culture for 24 h, the cells were washed twice with cold PBS and fixed in prechilled methanol/acetone (1:1, v/v) for 5 min on ice. Fixed cells were permeabilized in PBS containing 0.2% Triton X-100 for 10 min, and blocked with 2% bovine serum albumin in PBS for 20 min. The cells were incubated with anti-mortalin antibody at room temperature for 1 h, washed with the PBS containing Triton X-100, and then incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG for 30 min. After extensive washings with the same buffer, the cells were overlaid with Fluoromount (Difco), and examined under a Zeiss IM-35 microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a CELLscan System (Scanalytics, Billerica, MA).

Northern Blot Analysis—Total RNA samples (15 μg per lane) were subjected to electrophoresis in 1% formaldehyde-agarose and transferred to a nylon membrane (Hybond-N^+, Amersham). The blots were hybridized at 65°C with probes in a mixture consisting of 0.43 M Na-P_I, 7% SDS, 1% bovine serum albumin, and 20 mM EDTA for 16 h. The probes were amplified by polymerase chain reaction from the cDNA sequences of fibronectin (*3*), collagenase I (*4*), $p21^{waf1/cdi-1}$ (*23*), and GAPDH cDNA (*24*), and labeled with [α-^{32}P]dCTP using a random priming kit (Amersham). The membrane was washed twice at 65°C for 30 min in 2×SSC and 0.1% SDS and twice in 0.1×SSC and 0.1% SDS, and subjected to autoradiography as described previously (*24*).

Western Blot Analysis—Cell extracts were prepared as described (*25*). Samples were subjected to 12% SDS polyacrylamide gel electrophoresis, blotted onto a PVDF-membrane, and probed with specific rabbit polyclonal antibodies (Fl-393 for p53, H-156 for $p16^{ink4a}$, and H-1649 for $p21^{waf1/cdi-1}$, Santa Cruz Biotechnology, Santa Cruz, CA). The specific antibodies were detected with a chemiluminescence detection kit (BM chemiluminescence western blotting kit, Boehringer Mannheim) according to the supplier's manual. Chemiluminescence was detected by exposure to X-ray film (FUJI-RX, FUJI Film, Tokyo).

Analysis of Terminal Restriction Fragments—Genomic DNA samples (3 μg) were digested with the restriction endonuclease *Hin*fI (Boehringer Mannheim), and resolved by electrophoresis in a 0.5–0.7% agarose gel in Tris-acetate-EDTA buffer (pH 7.5) (*22*). DNA was transferred onto a nylon filter (Hybond-N^+, Amersham) after depurination, and the filter was hybridized with oligonucleotide [TTAGGG]$_3$. End-labeling was carried out with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolab) at 37°C in a mixture containing 0.5 M Na-P_I, 7% SDS, and 1 mM EDTA for 16 h. The filter was washed twice at 25°C in 2× SSC containing 0.1% SDS for 5 min, twice at 25°C in 0.1× SSC containing 0.1% SDS for 20 min, and once at 37°C in 0.1×SSC containing 0.1% SDS for 20 min, and then exposed to X-ray film.

Telomerase Assay—Telomerase activity was assayed by PCR-based TRAP assay as described previously (*26, 27*). Cells were harvested by trypsinization, washed with ice-cold phosphate-buffered saline, suspended in ice-cold CHAPS-lysis buffer, and finally incubated on ice for 30 min. TRAP reactions contained 1×TRAP buffer (*26*), 50 μM dNTPs, 0.1 μg TS substrate primer [5′-AATCCGTC-GAGCAGAGTT-3′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB)], 0.1 μg ACX return primer (5′-GCGCGG(CTTACC)$_3$CTAACC-3′), 0.1 μg NT internal control primer, 0.01 μmol TSNT internal control, 2 U Taq DNA polymerase (Perkin Elmer), and 2 μl CHAPS cell extract in a total volume of 50 μl. Each TRAP reaction mix was incubated at 30°C for 10 min, heated at 94°C for 5 min, and subjected to 30 PCR cycles of 94°C for 30 s and 60°C for 30 s. PCR products were separated by electrophoresis in 12% non-denaturing polyacrylamide gels and an autoradiogram was prepared.

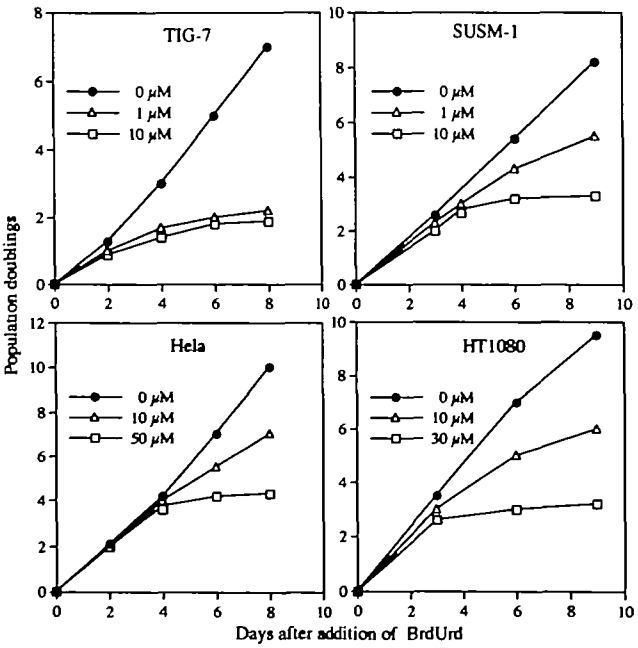


Fig. 1. **Growth curves of TIG-7 and immortal human cell lines.** TIG-7 at 38 PDLs, HT1080, SUSM-1, and HeLa were cultured in the presence of BrdUrd at the concentrations indicated.

RESULTS

Induction of Cell Cycle Arrest—We monitored the growth of TIG-7 and several human immortal cell lines (SUSM-1, HT1080, and HeLa) during culture in the presence of BrdUrd. All lines gradually stopped dividing in a dose-dependent manner (Fig. 1). BrdUrd concentrations differed by up to 5-fold to give the same effect. There was no apparent difference between human and rodent cells. The simultaneous addition of deoxycytidine did not reverse the effects of BrdUrd. We performed cytofluorometric analysis to determine where the cells were arrested in the cell cycle. In every case examined, the majority of cells arrested at the G_1 phase of the cell cycle, leaving a small fraction of cells at the G_2 range. We do not know whether this small fraction of cells represents G_2 cells or G_1 cells with increased ploidy.

Morphological Changes and Senescence-Associated β-Galactosidase—We observed morphological alterations and assayed senescence-associated β-galactosidase in various cell types cultured in the presence of appropriate concentrations of BrdUrd. Normal human diploid fibroblasts TIG-7 and MEF showed a flat and enlarged cell shape upon the addition of BrdUrd, and a strong induction of senescence-associated β-galactosidase just as they entered senescence under normal culture conditions (Fig. 2).

We then tested various immortal cell lines. Human lines

Fig. 3. **Changes in morphology and the induction of senescence-associated β-galactosidase in immortal cell lines.** Cells were cultured for 2 weeks in the presence and absence of BrdUrd at the concentrations indicated. Microphotographs were taken as in Fig. 2. a and a1,VA13 − and + 20 μM BrdUrd; b and b1, SUSM-1 − and + 10 μM BrdUrd; c and c1, HT1080 − and + 30 μM BrdUrd; d and d1, HeLa − and + 50 μM BrdUrd; e and e1, HCT116/80S14 − and + 10 μM BrdUrd; f and f1, rat 3Y1 − and + 30 μM BrdUrd; g and g1, Chinese hamster CHO − and + 30 μM BrdUrd; h and h1, mouse NIH3T3 − and + 30 μM BrdUrd, respectively. Scale bar, 100 μm.

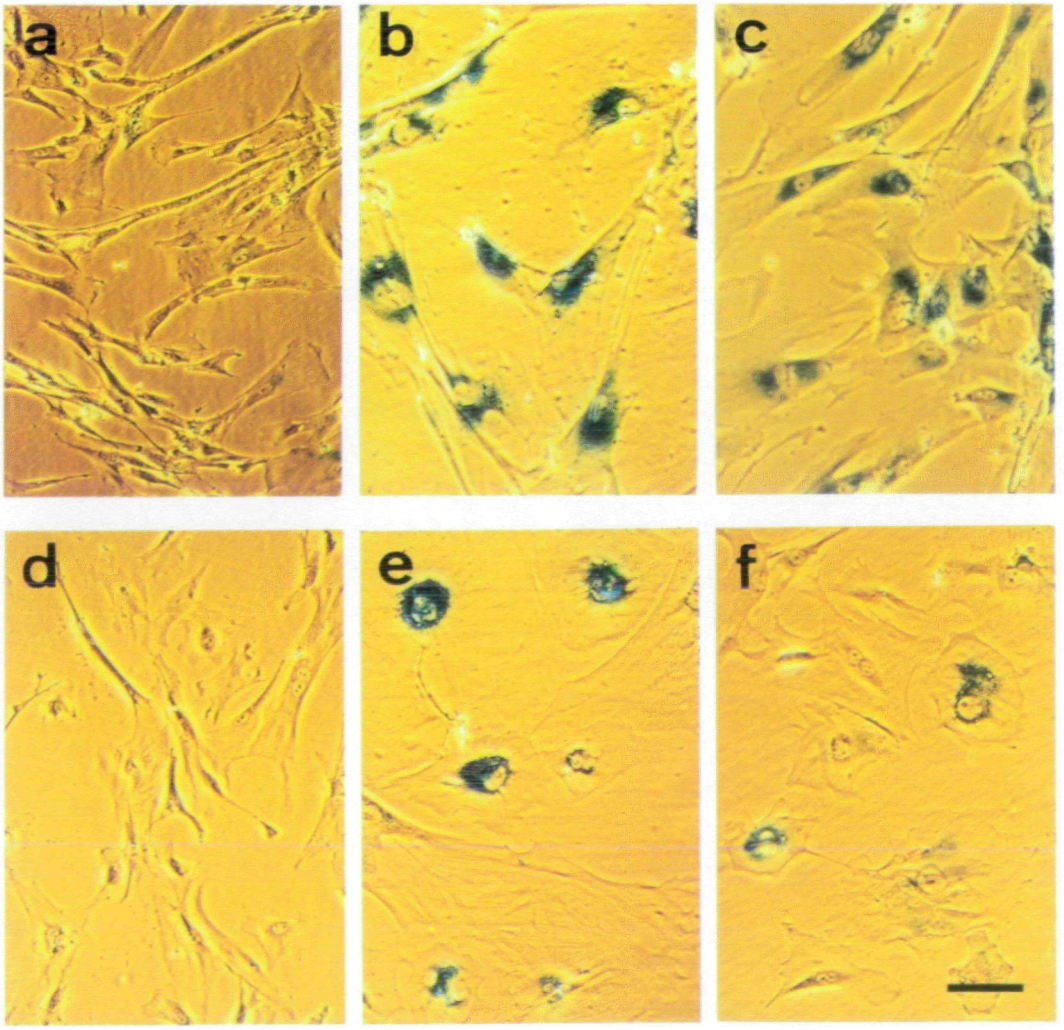
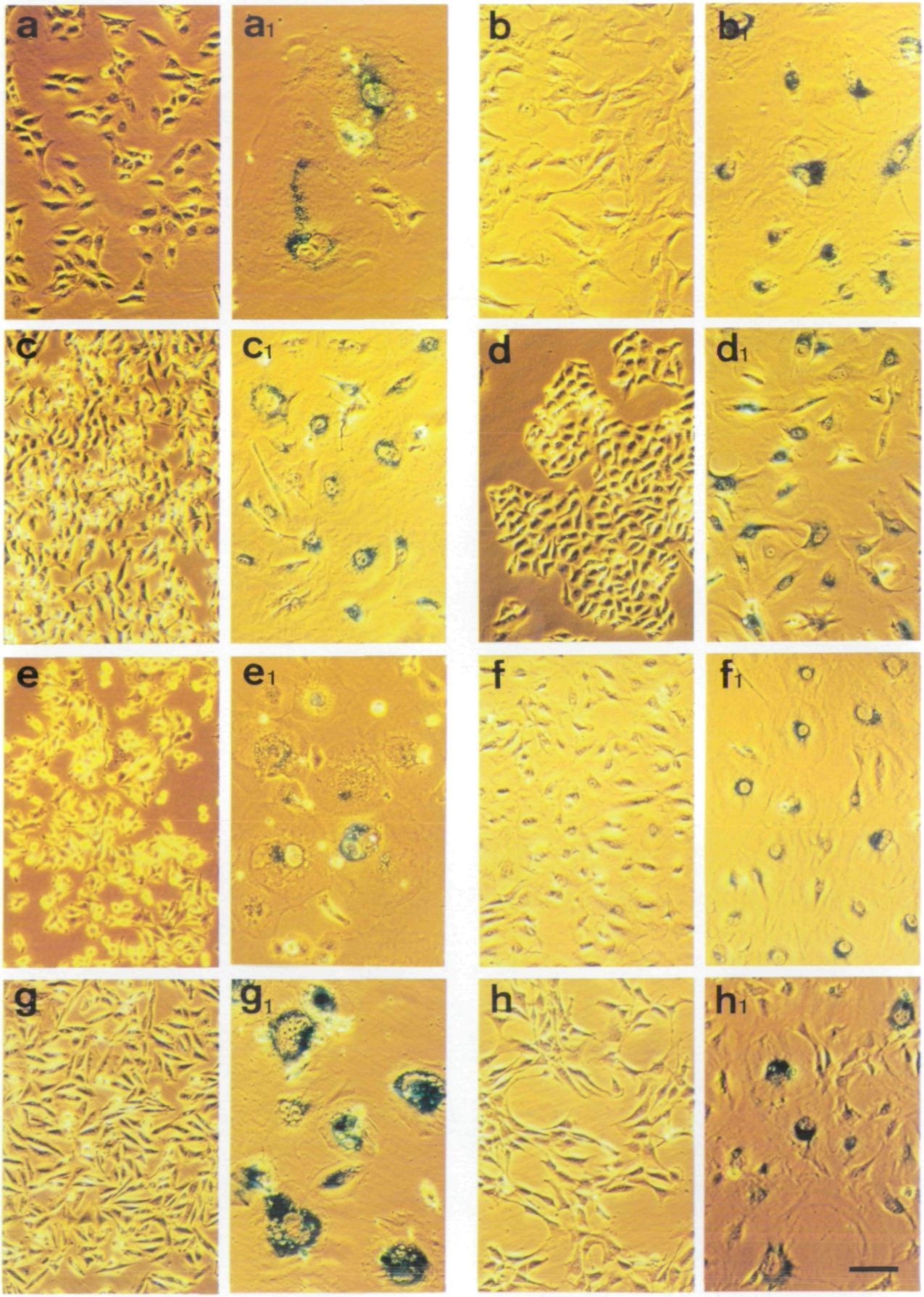


Fig. 2. **Changes in morphology and the induction of senescence-associated β-galactosidase in normal cells.** TIG-7 at 38 PDLs and MEFs at 3 PDLs were cultured for 2 weeks in the presence and absence of BrdUrd at the concentrations indicated. These cells enter senescence at 72 and 13 PDLs, respectively, under normal culture conditions. Microphotographs were taken after staining with β-galactosidase as described in "MATERIALS AND METHODS." a, TIG-7 at 40 PDLs; b, TIG-7 at 38 PDLs +10 μM BrdUrd; c, TIG-7 at 72 PDLs; d, MEFs at 5 PDLs; e, MEFs at 3 PDLs +10 μM BrdUrd; f, MEFs at 13 PDLs. Scale bar, 100 μm.



include SUSM-1 (4-nitroquinoline-1-oxide-treated liver fibroblasts), KMST-6 (γ-ray-irradiated embryonic fibroblasts), VA13 (SV40-transformed fibroblasts), HT1080 (fibrosarcoma), HeLa (cervical tumor), TE85 (osteosarcoma), MeT5A (SV40-transformed mesothelial cells), HCT116 (colon carcinoma), and HCT116/80S14 ($p21^{waf1/sdi-1}$ negative mutant). Rodent lines include mouse NIH3T3 (embryo) and L929 (skin), rat 3Y1 (embryo), and Chinese hamster CHO (ovary) and V79 (lung). All of these lines responded to BrdUrd, showing a flat and enlarged cell shape, and a strong induction of senescence-associated β-galactosidase (Fig. 3).

Behavior of the Mortality Marker Mortalin—Mortalin is a member of the HSP70 family and is distributed homogeneously in the cytoplasm of mortal types of cells (*28*). Interestingly, it displays four perinuclear distribution patterns in immortal human cell lines (*29*). Due to these properties, it has been used as a marker to distinguish between cellular mortality and immortality.

We cultured HT1080, HeLa and SUSM-1 cells in the presence and absence of BrdUrd, and examined mortalin distribution by immunostaining with anti-mortalin antibody. Under normal culture conditions, the patterns show the granular juxtanuclear cap, a granular gradient from the nuclear membrane to the cell membrane, and perinuclear fibres (*29*) (Fig. 4, a-c). When cultured with BrdUrd for 2 weeks, all showed a cytosolic distribution pattern characteristic of mortal cells (Fig. 4, a1-c1). These results suggest that the cell cycle arrest induced by BrdUrd is associated with a loss of indefinite division potential.

Induction of Senescence Markers—We monitored mRNA levels for $p21^{waf1/sdi-1}$, fibronectin, and collagenase I, which are frequently used as senescence markers in normal types of cells. We used HeLa cells as this line is the most widely used among human immortal cell lines. The mRNA levels of both $p21^{waf1/sdi-1}$ and fibronectin began to increase two days after the addition of BrdUrd and reached strikingly high levels (Fig. 5). The level on day 8 of $p21^{waf1/sdi-1}$ mRNA was 80 times and of fibronectin mRNA more than 1,000 times higher than their levels on day 0. The early onset of induction is in agreement with the finding that the cells are destined to stop dividing by 2-3 days after the addition of BrdUrd. Collagenase I mRNA began to increase

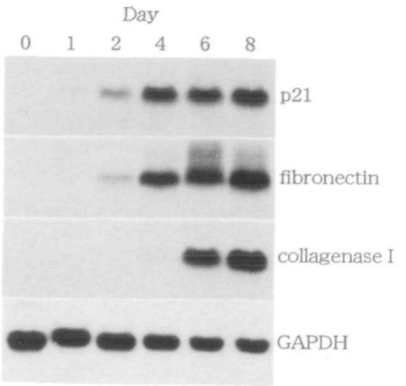


Fig. 5. **Northern blot analysis of $p21^{waf1/sdi-1}$, fibronectin, and collagenase I mRNAs in HeLa cells.** Cells were cultured in the presence of 50 μM BrdUrd for the times indicated. The mRNA levels were determined by Northern blot analysis as described in "MATERIALS AND METHODS." GAPDH: glyceraldehyde monophosphate dehydrogenase.

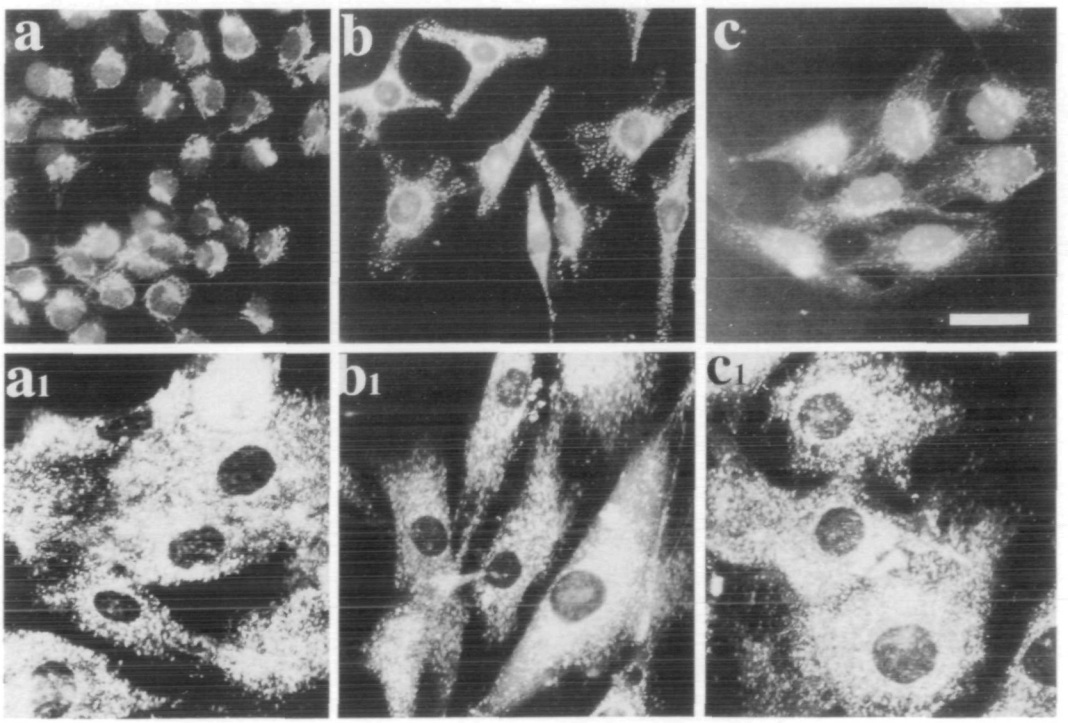


Fig. 4. **Distribution of mortalin in immortal human cell lines.** Cells were cultured for 2 weeks in the presence and absence of BrdUrd at the concentrations indicated. Mortalin was immunostained with anti-mortalin antibody and microphotographs were taken under a fluorescence microscope as described in "MATERIALS AND METHODS." a and a1, HT1080 − and + 30 μM BrdUrd; b and b1, HeLa − and + 50 μM BrdUrd; c and c1, SUSM-1 − and +10 μM BrdUrd. Scale bar, 100 μm.

more slowly than the others, but the level on day 8 was 850 times higher than that on day 0.

These results strongly suggest that HeLa cells immediately enter a senescence-like state upon the addition of BrdUrd.

Changes in Cell Cycle Regulators—We examined the protein levels of $p21^{waf1/sdi-1}$, $p16^{ink4a}$, and p53 by Western blot analysis (Fig. 6), as these proteins have been shown to be up-regulated or activated in normally senesced human cells and have been implicated in senescence (*6–8*).

The level of $p16^{ink4a}$ in TIG-7 increased 3-4-fold upon culture in the presence of BrdUrd for 1 week. The basal levels of VA13 and HeLa were much higher than that of

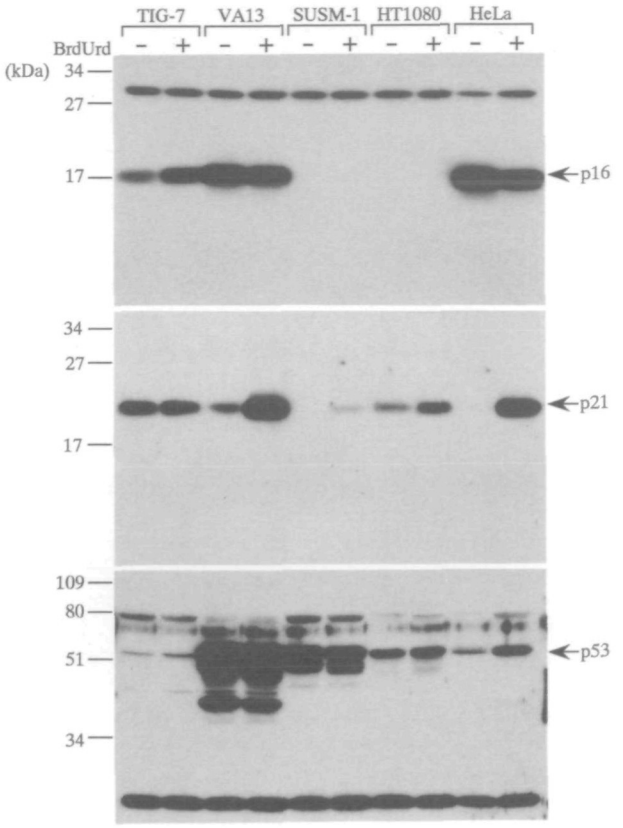


Fig. 6. **Western blot analysis of $p16^{ink4a}$, $p21^{waf1/sdi-1}$, and p53 in TIG-7 and immortal human cell lines.** TIG-7 at 38 PDLs (10 μM), VA13 (20 μM), SUSM-1 (10 μM), HT1080 (30 μM), and HeLa (50 μM) were cultured for 1 week in the presence and absence of BrdUrd at the concentrations indicated. Western blots were probed with specific antibodies to $p16^{ink4a}$, $p21^{waf1/sdi-1}$, and p53 as described in "MATERIALS AND METHODS." Arrows indicate the position of each protein. Molecular standards are shown on the left.

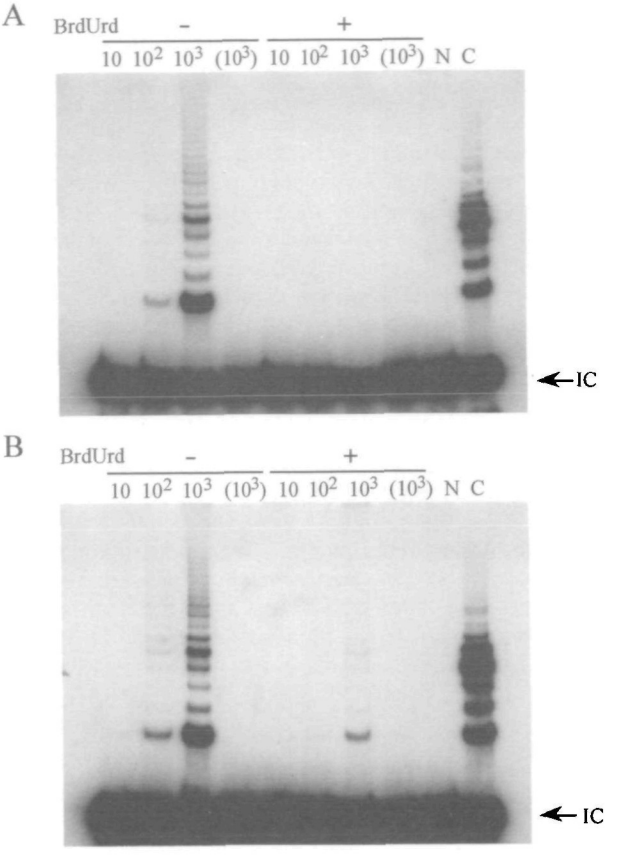


Fig. 8. **Telomerase activity in HeLa (A) and HT1080 (B).** HeLa (50 μM) and HT1080 (30 μM) cells were cultured for 2 weeks in the presence and absence of BrdUrd at the concentrations indicated. Cell extracts were prepared from the cells and telomerase activity was assayed in cell extracts equivalent to 10, 10^2, and 10^3 cells as indicated by the PCR-based TRAP assay as described in "MATERIALS AND METHODS." (10^3), N, and C above the lanes indicate heat-inactivated cell extracts equivalent to 10^3 cells, cell extraction buffer, and control telomeric sequence (R8) (*26*), respectively. The internal control (IC) is marked by an arrow.

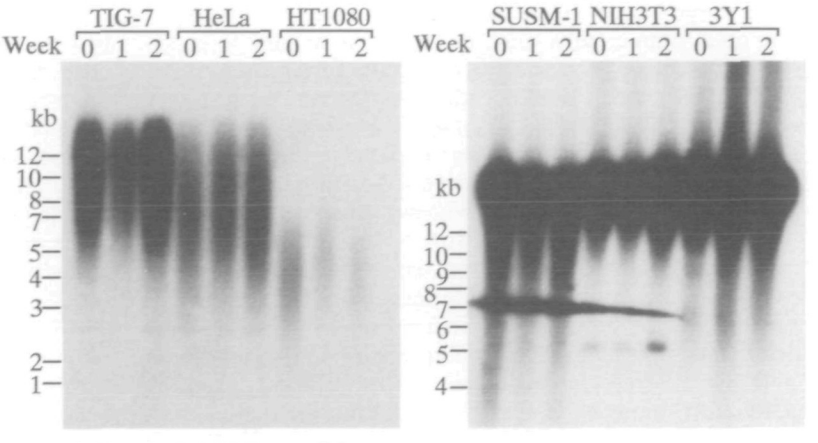


Fig. 7. **Telomere restriction fragments in TIG-7 and human and rodent immortal cell lines.** TIG-7 at 38 PDLs (10 μM), HeLa (50 μM), HT1080 (30 μM), SUSM-1 (10 μM), mouse NIH3T3 (30 μM), and rat 3Y1 (30 μM) were cultured for 0, 1 and 2 weeks in the presence of BrdUrd at the concentrations indicated. Genomic DNA samples were digested with endonuclease *Hin*fI, and subjected to Southern blot analysis with labeled [TTAGGG]$_3$ as a probe as described in "MATERIALS AND METHODS."

TIG-7, but did not increase upon the addition of BrdUrd. SUSM-1 or HT1080 did not show detectable levels of $p16^{ink4a}$.

The level of $p21^{waf1/sdi-1}$ in TIG-7 did not change significantly upon the addition of BrdUrd. This observation is in agreement with our previous finding that the level is not significantly increased in normally senesced TIG-7, although the mRNA level of $p21^{waf1/sdi-1}$ increases significantly (*19*). In the immortal cell lines examined, $p21^{waf1/sdi-1}$ levels increased more or less upon the addition of BrdUrd, although the basal levels differed widely.

The level of p53 was very low in TIG-7, and increased 2-3-fold upon the addition of BrdUrd, an increase also seen in normally senesced TIG-7 cells (*19*). In the immortal lines, the basal levels varied as much as those of $p21^{waf1/sdi-1}$. In VA13 and SUSM-1, which contain high basal levels of p53, the levels did not increase upon the addition of BrdUrd. It should be noted that p53 is functionally or genetically inactivated in these lines (*24, 30*). HT1080 showed a slight increase, while HeLa showed a significant increase upon the addition of BrdUrd.

Characterization of Telomeres and Telomerase—We examined whether accelerated telomere shortening occurs in TIG-7, HeLa, HT1080, SUSM-1, NIH3T3, and 3Y1 upon treatment with BrdUrd. First, we monitored terminal restriction fragments in TIG-7 cells cultured to 40, 50, 60, and 72 population doubling levels (PDLs). As expected, the average length of the fragments became shorter as the PDLs increased. When TIG-7 at 38 PDLs and immortal lines were cultured in the presence of BrdUrd for 2 weeks, the terminal restriction fragments were not shortened in any case (Fig. 7).

Then we examined telomerase activity in two telomerase-positive cell lines, HeLa and HT1080. In both lines, the activity decreased to less than 10% 2 weeks after the addition of BrdUrd (Fig. 8). It is thus possible that BrdUrd directly down-regulates the genes for the telomerase catalytic subunit or its associated proteins. However, this result may simply reflect the cell cycle arrest induced by BrdUrd since telomerase activity is shown to depend on the proliferative potential of the cells (*31*).

DISCUSSION

Accumulating data have revealed that normal human fibroblasts can enter senescence prematurely by various means, such as, UV- or X-ray irradiation, treatment with a DNA damaging agent or a particular cellular compound, or the ectopic expression of a particular gene. In contrast, immortal human cells undergo apoptosis or cell lysis by these treatments or resist them. These observations suggest that immortal human cells have lost the capacity to respond to these stresses to enter senescence. Contrary to this notion, we have found that BrdUrd induces a senescence-like phenomenon in all types of mammalian cells as judged by the behaviors of well-known senescence markers. If this is so, it may be that BrdUrd activates a common senescence pathway present in mammalian cells. In this respect, a few reports describe the effects of BrdUrd in terms of senescence. In chick chondrocytes, BrdUrd has been shown to induce the production of minor cartilage collagens that are found in cells cultured to enter senescence (*32*). In neonatal rats, the subcutaneous injection of BrdUrd has been shown to markedly decrease their mean lifespan with an increase in tumor incidence (*33*).

The immortal human cell lines we examined here include those genetically or functionally defective in $p21^{waf1/sdi-1}$, $p16^{ink4a}$, p53, or pRB, which are thought to be involved in cellular senescence. Further, $p16^{ink4a}$ or p53 do not change uniformly upon the addition of BrdUrd, although $p21^{waf1/sdi-1}$ increased to various degrees in the cell lines examined. These observations suggest that none of these proteins is involved in the BrdUrd-induced senescence-like phenomenon, although the expression of $p21^{waf1/sdi-1}$ seems to coincide with this phenomenon. It is now thought that there exist multiple pathways for normal cells to enter senescence. According to this scenario, BrdUrd might activate a novel senescence pathway that is common to both normal and immortal cells. Alternatively, BrdUrd might activate or suppress particular genes that play critical roles in senescence and operate downstream of a stress-response machinery present in normal and immortal cells.

BrdUrd is incorporated into DNA in place of thymidine and shown to change the interaction between DNA and proteins (*34-36*). This change is thought to alter the expression of a class of genes by an unknown mechanism, thereby leading to the induction or inhibition of differentiation in mammalian cells. Despite numerous reports, the molecular mechanism underlying these phenomena remains to be characterized. Recently, we identified many genes whose expression immediately increases or decreases upon the addition of BrdUrd to immortal human cells. As expected, some of these genes have been found to be related to senescence and differentiation in normal cells (Suzuki, T. *et al.*, manuscript in preparation). We have also constructed a BrdUrd-dependent expression system for the green fluorescent protein gene. These results will help in gaining an understandings of the molecular mechanism of the action of BrdUrd, especially in terms of the induction of senescence.

On the other hand, it is well known that 5-bromouracil incorporated into DNA is converted to uracil by irradiation with light, and this may cause a nick or gap in the DNA during the process of excision repair. In fact, the rate of sister chromatid exchanges slightly increases in cells cultured in the presence of BrdUrd (*37, 38*). However, such DNA-damage, if any, does not seem to contribute much to the phenomenon described here because DNA damage-inducible genes, except for the $p21^{waf1/sdi-1}$ gene, were not induced in the above experiment (Suzuki, T. *et al.*, unpublished data).

Finally, the methodology described in present study provides several advantages in the study of senescence. It enables us to obtain a large number of homogeneously senescent cells in a short time from immortal cells instead of normal cells. It is difficult to obtain these cells from normal human cells due to heterogeneity in division potential. It also makes it possible to study senescence genetically using standard rodent cell lines. These and other advantages described here will lead to an understanding of the molecular basis for cellular senescence and immortalization.

We thank B. Vogelstein of Howard Hughes Medical Institute for providing us with the HCT116 cell line and its derivatives.

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