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Cellular aging, destabilization, and cancer

(senescence/neoplastic transformation/lipofuscin/age pigment)

HARRY RUBIN, MING CHOW, AND ADAM YAO

Department of Molecular and Cell Biology and Virus Laboratory, Stanley Hall, University of California, Berkeley, CA 94720-3206

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ABSTRACT **Three major characteristics of aging in animals are a slowdown of cell proliferation, an increase in residual bodies associated with age pigments, and a marked increase in the likelihood of neoplastic transformation. The 28 L subline of the NIH 3T3 line of mouse embryo fibroblasts exhibits all these characteristics when held at confluence for extended periods. The impairment of proliferation is the first behavioral characteristic detected in low density subcultures from the confluent cultures, and it persists through many cell generations of exponential multiplication. There is an equal degree of growth impairment among replicate cultures (lineages) recovered after each of 2 successive rounds of confluence, although heterogeneity appears after the third round. The growth impairment pervades the entire cell population of each lineage. The degree and duration of impairment increase with repeated rounds of confluence. A marked increase of residual bodies characteristic of age pigments occurs in the cytoplasm of all the cells kept under prolonged confluence. Neoplastic transformation first appears as foci of multilayered cells on a monolayered background of nontransformed cells. The transformed cells arise at different times in the lineages and originate from a very small fraction of the population. The transformed cells selectively overgrow the entire population in successive rounds of confluence leading to an increase in saturation density of each lineage at different times. Under cloning conditions, isolated colonies of transformed cells develop more slowly than colonies of nontransformed cells but eventually reach a higher population density. The regularity of persistent growth impairment among the lineages and the appearance of large numbers of residual bodies in all the cells of each population are more characteristic of an epigenetic process than of specific local mutations, although random chromosomal lesions cannot be ruled out. By contrast, the low frequency and stochastic character of neoplastic transformation are consistent with a conventional genetic origin. The advent in long-term confluent NIH 3T3 cultures of three cardinal characteristics of cellular aging *in vivo* recommends it as a model for aging cells.**

The overall incidence of cancer in humans increases exponentially with age beginning at about 30 years (1, 2). Specific examples of such a Gompertzian relationship are to be found, for example, in mortality rates from lung and prostate cancer (3, 4). Chromosomal aberrations and local mutations are found in profusion in cancer, and one explanation for the increase in cancer with age is the requirement for five to seven such genetic changes to produce the malignancy (5, 6), although the number may be as high as 10 in prostate cancer (7). That this is not the full explanation for the age dependence of cancer is indicated by the 50-fold increase in frequency of chronic myelogenous leukemia between the ages of 15 and 70 years (8), since there it involves only a single chromosomal translocation (9). Similar evidence of such an effect is the 100-fold increase in the incidence of Down syndrome in the offspring of mothers between the ages of 20 and 45 years (10). This condition is also the result of a single chromosome change, either trisomy of short chromosome 21 or translocation of its long arm to chromosome 13, 14, or 15. These marked increases in conditions caused by a single chromosomal change indicate that the frequency of some disease-related chromosomal aberrations increases markedly with age. Large increases with age in the frequency of chromosome aberrations have been reported in humans (11, 12), dogs (13), and rodents (14–16). It is therefore reasonable to assume that the multiple genetic changes generally associated with cancer as well as the cancers themselves are related to destabilization of cells with the age of the organism.

It would be desirable to define the effects of aging on neoplastic transformation in culture. Normal fibroblasts from human donors have a limited lifespan in culture (17), which has a weak inverse correlation with the age of the donor (18–21). Since the growth rate of the cells decreases with the number of population doublings, resulting eventually in their reproductive death, there is no opportunity to observe the effect of age on the frequency of neoplastic transformation. The growth rate of mouse cells declines with successive passages in culture, but the cells go through a crisis from which permanent lines are established (22). The growth of some mouse cell lines is inhibited at confluence and they are nontumorigenic while others behave like neoplastic cells (23). As a general rule, those lines subcultured at low density like the NIH 3T3 line (24) are of the former category, and those subcultured at high density are of the latter (23). While the inverse relation between human donor age and average lifespan of the cells in culture has been repeatedly observed (18–20), reflecting some difference in cells from young and old donors, the loss of viability itself is not characteristic of *in vivo* aging. There is no generalized age-associated loss *in vivo* of stem cell ability to divide followed by dying out of these cells (25). Labeling studies *in vivo* of mouse tongue epithelium showed an average of 565 doublings over a lifespan (26) compared to the 14–28 doublings *in vitro* that precede development of a cell line from mouse embryo fibroblasts (22, 27). Early passage cells cultured from a human fetus have a much lower RNA and protein content and volume than late passage cells of the same origin, while there is no difference in these properties between early passages of cells cultured from young and old men (20). Although cells cultured from young men multiply faster than cells from old men, the difference between the two age groups is much smaller than that between early and late passage human fetal fibroblasts (20). The reduction in growth rate in culture of cells from old men is of similar magnitude to the reduction measured in aging mice (28, 29). Thus, the "Hayflick limit" on the number of divisions of human cells in culture (17) may serve as an indicator of differences in the initial state of the cells from young and old donors and of their survival in culture, but it does not necessarily reflect a fixed limit on the

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Abbreviations: 1°–4° assays, consecutive assays for focus formation and saturation density.

number of cell divisions *in vivo*, nor can it be said to accurately simulate the *in vivo* aging process. Ideally, to do so it would be necessary to induce differences comparable in type and magnitude to those found in cells recently cultured from young and old individuals (20). It would be an extra dividend for studying cellular aging if an experimental treatment could be shown to increase the cytoplasmic concentration of age pigments and the likelihood of neoplastic transformation.

Toward these goals two sublines of NIH 3T3 cells have been isolated after repeated passage at low density, which maintain a constant growth rate and do not produce transformed foci in a standard assay (30, 31). By the very fact of their derivation from an established line, these sublines differ from cells isolated directly from the animal. However, they can be formally treated as normal in growth behavior since their growth is strongly inhibited by contact at confluence. It may then be asked whether the cells exhibit the criteria of aging and neoplastic transformation if maintained under the constraint of confluence for prolonged periods, in analogy to the slowly dividing state of the aging organism. We had previously shown that repeated periods of prolonged confluence of NIH 3T3 cells invariably result in neoplastic transformation (32, 33). Recently, we found that there is a reduction in growth rate of cells passaged after prolonged confluence that persists for many cell generations and a destabilization that predisposes them to neoplastic transformation (30, 31). The persistent reduction in growth rate and destabilization are analogous to the heritable damage induced in cells by x-irradiation (34–36) or treatment with carcinogenic polycyclic hydrocarbons (37). They are consistent with the suggestion that a lengthy period of quiescence in culture produces sustained effects on cell growth similar to those produced by aging in adult animals (38).

Another concomitant of aging is the increase in age pigment or lipofuscin granules especially prominent in fixed postmitotic cells but also found, albeit to a lesser extent, in some dividing cells (39). The granules are associated with residual bodies and are considered secondary lysosomes that result from incomplete digestion of cytoplasmic organelles (40). These structures are found in large numbers in cultures of human glial cells when they approach the "Hayflick limit" of multiplication or are inhibited at confluence for 3 weeks or more (40).

The present experiments were designed to critically test the thesis that the persistent impairment of proliferation produced by prolonged confluence simulates aging in producing an enduring, cumulative slowdown in growth of the entire population of cells accompanied by an instability that results in neoplastic transformation in a small fraction of them. The basic design of the test was to subject four parallel cultures from the same pool of cells to repeated rounds of prolonged confluence and to measure a variety of growth characteristics of each lineage after each round. In addition, cells from the first round of confluence were prepared for electron microscopy and examined for cytoplasmic structures associated with aging. The results confirm the proposal that prolonged confluence induces the major growth and morphologic characteristics associated with cells in aging organisms.

MATERIALS AND METHODS

Cells and Culture Methods. The 28 L subline of NIH 3T3 cells was used throughout (31). It had been subcultured weekly at cloning densities (400 cells per 56-cm^2 plastic Petri dish) for 19 weeks and then cryopreserved in liquid nitrogen for almost 2 years. Freshly thawed cells were seeded at 10^5 in each of six 21-cm^2 plastic Petri dishes (Falcon) and at 4×10^4 in two 56-cm^2 dishes in molecular, cellular, and developmental biology medium 402 (41) containing 10% calf serum for maximal growth rate. The 21-cm^2 cultures became confluent in 4 days, and four of them (nos. 1–4) were incubated as separate lineages at 37°C for a total of 2 weeks before use in most of the experiments to be described. The remaining two (nos. 6 and 8) were incubated for 3 weeks and used in the experiment on colonial growth recorded in Fig. 4. Both sets were considered 1° assays. They were subcultured at 10^5 cells for the standard focus and saturation density assay in 2% calf serum (CS) for 2 weeks for 2° assays. Serial repetition of the 2° assays at intervals of 2 weeks constituted 3° and 4° assays, the latter only for lineages nos. 6 and 8. Cultures from each assay were fixed in Bouin's reagent and stained with 4% Giemsa to count transformed foci (30–32). Sister cultures from each assay were treated with trypsin and counted to establish their saturation density. The 56-cm^2 cultures were kept at low density by subculture every 2–3 days at 4 and 2×10^4 cells, respectively. They served as controls for the postconfluent cultures in all the measurements of growth and neoplastic transformation. Following each assay, some of the cells were subcultured for 2 or 3 days at 4×10^4 per 56-cm^2 dish to recover from the direct inhibitory effects of prolonged confluence. Each of the lineages was then distributed in eight replicates at 10^4 cells per 56-cm^2 dish in 10% CS to initiate growth curves in which two cultures were counted on each of 4 successive days. An auxiliary control (C′) accompanying the 2° and 3° serial assays of the lineages consisted of cells that had been through only a single assay in 2% CS, which is less effective than 10% CS in producing persistent impairment of proliferation at low density and neoplastic transformation (31).

In addition to the growth curve, cells from the 2- to 3-day recovery period of each assay were seeded at 100 per 21-cm^2 dish in 10% CS for colony formation. Colonies were stained at 6 and 7 days with 4% Giemsa stain. The 6-day colonies were analyzed for area and density by a computer program that processed the images produced by a Hewlett–Packard Scanjet Plus flatbed optical scanner (42). The area and density of each colony were multiplied to define quantitatively the colony size. The 7-day colonies were counted by naked eye to determine colony-forming efficiency. In a single case, cells from lineages nos. 6 and 8, which had been through a 4° assay, as well as controls were seeded on 12 21-cm^2 dishes in 10% CS. Two of these were stained at 6 days for colony formation and 2 were treated with trypsin for cell counting. The remaining dishes were switched to 2% CS to differentially favor the growth of transformed cells, and both staining and cell counting were repeated at 9 and 15 days. The results were expressed as the average number of cells per colony. The number of colonies per dish at 9 days was used to calculate the number of cells per colony at 15 days to avoid secondary colonies that arose from detachment and reattachment of cells from the primary colonies and became prominent at 15 days. All cell counts were made in a model ZM Coulter electronic counter.

Electron Microscopy. Cultures in 21-cm^2 dishes were initiated in 10% CS with 10^5 cells from the low density passages of the 28 L cells. They were prepared for electron microscopy at 3 and 14 days, which were, respectively, 1 day before and 10 days after reaching confluence. The cultures were fixed overnight *in situ* with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) followed by 30 min in 0.5% uranyl acetate. They were then dehydrated in a graded ethanol series, embedded in epon/araldite resin, and sectioned en face on a Reichert Ultracut E using a Diatome diamond knife. The sections were collected on carbon/formvar-coated copper grids and stained with 70% methanolic uranyl acetate for 10 min and then with Reynolds lead citrate for 5 min. Samples were viewed on a JEOL 100 CX transmission electron microscope operated at 80 kV.

RESULTS

Saturation Densities and Focus Formation After Successive Rounds of Confluence. Increase in saturation density is an

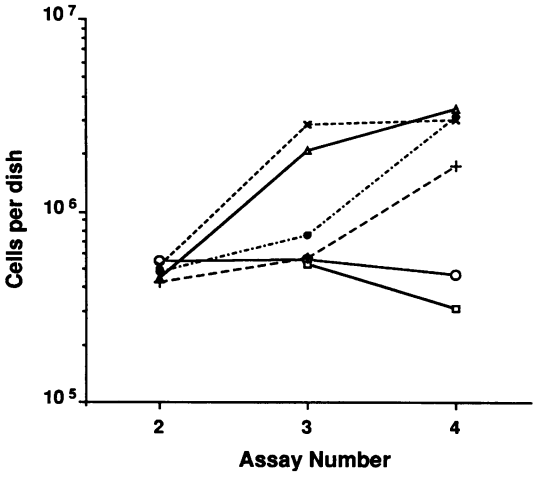

FIG. 1. Saturation densities at the termination of serial assays. The 2°, 3°, and 4° assays of the four lineages were accompanied by 1° assays of cells that had been kept in continuous, exponential multiplication by frequent low density subcultures (C, control) and by 2° assays of the cells that had been through only a 1° assay for 2 weeks in 2% CS (C′, auxiliary control). The cell counts at 2 weeks from the assays are the saturation densities shown in the figure. ○, C; □, C′; △, no. 1; x, no. 2; ●, no. 3; +, no. 4.

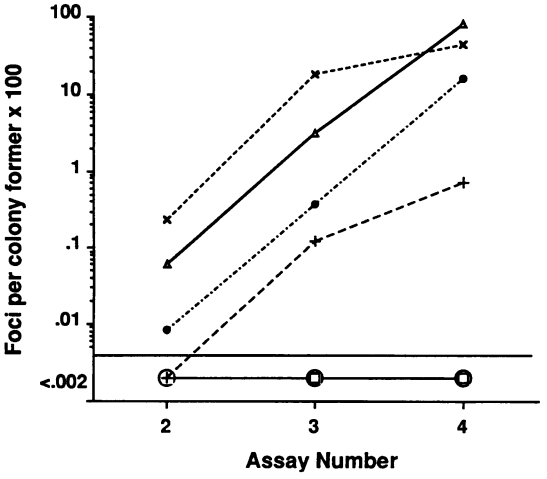

FIG. 2. Transformed focus formation in serial assays. In addition to the straight assay of 10^5 cells for each sample, serial 1:10 dilutions of the cells were made, mixed with 10^5 control cells, and seeded for assay in 2% CS. The cells were also seeded without mixture at 10^2 cells per dish in 2% CS and incubated for 12 days to determine cloning efficiency. Foci were counted on the stained assay dishes and plotted as the percentage of clonable cells that could form foci. Symbols are the same as in Fig. 1.

indicator of the capacity of cells to continue multiplying after they have reached confluence on the culture dish and is a precursor of their capacity to form tumors in animals. Saturation densities of the four lineages of the 28 L subline of NIH 3T3 cells when subcultured in 21-cm^2 dishes after successive rounds of prolonged confluence are shown in Fig. 1. All of the four lineages subcultured after a 2-week 1° assay period in 10% CS (≈10 days at confluence) grew to a slightly *lower* saturation density in a 2° assay than control cells that had never experienced the constraint of confluence. After a second round of confluence, lineages 1, 2, and 3 multiplied to approximately 4, 6, and 1.5 times the control saturation density in a 3° assay, while lineage 4 showed no significant increase. By the 4° assay, the saturation density of lineage 3 reached the high saturation density of lineages 1 and 2, while lineage 4 lagged somewhat behind. Therefore, the saturation density of all the lineages increased with repeated constraints of confluence, but it did so at different times.

The numbers of transformed foci produced in assays 2°, 3°, and 4° are shown in Fig. 2 where they are expressed as percentage of clonable cells that produce foci. The sequence of expression of focus formation follows an order similar to that of saturation density although the number of foci spans a much wider range. Taken together the data of Figs. 1 and 2 indicate that transformation, expressed initially as focus formation in a small fraction of cells and later as an increase in saturation density, is a sporadic and low frequency ($\approx 10^{-8}$ per cell per hr) event in confluent cultures.

Growth Rates of Cells After Successive Rounds of Confluence. The growth rates of all four of the lineages retrieved from the 1° assay were 16–18% lower than that of the control cells that had never experienced the inhibitory effects of confluence (Fig. 3*A*). The growth rates of the lineages recovered from the 2° assay were again similar to one another and ≈20% lower than the standard control (Fig. 3*B*). An auxiliary control of the cells was used that had recovered from one round of confluence in only 2% CS. Although the number of these cells present on the first day after seeding was less than the standard control, the subsequent growth rate was the same. The growth rates of the lineages 1 and 3 recovered from the 3° assay were

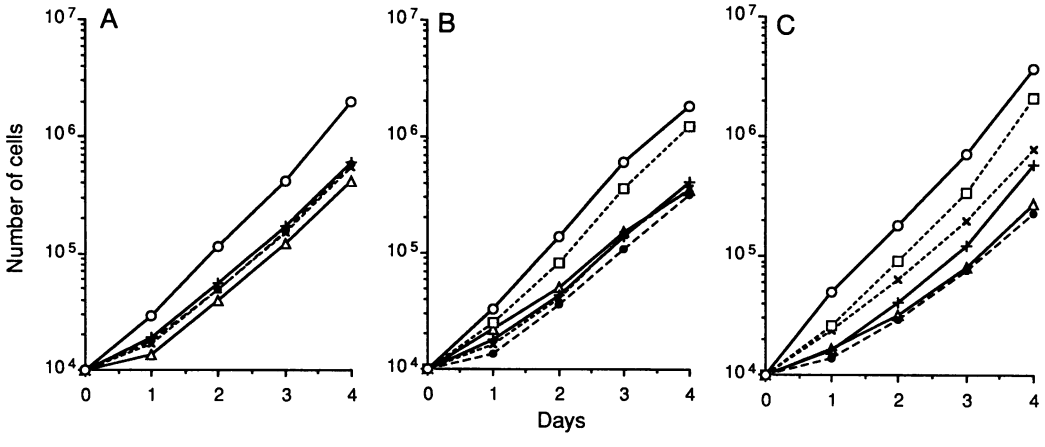

FIG. 3. Growth curves of cells subcultured from each assay. At the termination of the 1°, 2°, and 3° assays, the cells of each category were subcultured once at low density in 10% CS for 2 days (*A* and *C*) or 3 days (*B*) and then reseeded at 10^4 in eight 56-cm^2 dishes for growth curves. Cell counts were made at 24-hr intervals for 4 days. In addition, 100 cells were seeded in 10% CS for 7 days to determine colony-forming efficiencies, and for 6 days to determine average colony sizes. (See text.) Growth curves are of cells recovered from 1° assay (*A*), 2° assay (*B*), and 3° assay (*C*). Symbols are the same as in Fig. 1.

≈35% lower than the standard control, while those of lineages 2 and 4 were ≈18% lower (Fig. 3*C*). The results from the 3° assay, therefore, reveal considerable heterogeneity among the lineages in the impairment of proliferation. Once again the auxiliary control yielded fewer cells than the standard control on day 1 but multiplied at the same rate thereafter.

The results showed that there is a progressive impairment of proliferative capacity in cells with successive rounds of prolonged confluence. Since the initial slope of the growth curves is reduced, it is apparent that multiplication of the entire cell population has been reduced. The similarity of the reduced multiplication rates among the four lineages after each of the first two rounds of confluence is consistent with a population-wide effect. The appearance of heterogeneity of multiplication rate among the lineages after the 3° assay suggests that there was heterogeneity within each population, with selection in lineages 2 and 4 of those cells that were resistant to further impairment of proliferation.

The persistent reduction in growth rate was only part of the deleterious effect of long-term confluence. The colony-forming efficiency of the lineages, given a 2- to 3-day recovery period after confluence averaged about half the 60–80% efficiency of the controls, indicating that some cells were capable of no, or only limited, reproduction. Those colonies that developed at 6 days to a size scored by the scanner were less than half the standard control sizes. The auxiliary control had about the same cloning efficiency as the standard control, and the colony sizes were only slightly less than that of the control.

It is noteworthy that the extent of transformation in a lineage is not correlated with the degree of growth impairment. Lineages 1 and 2, which were the most transformed of the four lineages in both the 2° and 3° assays (Figs. 1 and 2), were, respectively, among the slowest and the fastest growing after the 3° assay (Fig. 3*C*).

To determine unequivocally whether slow, initial multiplication is a common characteristic of transformed cells, two strongly transformed lineages were cloned after recovery from a 4° assay, and their colonies were compared with those of the standard control at various times. The multiplication of the clones was maximized for 6 days by incubation in high (10%) CS to illustrate their initial growth rate and then switched to 2% CS to differentially favor the growth of transformed cells during the additional incubation periods. The appearance of the stained colonies is shown in Fig. 4. The colonies from transformed lineages nos. 6 and 8 were barely detectable at 6 days, while those of the control were clearly visible. After switching at 6 days to 2% CS, the control colonies increased in area but decreased in staining intensity as the cells flattened and their metabolism subsided. By contrast, all the colonies of the transformed lineages continued to increase in area, density, and staining intensity. The ratio of cells per colony of the transformed lineages nos. 6 and 8 to cells per control colony was only 0.26 and 0.20 at 6 days but it increased to 7.53 and 12.79 at 15 days.

Electron Microscopy. The electron micrographs of Fig. 5 confirm that large numbers of residual bodies, which are the repository of age pigments (43), accumulate in cells incubated for 2 weeks in a 1° assay. They occupy a large fraction of the cytoplasm in the quiescent cells (Fig. 5*B*), but none are seen in actively multiplying cells of the control (Fig. 5*A*). The cells shown were representative of their respective populations in both cases and indicate that age pigment formation is a populationwide response to the growth constraint of confluence.

DISCUSSION

One of the goals of this work was to establish the nature of the persistent impairment of proliferation of NIH 3T3 cells subcultured after prolonged confluence and its relationship to the neoplastic transformation that also occurs in these cells. The degree of growth impairment was found to be the same in each of four parallel lineages after one and two rounds of confluence and to involve the entire population of cells as indicated by the reduced slope of the postconfluent growth curves and the pervasive reduction in size of short-term colonies (Fig. 4). The uniformity and pervasiveness of the response are indicative of an epigenetic rather than a specific mutational effect, but chromosomal damage other than classical mutations cannot be ruled out. For example, it has been reported that there is a continuous reduction in ribosomal DNA in brain and cardiac muscle with age in humans and dogs, which could account for the loss of function in these tissues (44). Whatever the cause of the persistent growth impairment, it certainly is a regular feature of cellular aging in humans (20, 21) and mice (28, 29, 45). The pervasiveness of the reduction in growth rate of cells in our postconfluent cultures and of cells in aging organisms suggests a relationship of our treatment to the aging process.

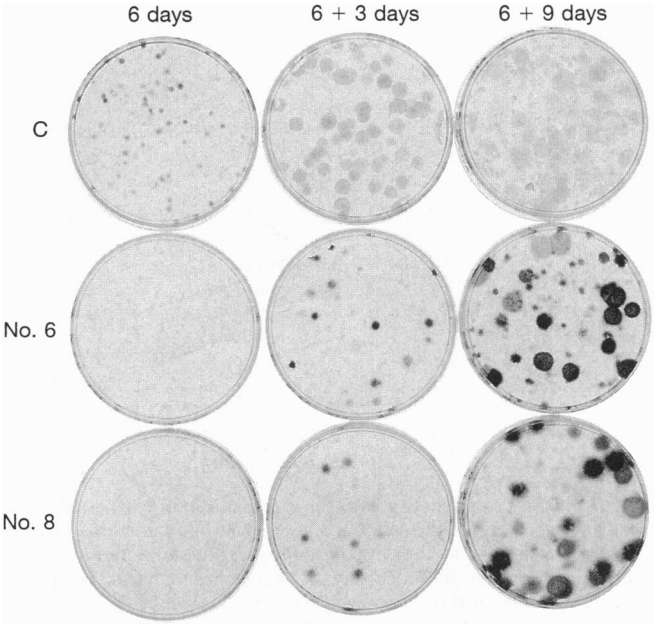

FIG. 4. Early and late colony development by control and transformed cells. Lineages nos. 6 and 8 originated in a 1° assay of 3 weeks in 10% CS and had undergone three further serial assays (2°, 3°, and 4°) in 2% CS. After the 4° assay, they were subcultured once at low density for 2 days to recover from confluence, and 50 cells of each lineage, as well as the controls, were seeded in 10% CS. Dishes of each were fixed and stained at 6 days or treated with trypsin and counted. The remaining dishes were switched to 2% CS and processed 3 and 9 days later. Days of sequential incubation in 10% CS (6 days) and 2% CS (6 + 3 days and 6 + 9 days) are indicated. See text for cell counts per colony relative to controls.

Unlike impaired proliferation, neoplastic transformation is a low frequency, irregular occurrence in our cultures and is consistent with a requirement for one or more genetic events. It is found only after the populationwide impairment of proliferation, which persists after transformation. The results indicate that the condition of prolonged constraint of confluence that induces the impairment of proliferation also destabilizes the cells to greatly increase the probability of neoplastic transformation. Such transformation in the form of human cancer is strongly correlated with age (1–4, 7). Prostate cancer, the second most common cause of death in men, exhibits the steepest increase with age of any human cancer (4, 7). Any departure at very old age of prostate cancer mortality from the Gompertzian dynamics of age-related mortality can be explained by competitive influence of other diseases (4). An

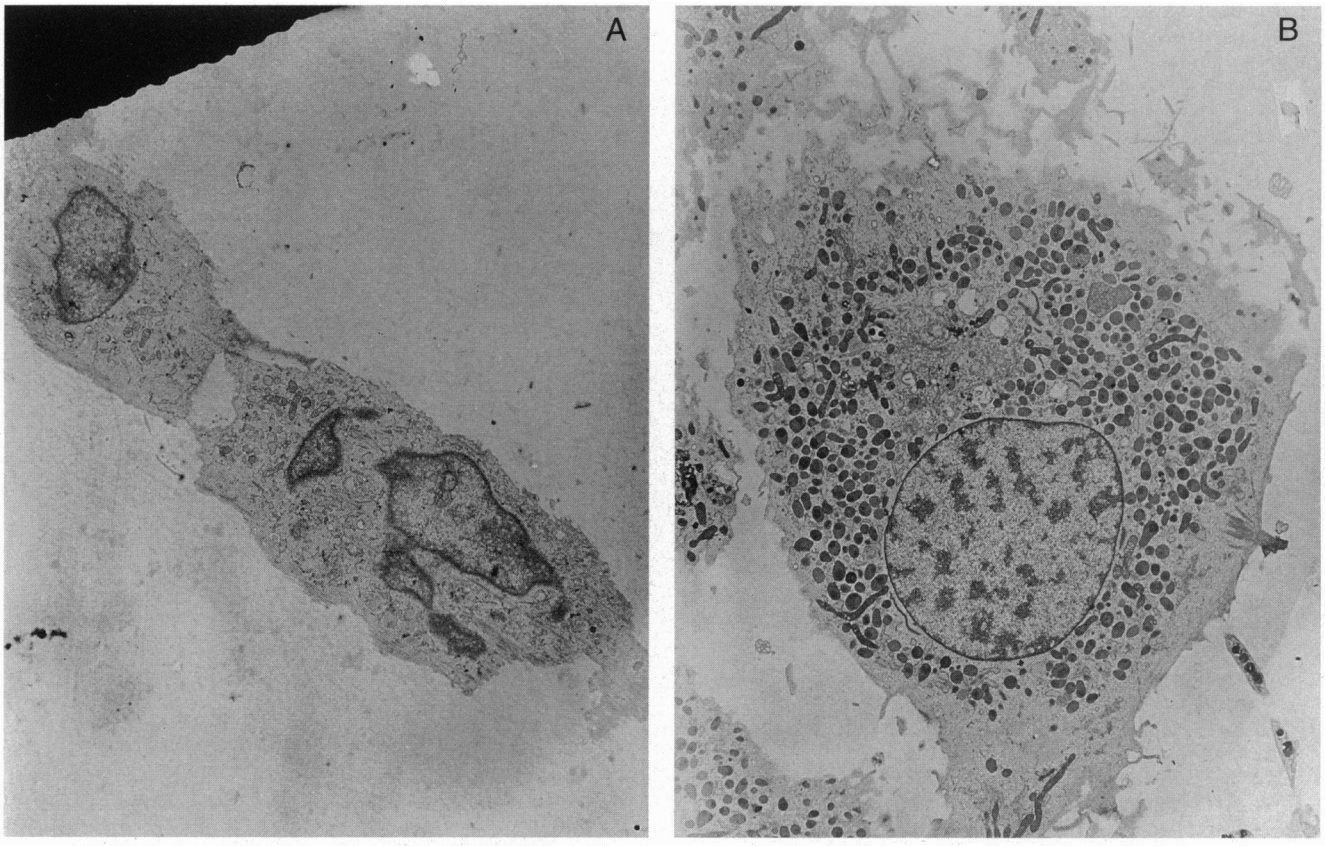

FIG. 5. Accumulation of residual bodies in cells after prolonged confluence. Cells in *A* had been in continuous low density passage and those in *B* had been confluent for 10 days.

increase with age in the susceptibility of skin to carcinogenesis has been clearly demonstrated by transplanting skin from old and young mice to a common young host and exposing the transplants to carcinogens (46). It has also been shown that cultures of bladder epithelium from old mice are far more susceptible to transformation by chemical carcinogens than are bladder cultures from young mice (47). Even the untreated bladder epithelium from old mice transformed at a much higher rate than the carcinogen-treated cultures from young mice. Our own finding of a great increase in frequency of transformation on the NIH 3T3 cells with prolonged confluence therefore lends weight to the thesis that this treatment simulates the *in vivo* process of cellular aging.

The authenticity of the model is further reinforced by the appearance in the cytoplasm of large numbers of residual bodies with prolonged confluence. These bodies are analogous to the age pigments known as lipofuscin (43). They have been demonstrated in normal human glial cells after 3 or more weeks of confluence (40). Lipofuscin is thought to arise from lipid peroxidation and the polymerization of substances rich in phospholipids—e.g., mitochondrial membranes—taken into secondary lysosomes that cannot fully hydrolyze them (43).

While it might be argued that an established, aneuploid line of cells is an inappropriate model for cellular aging *in vivo*, it should be noted that prolonged confluence has been shown to destabilize the genetic material of normal human fibroblasts (T. Ignatova, personal communication) and near diploid hamster cells (C. Vidair, personal communication), as well as inducing the formation of age pigment in normal human glial cells (40, 43). In addition, it has been shown that prolonged confluence shortens the *in vitro* lifespan of normal chicken embryo cells (48). It can therefore be concluded that the response of NIH 3T3 cells to confluence is a general characteristic of cultured cells. The NIH 3T3 model system has the virtues of allowing controlled induction of the aging effects and the long-term survival necessary to analyze the relation of cellular aging to neoplastic transformation. The results obtained with the system indicate that cellular aging does not reflect a fixed limit on the number of cell divisions but represents accumulation of metabolically related damage to the cells, which leads to their gradual decline and destabilization.

Note Added in Proof. We have recently found that residual bodies or age pigments like those in Fig. 5*B* disappear from confluent cultures when they undergo neoplastic transformation, just as they do in carcinoma of the prostate in aging men (49).

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