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# Heritable, population-wide damage to cells as the driving force of neoplastic transformation

**(apoptosis/free radicals/lysosomes/proteases/nucleases)**

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ABSTRACT **Prolonged incubation of NIH 3T3 cells under the growth constraint of confluence results in the death of some cells in a manner suggestive of apoptosis. Successive rounds of prolonged incubation at confluence of the surviving cells produce increasing neoplastic transformation in the form of increments in saturation density and transformed focus formation. Cells from the postconfluent cultures are given a recovery period of various lengths to remove the direct inhibitory effect of confluence before their growth properties are studied. It is found that with each round of confluence the exponential growth rate of the cells at low densities gets lower and the size of isolated colonies of the same cells shows a similar progressive reduction. The decreased growth rate of cells from the third round of confluence persists for >60 generations of growth at low density. The proportion of colonies containing giant cells is much higher after a 2-day recovery from confluence than after a 7-day recovery. Retardation of growth at low density and increased saturation density appear to be two sides of the same coin: both occur in the entire population of cells and precede the formation of transformed foci. We propose that the slowdown in growth and the formation of giant cells result from heritable damage to the cells, which in turn drives their transformation. Similar results have been reported for the survivors of x-irradiation and of treatment with chemical carcinogens and are associated with the aging process in animals. We suggest that these changes result from free radical damage to membrane lipids with particular damage to lysosomes. Proteases and nucleases would then be released to progressively modify the growth behavior and genetic stability of the cells toward autonomous proliferation.**

In a series of articles in the late 1930s Haddow (1, 2) reported that the growth of transplanted tumors of the rat and spontaneous tumors (mainly mammary carcinomas) of mice was inhibited in a lasting manner by intraperitoneal inoculation of carcinogenic polycyclic hydrocarbons. Related noncarcinogenic compounds were ineffective. He proposed that carcinogenic treatments, including ionizing radiation, "operate by producing retardation of the growth of normal cells, which eventually react to give a new cell race with an increased rate of division" (3). This conclusion was later reinforced by studies of Haddow *et al.* (4) with 4-aminostilbene and its derivatives. It was subsequently discovered that x-irradiation of cells in culture results in a retardation of growth among most of the survivors that persists for months through many cell generations (5, 6). In addition, x-irradiation produces delayed reproductive death expressed in the occasional formation of poorly viable giant cells (7, 8). A high proportion of x-irradiated cells gives rise to cells that produce a low frequency of neoplastically transformed foci when the cells are grown to confluence (9, 10). Since growth retardation precedes transformation, the results with ionizing radiation are consistent with Haddow's hypothesis of a primary role for such retardation in the genesis of tumors.

We have shown that spontaneous transformation of cells in culture is related to the retardation of growth that accompanies prolonged incubation either in suspension, in confluent cultures, or in low concentrations of serum (11–15). The transformation itself is not usually expressed in cells from confluent cultures until there have been one or more rounds of growth to and incubation at confluence (16, 17) with the implication that the postconfluent cells bear a lesion related to the growth retardation of confluence. We recently noted the appearance of large numbers of cells detaching from the cell sheet into the medium beginning a few days after confluence was reached. Since most of the floating cells were dead, it was apparent that confluence was not only inhibiting growth but also damaging cells and that the viable cells remaining attached to the substratum might bear evidence of such damage. Microscopic examination of the cell sheet revealed some cells with marginated chromatin typical of the earliest stages of apoptosis (18). We therefore initiated a series of experiments examining the growth rate on passage of postconfluent cells and found that there was a reduced growth rate at low population densities that persisted for many cell generations in high serum and low cell density passages and was accompanied by the production of poorly viable and nonviable giant cells in the first few days of passage from confluence. Attention is drawn to the striking similarity of growth behavior between the postconfluent cells and those that survive x-irradiation. The significance of these observations for Haddow's hypothesis of the mechanism of carcinogenesis is discussed and related to the strong correlation of age with cancer.

## MATERIALS AND METHODS

The $17_{3c}$ subline of NIH 3T3 cells was used in this study. It was routinely maintained in 100-mm plastic culture dishes (Falcon) in 12.5 ml of growth medium consisting of 90% molecular, cellular, and developmental biology 402 medium (MCDB 402) (19) and 10% (vol/vol) calf serum (CS). The subline was passaged every 2 or 3 days with seedings of 4 or $2 \times 10^4$ cells, respectively, to maintain cells always in exponential growth at low densities. It had been through about 600 passages at the beginning of these experiments.

The procedure used for a primary (1°) assay of cells for transformed focus formation is to seed $10^5$ cells in MCDB 402 containing 2% CS on 60-mm culture dishes and incubate them for 2–3 wk. Successive repetitions of this procedure are termed 2° and 3° assays. The 1° cells had been through one assay of 2 wk, the 2° cells had been through successive assays of 3 and then 2 wk, and the 3° cells had been through successive assays

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Abbreviations: CS, calf serum; C cells, control cells; 1°, 2°, 3° assays, consecutive assays at confluence for transformed foci and saturation density; MCDB 402, molecular, cellular, and developmental biology 402 medium.

Table 1. Growth and focus formation of cells assayed after successive rounds of confluence

| Postconfluent recovery period,* days | Previous rounds of confluence | | | |
|---|---|---|---|---|
| | Control | 1° | 2° | 3° |
| | % cells producing foci | | | |
| 0 | <0.001 | 0.03 | 2.1 | 34.3† |
| | Saturation density,‡ cells × $10^{-4}$ per $cm^2$ | | | |
| 0 | 2.57 | 3.04 | 5.40 | 17.09 |
| 9 | 2.52 | 2.33 | 3.57 | 4.62 |
| | Initial growth rate, population doublings per day,§ 1–3 days | | | |
| 2 | 2.13 | 1.88 | 1.69 | 1.54 |
| 7 | 2.34 | 2.17 | 2.14 | 1.85 |
| | Relative colony size | | | |
| 2 | 1.0 | 0.52 ± 0.06¶ | 0.40 ± 0.04¶ | 0.32 ± 0.06¶ |
| 7 | 1.0 | 0.92 ± 0.11¶ | 0.67 ± 0.06¶ | 0.47 ± 0.06¶ |

*The 0, 2, 7, and 9 days of recovery from confluence correspond to 0, 1, 3, and 4 passages at low density. Controls were cells that had been kept continuously in frequent low density passages that never allowed to reach confluence.

†30% of the cells from the 3° assay produced large, light foci and 4.3% produced small, dense foci.

‡Saturation density 14 days after seeding $10^5$ cells per 60-mm dish in MCDB 402 with 2% CS.

§Population doublings per day = 3.32·log(cell no. at 3 days/cell no. at 1 day)/2.

¶Mean ± SE.

of 2, 3, and then 2 wk. Where large numbers of transformed cells with crowded foci were anticipated, the cells were diluted in 10-fold steps and mixed with $10^5$ cells of the routine passage—which by themselves do not produce transformed foci—to obtain discrete, countable foci. At the end of each assay one culture of the $10^5$ seeding and both dishes of each dilution were fixed with Bouin's reagent, washed, and stained with 4% Giemsa stain. The cells of the other dish were suspended by incubation with 0.01% trypsin in 0.5 mM EDTA in Tris/saline buffer. They were electronically counted to determine the saturation density of the culture and then used for the next consecutive assay.

The growth rates of cells from confluent cultures were determined by first passaging them at 2- to 3-day intervals at the same low densities in 10% CS as in the routine passages described above to permit their recovery from the direct inhibitory effects of confluence. They were then trypsinized and seeded at a density of $10^4$ cells per 100-mm dish in 10% CS and counted at daily intervals to 7 days. The medium was completely replaced on days 3, 5, and 6. Controls for all experiments were cells from standard passages that had been kept in continuous exponential multiplication throughout. When cells were to be cloned they were diluted for a seeding of $10^2$ cells in 60-mm dishes with MCDB 402 containing 10% CS. They were incubated 5 days to allow development of visible colonies before fixation and staining as above. Colonies containing 200 or more cells were counted by naked eye on dishes that had been inverted over a light box.

The area and density of the stained colonies were analyzed by a program developed on a Macintosh II computer using images produced by a Hewlett-Packard Scanjet Plus flat bed optical scanner (20). Density readings on areas of the dish devoid of colonies were subtracted from density readings of the colonies. The average size of colonies on a dish was determined by multiplying their average area by average density and expressed as the fraction of values for the control cells.

## RESULTS

**Focus Formation and Growth Assayed After Successive Rounds of Confluence.** Control cells (C cells) that had been kept in continuous exponential growth by frequent passage at low density produced no transformed foci when grown to confluence in the standard assay, but foci were produced in increasing number with each successive round of extended incubation at confluence (Table 1). Reassay of cells from the 3° assay yielded 34.3% focus formers, which was about equal to the 32% colony-forming efficiency of these cells, indicating all the clonogenic cells were transformed. The saturation density of the cells also increased progressively when the cells were reseeded directly 0 day of recovery after each round of confluence (Table 1) as is evident from the photograph of Fig. 1. If the postconfluent cells were serially passaged at low density for 9 days before seeding, there was a decrease in saturation density of the cells from the 2° and particularly the 3° assay (Table 1). The decrease is reflected in the appearance of these cultures in Fig. 1, where some of the denser individual foci can be seen in the 3° assay but the lighter ones are too numerous to count. Standard assay with higher dilutions of cells after another week of low density passage revealed that about 10% remained focus formers.

Each of the postconfluent groups was passaged once to recover from the direct inhibitory effects of confluence and reseeded along with the control group to establish growth curves. Fig. 2 shows that the initial growth rates of the cells decreased progressively with increased rounds of confluence.

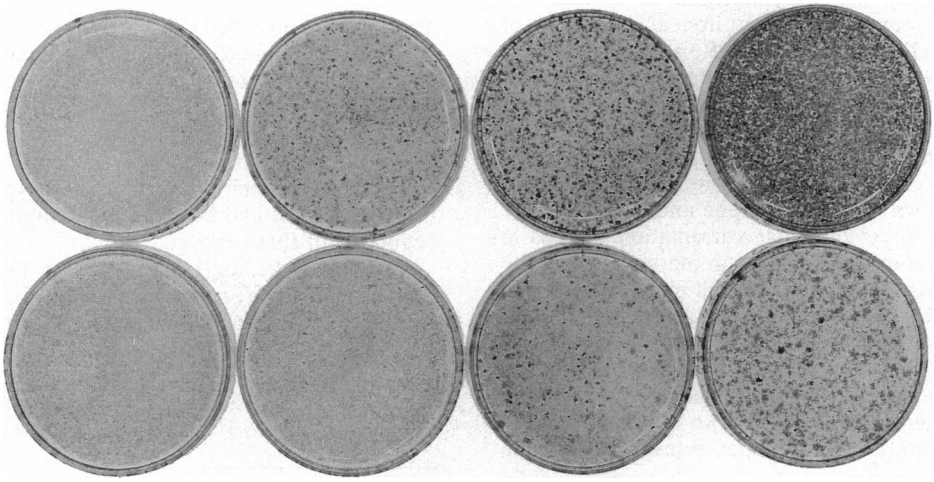

FIG. 1. Appearance of assay dishes from C cells and cells that had previously been through 1°, 2°, and 3° assays. Cultures were stained after 14 days of incubation in MCDB 402 containing 2% CS. Top row, cells that were assayed directly from the previous assay; bottom row, cells that were assayed after four low-density passages over a 9-day period. The cultures are, from left to right, controls, 1°, 2°, and 3° cells.

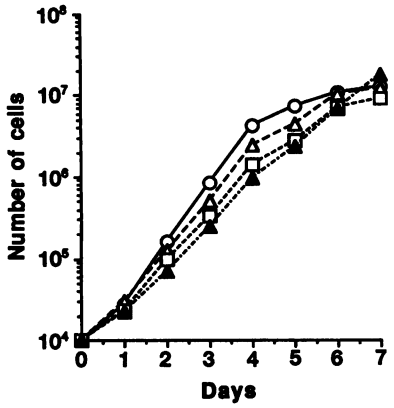


FIG. 2. Growth rates of cells after successive rounds of prolonged confluence plus a 2-day recovery period of passage at low density before reseeding. ○, Controls; △, 1°; □, 2°; ▲, 3°.

The growth rate of all groups decreased as they became more crowded beyond day 4, but 3° cells were least inhibited so they surpassed the others in number by day 7. The initial growth rates are numerically represented as population doublings from days 1 to 3 (Table 1). This is when the cells have recovered from trypsinization and before they can be inhibited by mutual contact. The results show step-wise decreases of 0.15 to 0.25 population doublings per day with each successive round of confluence for cells from the 2-day recovery. The differences were lessened with cells that had undergone a 7-day recovery period before reseeding for growth curves (Table 1). Comparison of results in Table 1 (initial growth rate vs. relative colony size) shows a negative correlation between initial growth rate and saturation density.

At the same time the growth curves of populations were initiated, $10^2$ cells were seeded for colony formation, and the individual colonies were measured by the scanning technique. The results in the scatter diagrams of Fig. 3 indicate a continuous decrease in the density of the individual colonies of cells, accompanied by a less striking decrease in their area. The average colony size, taken as the product of area and density, is presented relative to the control values in Table 1. For the 2-day recovery, there is a highly significant, progressive decrease in average colony size of the three postconfluent groups according to the number of rounds of confluence each had been through. The colonies initiated after 7 days of recovery from confluence were closer to the control sizes, but the difference from C cells was still highly significant in the case of the 2° and 3° cells.

**Heritability of the Growth-Retarded State.** The persistence of the growth-inhibitory effects of prolonged confluence after low-density passage of the cells is shown in Fig. 4. The growth rates were determined on the basis of cell yield at each passage.

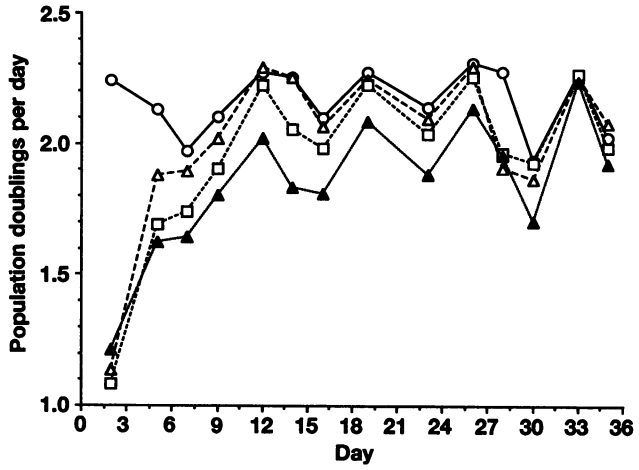


FIG. 4. Growth rates of controls and postconfluent cells in repeated low-density passages. Symbols are the same as in the Fig. 2 legend. Time on the abscissa represents the days after beginning low-density passages originating from confluent cultures.

Approximately 15 divisions of the 1° group and 50 divisions of the 2° group were required for a restoration of the original growth rate of the cells, and even then the restoration was not consistent in later passages. Cells of the 3° group did not return to their original growth rate in >60 divisions. The results indicate that the damage inflicted by prolonged incubation at confluence can be inherited through many cell generations of exponential growth.

**Production of Giant Cells.** Microscopic examination of the colonies produced by the postconfluent cells reveals a marked increase in the production of giant cells (Table 2). Unlike the progressive change in growth properties of the cells, the increase in giant cell formation remains constant in the cells through successive rounds of confluence, each followed by a 2-day recovery period. After a 7-day recovery, there is much less giant cell formation, although it may still be significant in the 2° and 3° groups. The marked difference in microscopic appearance of control and 3° assay colonies is apparent in the photographs of Fig. 5. The low-power photographs illustrate the greater heterogeneity and lower density of 3° colonies than control colonies. The higher-power photograph on the right shows a mixed 3° colony that appears to be continually generating giant cells. Comparison with the control colony indicates that even the cells from the 3° assay colony that cannot be classified as giant cells are abnormal in appearance and arrangement. The implication is that they may all be abnormal.

## DISCUSSION

We show here that prolonged incubation of cells under the constraint of confluence, which promotes neoplastic transfor-

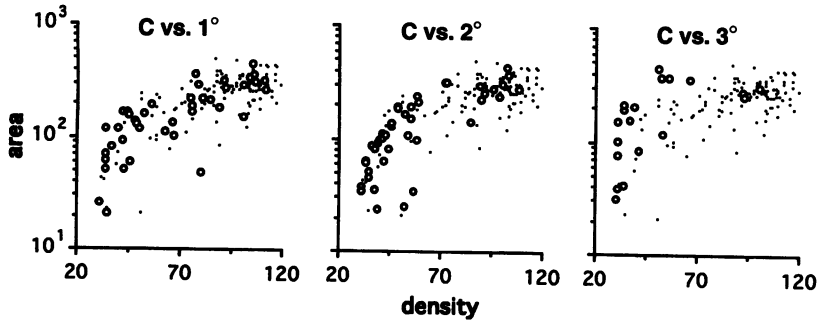


FIG. 3. Area and density of colonies from seeding cells from controls and 2-day recovery of 1°, 2°, and 3° assays. The same set of control colonies is shown as dots in every panel for comparison with the 1°, 2°, and 3° cells (○). Colonies developed for 5 days in 10% CS before fixation, staining, and computer analysis of images produced by the scanner. Arbitrary units for area and density are taken directly from the scanner.

Table 2. Fraction of colonies containing giant cells

| | 2-Day recovery | | | 7-Day recovery | | |
|---|---|---|---|---|---|---|
| Colony | Normal | Mixed | Giant | Normal | Mixed | Giant |
| C | 0.94 | 0.04 | 0.02 | 0.86 | 0.07 | 0.07 |
| 1° | 0.44 | 0.21 | 0.35 | 0.88 | 0.02 | 0.10 |
| 2° | 0.46 | 0.15 | 0.39 | 0.74 | 0.05 | 0.21 |
| 3° | 0.52 | 0.07 | 0.41 | 0.69 | 0.22 | 0.09 |

Colonies were the same as those used to determine colony size in Table 1, Relative colony size. Classification was based on inspection at 40× magnification in a stereomicroscope. Mixed colonies had three or more giant cells in colonies that had a majority of "normal"-looking cells. Giant "colonies" consisted only of giant cells and were included even when occurring as isolated individuals.

mation, also inflicts damage that results in a reduction in their growth rate when the cells are passaged at low density. The cells retain the capacity for exponential growth, but the reduced rate persists through many generations of low-density passage. The retardation of growth is cumulative, becoming more pronounced after successive rounds of prolonged confluence. Another sign of damage is a marked increase in the production of giant cells, though this is most evident in the first few days after release from confluence and shows no strong trend to increase with successive rounds of confluence. These signs of cell damage are similar to those described for the survivors of x-irradiation (5–8) but occur as a result of the self-constraint of cell contact rather than the application of exogenous agencies. Ionizing radiation also results in the transformation of a small fraction of the cells, most likely in those that exhibit the reduced growth rate, but no direct connection has been reported between the phenomena. It is clear in the present case, however, that the very cells that have a reduced growth rate are the ones that attain a higher saturation density. This is evident from the fact that the reduced growth rate is manifest in the initial slope of the growth curves, which indicates that the bulk of the population is altered, and in the scans of 3° colonies in Fig. 3 that show a reduced density of most of the colonies. The reduction in growth is combined with an increase in saturation density, which in turn results in a further decrease in growth rate in the following passages. Increased saturation density can be considered an incipient stage of neoplastic transformation and is followed by the appearance of transformed foci or overt transformation (17) arising from a small fraction of the cells. So the damage to the population as a whole is a precursor to the full transformation in a small fraction of the population. The transformed cells eventually dominate the population in confluent cultures by virtue of their defining property of continuing growth at confluence. The implication of our results and those of x-irradiation is that an entire field of cells is altered in an incipient manner by carcinogenic treatments, thereby increasing the probability for progression of a few cells to tumor formation. The results therefore provide direct support for Haddow's proposal that the primary effect of carcinogens is persistent growth retardation followed by adaptive change in a few cells that results in tumor formation (1–4).

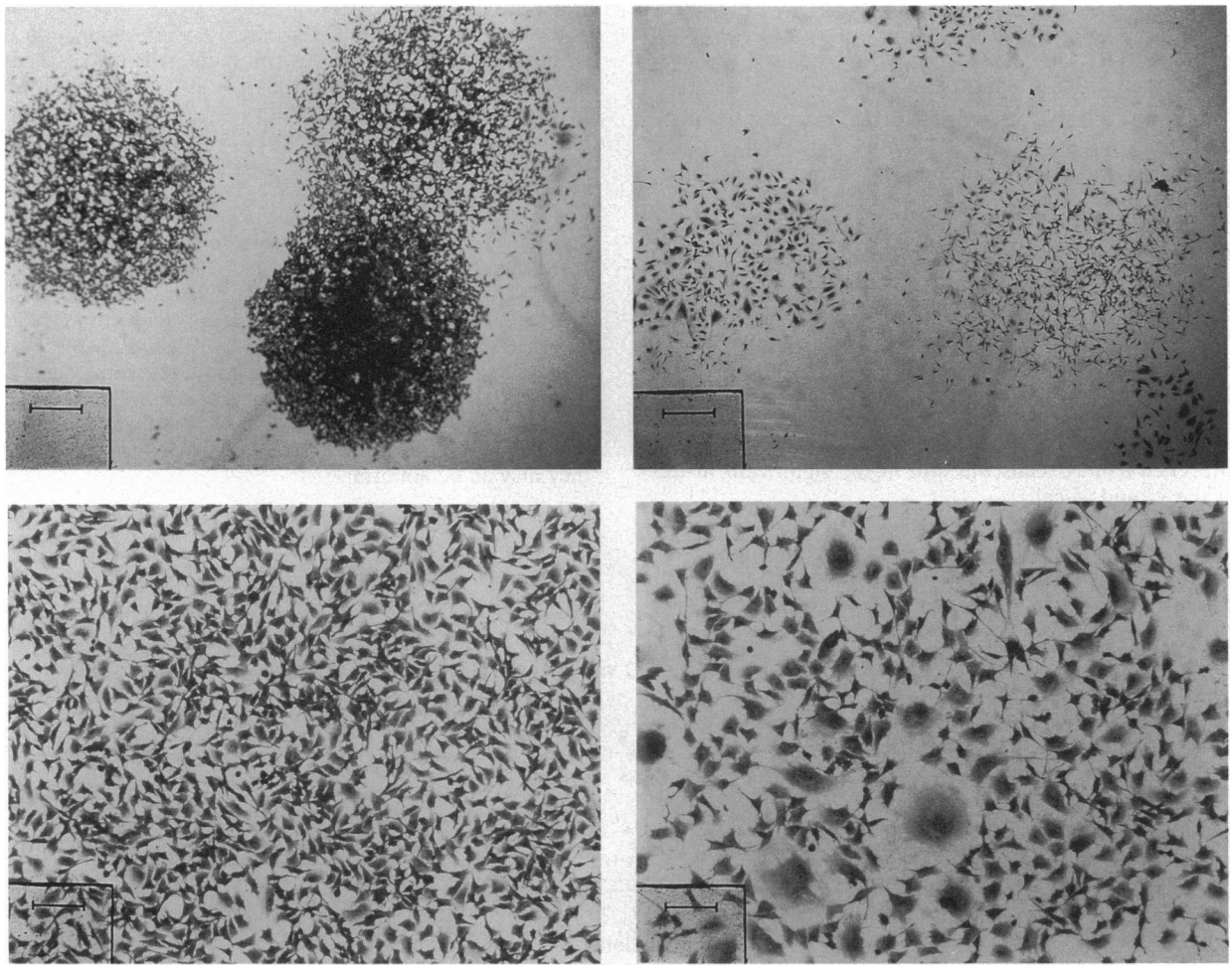

FIG. 5. Microscopic appearance of colonies from control (*Left*) and 3° assay (*Right*). (Bars in *Upper Left* and *Right* = 400 μm; bars in *Lower Left* and *Right* = 100 μm.)

We now consider what the nature of the change might be that simultaneously produces growth retardation and increased saturation density. It is a change that must occur cumulatively and in the entire population of exposed cells. Such pervasive and finely graded change is incompatible with the low frequency and discrete nature of local mutations. A hint about the source of the change comes from the observation that carcinogenic hydrocarbons are mainly sequestered in lysosomes (21), which are the repository of nucleases and proteases. X-irradiation is thought to initiate apoptosis through the production of free radicals, which damage membranes through combination with the double bonds of phospholipids (22). Lysosomal membranes are extremely labile to free radical damage, as indicated by release of bound enzymes (23). Transformation induced by x-irradiation or chemical carcinogens in cultured cells can be blocked by protease inhibitors long after the initial exposure (24, 25). Proteases inhibit growth of cells at low density and stimulate growth at high density (26). A serine protease is elevated in primary tumor cells, virus-transformed cells, and a number of tumorigenic cell lines (27). Treatment of human colon cancers with a tumor promoter induces a protease in amounts proportional to the malignancy of the tumors (28). Carcinomas of the hamster cheek pouch induced by a polycyclic hydrocarbon exhibit a large increase in proteolytic activity, as do the surrounding normal-appearing tissues that had been exposed to the carcinogen (29). An inactivating mutation in a key protease of yeast results in failure of other proteolytic and hydrolytic enzymes to be processed to an active form (30). If a mutant cell is exposed to wild-type cytoplasm through mating or heterokaryon formation, the mutant progeny maintain indefinitely the wild-type content of active proteases and hydrolases normally dependent on the now inactive protease. The yeast system demonstrates that an altered protease phenotype can be transmitted to progeny by epigenetic means without involvement of cytoplasmic genes (31). We propose therefore that the inhibition of cell metabolism and growth that occurs at confluence in cell culture engenders free radicals that damage the membrane of lysosomes and liberate nucleases and proteases. These in turn retard the growth of cells at low density, stimulate the growth at high density, and over extended periods of time produce the genetic lesions that are commonly detected in tumors. The latter may stabilize by genetic means a behavior that is basically epigenetic in origin. Once genetic fixation has occurred, lysosomal lability is dispensable, and not all transformed clones need exhibit retarded low density multiplication, though most apparently do (32). The hypothesis can be extended to the carcinogenic action of ionizing radiation and carcinogenic hydrocarbons.

In view of the strong correlation between aging and carcinogenesis (33) it is of interest that the cellular hallmark of aging is the appearance of residual bodies and lipofuscin granules, which are degenerate forms of lysosomes (34). These are scanty in rapidly dividing cells but accumulate continuously in long periods of confluence (34). The latter conditions are, of course, precisely those shown here to generate persistent slowdown of growth and neoplastic transformation. Another characteristic of aging tissue is a slowdown of cellular growth rate (35), again analogous to our results. It is therefore plausible that damaged lysosomes contribute to the slowdown in growth and the increased risk of cancer in older animals and humans.

**Note Added in Proof.** The proposed increase in hydrolytic enzyme activity in growth-constrained cells is reminiscent of the increase in protease activity found in bacteria and yeast during transition from exponential growth to stationary phase (36).

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