Review

Selected Cell and Selective Microenvironment in Neoplastic Development

Harry Rubin

Department of Molecular and Cell Biology and Virus Laboratory Life Sciences Addition, University of California, Berkeley, California 94720-3200

"Unlike the situation in earlier periods, clarity does not reside in reduction to a single, directly comprehensible model, but the exhaustive overlay of different descriptions that incorporate apparently contradictory notions."


Abstract

Recent analysis of genetic alterations in human cancer points to a major role for selection in neoplastic development but provides few details about the dynamics of the process. Many such details, however, have emerged from quantitative studies of spontaneous transformation among mammalian cells in culture. The chief insight of these studies is that there is a continuous generation of variants in proliferative potential among growing cells that provides the substratum for progressive development to a frankly neoplastic state when selective growth conditions are persistently applied. Much of the selection occurs before the cells are capable of producing discrete neoplastic foci. The varied observations in cell culture draw attention to analogous features of carcinogenesis in experimental animals and the development of human cancer.

Introduction

There is an extensive literature on the role of genetic destabilization in the development of cancer, and it seems to be widely assumed that it is the driving force in the process. Recent computer modeling, based mainly on the large body of genetic studies in colorectal cancer, argues strongly that selection plays the dominant role in the early stages of cancer with the notable exception of those familial cancers in which there is a defect in one allele of a mismatch repair gene (1, 2). Increases in natural mutation rates in sporadic cancers are usually found during later stages of tumor development (3, 4) and are not considered important in early stages. However, they may play a role in the exponential increase of cancer rates in aging people if somatic mutations increase exponentially with age due to the accumulation of mutations in mismatch repair genes (5). In either case, there has to be selective growth of altered cells for tumors to develop, and little is known about the conditions that underlie that selective growth in vivo or the cellular diversity on which it operates.

By contrast, a considerable body of information has accumulated on the role of selection in progressive neoplastic transformation in cell culture including the frequency of selectable variants, the culture conditions that drive their selection, and the progressive changes in phenotype that are associated with transformation. The information is complex and has developed incrementally for over a decade. This review aims to put the pieces together in a comprehensive picture and relate it to carcinogenesis in animals and man with particular attention focused on the role of selection in the process.

Selection in Transformation of Primary Cultures

Most of the knowledge about selection in neoplastic transformation in culture comes from the process known as spontaneous transformation. It was first discovered in experiments on chemical carcinogenesis of cells from mouse and rat embryos, in which it was found that the untreated controls eventually produced tumors on inoculation in syngeneic animals if the cultures had been of sufficient duration (6, 7). It was later found that increases in the saturation density of mouse embryo cells occur much more rapidly when cells are passaged at higher population densities (8). Increase in saturation density is associated with capacity to produce tumors in syngeneic mice: both changes occurred in BALB/c mouse embryo cells passaged at high density but not in those passaged at low density (9). In another study using clonal cultures from hybrid mouse embryos, tumorigenic capacity developed from both high density passages and LDPs but did so a few months earlier in the former (10). Therefore, in both cases, neoplastic transformation was favored in cultures passaged at high density, which suggests selection of spontaneously occurring variants with neoplastic potential that is associated with increased capacity to escape contact inhibition. It cannot be ruled out, however, that the earlier appearance of transformed cells in high density passages resulted from the larger number of cells continually present in the high density cultures as compared with the low density cultures, which would increase the probability of detecting neoplastic variants in the former.

Selection in Transformation of Established Cell Lines

Fibroblasts. A permanent line of cells was established by LDPs of fibroblasts from BALB/c mouse embryos (11). These cells, which were designated the Balb/3T3 line, maintained a normal sensitivity to contact inhibition of multiplication at high population densities. They rarely underwent neoplastic transformation when passaged at low density (12) but did so more often when suspended in soft agar (13). The transformed cells had a 10-fold increase in saturation density and were tumorigenic (14). The increased frequency of transformation in suspension that inhibits the growth of normal cells suggested a role for selection in the transformation. However, transformation was still too infrequent to draw any firm conclusion about the role of selection in the process.

The permanent line of nontransformed NIH 3T3 cells was established by LDPs of fibroblasts from embryos of partially inbred strain of mice (15). These cells were widely used as targets for transformation by putative oncogenes, but they developed transformed foci spontaneously if left at confluence for extended periods of time (16). Cells from large, dense foci were highly tumorigenic in nude mice (17). The spontaneous transformation of the NIH 3T3 cells occurred regularly at confluence, but this response to growth constraint decreased gradually in repetitive testing of cells that were kept at a maximal growth rate by frequent passage at low density in high (10%) concentrations of calf serum (18). After many such LDPs, several rounds of prolonged constraint at confluence were required before
transformed foci developed (19, 20). The reason for the reduction in susceptibility to transformation that resulted from LDP may be related to the observation that transformed cells usually multiply more slowly at low population density than nontransformed cells (21–23), with the possibility that cells more susceptible to transformation also exhibit this tendency and are selected against under maximal growth conditions at low density. If the LDPs were made in 10% fetal bovine serum, which reduced the growth rate of the cells to about 80% of that in calf serum, there was a marked increase in transformation (18). This indicates that selection for transformation and transformability is effective with even small differences in selective advantage when maintained over an extended period.

The role of selection in spontaneous transformation is also illustrated by LDPs when the concentration of calf serum is reduced from 10% to 2% (Fig. 1). There is a steady increase in saturation density of susceptible populations that is detectable when tested after each of the frequent LDPs in 2% calf serum over a 2-week period (24). No sign of an increase is seen in parallel LDPs of the cells in the standard 10% calf serum. To determine whether reducing serum concentration and maintenance at high population density have an additive effect in transformation, many cultures were allowed to grow to confluence in 2% calf serum without passage. At intervals of 2 or 3 days corresponding to the frequent LDPs, some of the cultures were transferred to determine their saturation density. The saturation density on transfer was found to increase steadily for 1 week while the number of cells in the source culture was still increasing. However, the saturation density on transfer did not increase further in the following week when growth of the source culture had markedly decreased (24). This showed that the somewhat reduced but still exponential growth in LDPs with 2% calf serum is far more effective in driving transformation than the stationary state produced at confluence in the same concentration of serum. It indicates that progressive transformation requires multiplication, albeit under conditions of constraint that permit selection of those cells that have a growth advantage over the majority population. However, a small minority of clonal populations may continue to multiply when the population as a whole reaches its saturation density; these selected cells would continue to accumulate mutations that would drive further selection, culminating in the formation of distinctive transformed foci, each derived from a single cell. The role of multiplication under constrained conditions in driving progressive transformation is illustrated by an experiment in which cells were grown to confluence in 2%, 5%, and 10% calf serum in a primary (1°) assay (20). The saturation density and the total number of cell divisions were proportional to the serum concentration. The cells from the 1° assays in each serum concentration were then serially assayed three times (2°, 3°, and 4° assays) in 2% serum. Although they were all in the same 2% serum concentration beyond the 1° assay, the saturation densities remained progressively higher as a function of their original serum concentration (Fig. 2). In addition, large, dense foci appeared in the later assays in proportion to the saturation density and therefore to the total number of divisions in the 1° assay (Fig. 3). Not only did this experiment confirm the transforming role of multiplication under constraint, but it also showed that...
selection operates on cells before they produce clearly visible, discrete foci, thus recalling the incipient state of neoplasia described by Foulds (25, 26).

The power of selection is enhanced by stepwise reductions in calf serum concentration from 10% to 0.25% (27). A one-step reduction of this magnitude prevents cell multiplication. Stepwise reductions, however, result in successive increases in saturation density on passage of the cells and an increase in the size and number of transformed foci produced in low serum concentrations. After a final reduction from 0.5% to 0.25% calf serum, there is at first only limited multiplication, but serial passage in the latter is accompanied by increasing capacities for multiplication and focus formation in that extremely low serum concentration. The cell populations that had adapted to multiplication in 0.25% serum lost that ability fairly rapidly when passaged in 10% serum. Because the population adapted to 0.25% serum multiplied more slowly in 10% serum than cells that had been passaged exclusively in the latter, there would be selection of any variants that could multiply faster in 10% serum with a loss of capacity to multiply in 0.25% serum. The results indicate there is a high rate of generation of genetic variants with different capacities for multiplication in various serum concentrations. The NIH 3T3 cells apparently have an unusual capacity for heritable adaptation to restrictive growth conditions because Swiss 3T3 cells do not exhibit such a capacity (28), and this may be related to the ease with which the former cells undergo spontaneous transformation.

The acute sensitivity of the NIH 3T3 cells to small differences in population density is illustrated by varying subconfluent densities over a narrow range in 2% calf serum and testing at intervals for an increase in saturation density (29). The rate of increase in saturation density tested every third LDP was proportional to seeding density. Although none of the LDPs reached confluence during each passage, there was contact between cells that increased with seeding density, and this apparently was sufficient to account for the increase in selectivity with population density. A critical test demonstrated that the increase in population density rather than the increase in cell number per se was responsible for the development of focus-forming ability (30).

Epithelial Cells. Most human cancers are of epithelial origin, so it is of particular interest to examine the role of selection in spontaneous transformation of epithelial cells in culture. This was done with an established line of diploid rat liver epithelial cells that multiplied at a maximum rate in multicellular islands that developed even at low density. The rate of multiplication slowed down at 1 week when the islands became confluent with one another (31). Cells were maintained either under nonselective conditions by weekly passages just as they reached confluence or under selective conditions by monthly passages that included 3 weeks of constraint at confluence. Experimental cells were also treated with the mutagenic carcinogen MNNG and cultured under selective and nonselective conditions, as were untreated controls. The MNNG-treated and control cultures under selective conditions started to initiate liver carcinomas on injection into syngeneic rats after about 16 and 20 cell doublings in culture, respectively. About five to six times as many cell doublings were required for the cultures under nonselective conditions to become tumorigenic, with no significant difference between the MNNG-treated and untreated cultures. The results demonstrated the powerful accelerating role of selection in developing the tumorigenic capacity of epithelial cells. They also showed that the appearance of tumorigenic variants occurred at almost as high a rate in untreated controls as in cultures treated with a mutagenic carcinogen. Evidently, the mutation rate was already high enough in the untreated cultures to provide neoplastic variants for selection; treatment with carcinogen added only marginally to the spontaneous rate. The fly in the ointment of this interpretation of the results is the possibility that long-term confluence is itself mutagenic. Whereas this possibility was once entertained for fibroblasts (16, 17), other considerations (13, 18, 24) eventually ruled it out, and it can be safely disregarded for the epithelial cells. Hence, we are left with the plausible conclusion that the rate of spontaneous variation is high in both fibroblast and epithelial cell cultures, and selection is the driving force in advancing the cells to the neoplastic state.

Cellular Diversity as Substrate for Selection

Primary Cultures. The average mutation rate of animal cells has been estimated to be about $1.4 \times 10^{-10}$ per nucleotide/per cell division, based largely on studies of cells in culture (32). The estimated rate of point mutations in the human germ-line, however, is about 200 times higher, and this does not include small duplications, rearrangements, or deletions (33). Mutations at micro- and minisatel-lites occur at 1 or more orders of magnitude higher (5). Because mutations accumulate with time, their frequencies in tissues are higher than their estimated rates (5, 34). More to the point regarding variation related to neoplastic transformation is the observation that many foci of altered cells appear in secondary cultures of baby mouse mesenchymal cells maintained in a quiescent state for 6–8 weeks (35). Exposure of primary cultures to fluorescent light or oxygen gave rise in the secondary cultures to much larger foci that continued to enlarge...
after the background cells had stopped multiplying. There was great variation in the arrangement of altered cells between the foci that overgrew the background in contrast to the similarity of altered cells within a particular focus. Although altered cells did not produce tumors in syngeneic mice, they had the morphology of transformed cells and were considered preneoplastic (36). This was an indication of selectable preneoplastic variants in a normal population of cells from very young animals.

Similar foci of increased population density also appeared in primary cultures of diploid rat embryo liver epithelial cells that were held at confluence for 1 week or longer (37). Such foci reappeared in secondary cultures that were held at confluence. When later passages of such diploid hepatocytes were subjected to selection under repeated rounds of prolonged confluence, they became tumorigenic in about 4 months (31) as described above in “Epithelial Cells.” They produced a diversity of tumor types (including hepatocellular carcinomas, cholangiocarcinomas, hepatoblastomas, and osteogenic sarcomas), but individual lineages yielded tumors with consistent and specific patterns of differentiation (38). It is plausible that the densely packed foci that appeared in the confluent primary cultures were precursors of the cells that became tumorigenic through the accumulation of independent transforming events during the selective regime. The foci are indicators of diversity in the growth capacity of cells in the original cultures of the liver epithelium, just as they are in normal fibroblast cultures (35), and probably provide the most favorable material for progressive selection of the neoplastic state. The implication of the diversity among spontaneously occurring foci of independent origin among fibroblasts and the diversity of tumors produced by separate lineages of transformed rat liver epithelia is that there were either intrinsic differences among the cells in each culture or that the stochastic accumulation of mutations differed among independent transformants. In either case, transformation reveals a higher level of variation than would otherwise be expected by visual inspection of cultures under nonselective conditions.

**Established Cell Lines.** The extent of variation detected in nontransformed and transformed sublines of the Balb/3T3 line of mouse embryo fibroblasts was so high as to be difficult to account for by conventional mutational rates. Each of the subclones isolated from a clone of nontransformed cells and a clone of transformed cells consistently differed from one another in a variety of morphological, growth, tumorigenic, and biochemical properties (39, 40). Cells from each of the subclones had a wide range of chromosome numbers, with averages around the tetraploid number (40). The colony-forming capacity in agar of a transformed clone was reduced 10-fold during tumor formation by the cells, and there was wide variation in this property among subclones from one tumor, but not from a second one (41). Differences in morphology, colony formation in agar, and tumorigenicity arose early in subclones of a transformed clone. Secondary subclones differed within each subclone, but not as much as they differed as a group from those derived from other subclones. Similarly high degrees of variability were later obtained among agar colonies derived from a clone of the Balb/3T3 cells and in subclones from those agar colonies (13). It was tempting at the time to speculate that the high degree of variability in growth properties within clonally derived populations was the result of epigenetic changes, but subsequent considerations from studies on spontaneous transformation in NIH 3T3 cells discussed below lent weight to a genetic interpretation.

Spontaneous transformation was much more regular in NIH 3T3 cells than in Balb/3T3 cells and could be efficiently measured by counting multilayered foci of transformed cells on a monolayered background of nontransformed cells (16). Fine features of morphology of the foci frequently allowed discrimination of variants, whereas the size and thickness of the foci were signposts of neoplastic progression that were reflected in their tumorigenic capacity (17), as noted previously with C3H 10T1/2 cells (21). Progression could also be followed by the procedure of serial assays at confluence, which revealed a sequence proceeding from no visible foci through foci of increasing size and population density.

Once the transformability of the NIH 3T3 cells had been reduced by frequent LDP to the extent that no dense foci appeared in a 1° assay, populations could be grown up from relatively small starting numbers, and the focus-forming capacities of each of many populations could be determined in 2° assays (42). This procedure allowed rough estimates of the heterogeneity of the competence for transformation in the original population. The results showed that a very low proportion of cells could progress rapidly to formation of many dense foci and that there was diversity among cells for selection under the growth-constraining conditions of the assay. As was the case for the Balb/3T3 cells, there was a wide range of variation in potential for focus formation among subclones of the same clone, with larger differences among subclones from different clones (43, 44). A wide range of differences among foci was detected by quantitative computer scanning of the area and density of the foci (45). As another indicator of variation, every cell in nontransformed populations proved to be unique in the precise distribution of chromosomes, including marker chromosomes (46). Therefore, chromosome variation was occurring at a much higher rate than conventional point mutations.

Some idea of the dynamics of progression in small populations can be obtained by seeding the cells in multiwell plates, growing them to confluence, and assaying many of the populations at successive intervals for focus formation with an excess of nontransformed cells. The wells were one-seventieth the area of the 21-cm² dish used for conventional assays, and the starting and final confluent cell numbers were reduced in proportion (47). Each series began with the production of small light foci from cells assayed from a few of the wells. The number of wells with cells that produced light foci increased rectilinearly with time and was followed by wells that produced foci of increasing size and density, until all wells were producing foci by 7–10 weeks. The result suggests that a high proportion of cells can give rise to focus formers if selective conditions are maintained long enough.

There are several problems in calculating rates of variation from the foregoing results. One is the heterogeneity of the cell population undergoing transformation, which is itself shifting even under nonselective conditions, as will be considered below. A second problem is the progressive nature of transformation. This presented difficulties even when light, small foci were considered the first indications of transformation. The difficulty was compounded when it was realized that selection was occurring under the constraint of confluence even before discrete foci appeared. This was already implicit in the observation using cells that had undergone extensive selection against transformable cells in frequent LDP. It then required several serial rounds of selection at confluence before foci became visible, indicating that selection was occurring without visible, discrete lesions in the early rounds (19). Such selection of incipiently transformed cells became apparent in a multilinage experiment with a particular batch of cells that had enough selectable cells to produce successive increases in saturation density with each serial round of confluence without producing foci in the early rounds (Ref. 20; Figs. 2 and 3). The excellent agreement among parallel quadruplicate lineages left no doubt about the accuracy of the saturation density measurements, and their agreement with the preceding total number of cell divisions reinforced the conclusion that early steps in transformation occurred before focal lesions were visible.

One way to estimate rates of variation is to start from cell populations expanded in a known number of divisions from single...
cells and maintained under the nonselective conditions of continuous exponential growth by frequent LDP. They could then be tested for early signs of focus-forming capacity at intervals by assaying aliquots of the cells in serial rounds of confluence. This was first done with 29 clones from cells newly thawed from the original, easily transformed stock of NIH 3T3 cells (48). Most of the clones produced light foci on assay of cells that had undergone 5 or 13 LDPs. The clones multiplied from a seeding of 2 × 10^4 cells to about 5 × 10^5 cells per culture at every passage or about 5 × 10^7 cell divisions. The rate of transformation to light focus formation would then be between 1.5 and 4 × 10^{-7} per cell division. These figures are about 10-fold higher than the values for spontaneous transformation estimated for dense foci in populations of C3H 10T1/2 cells (49). The difference may be due in part to the likelihood that many of the dense foci represent progression from light foci. The estimate for the light foci may itself be too low because there are subliminal stages that expand selectively and progress to transformed focus formation (20). The values would then be higher than those characteristic of mutation at a single locus (49) but not unexpected if mutation at each of many loci may afford selective advantage at confluence, and if chromosomal alterations are involved (5). The “many site” concept is consistent with the morphological diversity of spontaneously arising foci in secondary cultures of mouse fibroblasts (36) and NIH 3T3 cells (19) and the diversity of tumor types produced from independent lineages of early passage diploid rat liver epithelial cells that had undergone spontaneous transformation under selective conditions (38). One must also take into consideration the fact that different sublines derived from the original NIH 3T3 line have widely different susceptibilities to spontaneous transformation (18, 50), as do clones derived from the same culture (48, 51).

Population Drift of Transformability

It has already been pointed out that the NIH 3T3 cell line becomes less transformable if repeatedly passaged in frequent LDPs (18). To avoid such variability, many researchers do not use cells beyond a limited number of passages from the original frozen stock. However, this has the disadvantage of missing important features of the transformation process that are revealed with cells of different susceptibility to transformation such as its early incipient stages and progressive nature. It is therefore of interest to be aware of the different possible courses of evolution in populations under nonselective conditions and the possible contribution of intraclonal change in such evolution. To this end, six clones from the original, easily transformed stock of NIH 3T3 cells were passaged 56 times every 3 days at low density, and aliquots of cells were tested at six different passage levels for transformability in serial rounds of confluence (51). One of the clones produced a few tiny, dense foci in 1° assays after the early LDPs. Cells from these foci produced large, dense foci when reseeded in 2° assays. After many LDPs, this clone produced only light foci in the 1° assay that progressed to dense foci in the serial assays. Four of the clones produced only light foci in all of the 1° assays, although these always progressed to dense focus formation in the serial assays. The sixth clone behaved like the preceding four when assayed at the first five passage levels but progressed to dense focus formation in the 1° assay after the last passage. That clone behaved differently from previous experiments with uncloned cultures, which usually decreased in transformability after many frequent LDPs (18, 52). It was also unexpected because most transformed populations multiply more slowly at low density than their nontransformed progenitors (21–23, 49). The aberrant behavior of this clone serves as a reminder that all statements about transformability are probabilistic rather than categorical.

Clonal Expansion Advances Transformation

It is generally accepted that most human tumors arise from the expansion of a single clone (53–56). More than one cell in a population may give rise under selective conditions to cells at various stages of transformation in culture, but one of these subpopulations has a higher selective advantage than the others and eventually predominates (48, 57). Given the difference in transformability of clones derived from the same culture, it was of interest to know what clonal type would dominate in spontaneous transformation. This was fairly obvious in an easily transformed population containing some clones that became fully transformed much faster than the others (48) but was less apparent in a cell population that was relatively refractory to transformation. One such uncloned population exhibited no increase in saturation density or any sign of focus formation in five rounds of selection at confluence and only the barest suggestion of foci in the sixth round (58). Parallel assays of clones from that population revealed that some of them produced well-defined foci beginning in the third and fourth round of confluence. This seemed surprising at first glance because the polyclonal parental population must have had thousands of cells that could give rise to the neoplastically productive clones. However, only one-fifth of the clones were highly productive of foci, and then only after three or four rounds of confluence. The development of foci depends on the number of responsive cells present under the selective condition of confluence. The polyclonal parental population would have only one-fifth as many responsive cells at confluence as the neoplastically productive clones (which constituted only one-fifth of the total population) and therefore would not be expected to exhibit frank transformation until it was subjected to selective conditions five times longer than the clones, i.e., after 15–20 rounds of confluence. The experiment illustrates the importance of expansion of neoplastically productive clones in the genesis of transformation and, conversely, the importance of maintaining a balanced distribution of clones in preventing transformation. The latter point is reinforced by the evidence that normal cells surrounding transformed cells can, in some combinations, suppress expansion of the transformed cell (59–62).

The Nature of Transformation

In the early stages of systematic analysis of spontaneous transformation, it seemed that an epigenetic mechanism was at least as convincing as a genetic explanation of the phenomenon (16, 17). The fact that transformation was most frequently encountered when the cell population as a whole was quiescent seemed to argue against a mutational origin, which is conventionally correlated with cell division. Once a cell population was obtained that required several rounds of confluence for visible transformation (18), there seemed to be no evidence for selection in the early stages because there was nothing visible to select. Then there were indications that the transformation was reversible (17, 63) and that even tumors of varied origin in animals and humans would revert to normal under appropriate conditions (64–69). Later, however, it became apparent that cells were present that had a selective advantage at confluence but did not produce visible lesions (20). Multiplication and selection could occur in a minority population while there was no increase or even a decrease in the total number of cells. Apparent reversal could occur in LDP when there was contamination of a transformed clone with a few nontransformed cells because the latter usually multiplied faster at low densities than the former (23). Indeed, this raised the possibility that there was selection for reverted cells under optimal growth conditions. Reversion (as well as progression) could occur at a much higher rate than the common estimates of point mutations (32) if the chromosomal alterations so common in established cell lines play a
significant role in transformation, as they apparently do in human tumor development (70). However, when rigorous precautions were taken to isolate pure populations of transformed cells, the transformation proved to be irreversible (71). The sum of these qualifications and observations tipped the balance in favor of a mutational origin of the transformation, but one that usually occurred in a progressive manner in several steps.

The mutational view was reinforced by the finding that the few cells in a Swiss 3T3 line that could multiply in a sharply reduced concentration of serum were unable to maintain that capacity in subculture (72), unlike the NIH 3T3 cells in various stages of progression. The idea that nonspecific heritable damage to cells might increase the chance of transformation (23) was weakened by the failure of heritable damage induced by methotrexate to increase transformation of NIH 3T3 cells (73). It seems most likely therefore that spontaneous transformation is driven by selection of genetic variants produced in culture at the ordinary rate. Selection is the critical condition for the progressive emergence of those variants to the fully transformed state. The epigenetic components of transformation therefore are those selective conditions—suspension in soft agar, low serum concentration, fetal serum with low growth factor activity, and contact inhibition at confluence—that drive progressive transformation. As noted below, however, selective conditions in the organism are likely to be quite different than those in cell culture.

The Role of Selection in Experimental and Human Cancer

As noted earlier, interest in the role of selection in human neoplasia was heightened by recent computer models of colorectal cancer that indicated that selection is the driving force in the origin of most cases of this condition and probably of other human cancers as well (1, 2). However, there was already considerable evidence of a prominent role for selection in experimental cancer of animals. In cell transformation in culture, contact inhibition of confluence is the most frequently used selective condition; however, it cannot play that role in vivo, where epithelial cells are always in direct contact with each other. Studies of liver carcinogenesis in rodents reveal evidence of selection. Tens of thousands of microscopic foci of enzyme-altered cells appear in rat liver soon after carcinogenic treatment begins (74). Up to 10% of them progress to macroscopic nodules, most of which remodel into normal-looking structures (68), but a few progress to carcinoma (75). Careful examination of livers in untreated rats reveals the presence of small numbers of enzyme-altered microscopic foci that increase sharply with age to a maximum of about 100 (76, 77). These foci resemble the many thousands seen after carcinogenic treatment. Treatment with promoter alone, which is by definition nonmutagenic, raises the number of foci to about 1000 (78). A much higher percentage of cells in the enzyme-altered foci of untreated rats are synthesizing DNA than cells in the surrounding areas (77), and this advantage persists in rats treated with promoter alone (78). The presence of the foci in normal rat liver and their increase with promoter treatment implies that there are incipient foci in the normal liver that are selected by the promoter because of their growth advantage. Full carcinogenic treatment either creates more of them by inducing mutations or is an even more efficient selective agent than the promoters. Farber (79) has proposed a resistant hepatocyte model of liver carcinogenesis in which cells in foci and nodules are selected while the surrounding cells are suppressed by the carcinogenic growth.

Contrary to the conventional wisdom that promoters do not produce tumors of the skin, long-term repetitive treatment of SENCAR mice with promoters alone produced occasional papillomas and carcinomas (80). Most of them had a mutation in the same codon of the c-Ha-ras oncogene, suggesting that the promoters selectively induced expansion of epidermal cells already bearing this mutation. There are also a number of instances in which carcinogens apparently selected cells with spontaneous ras mutations for tumor growth (81). More recently, Ha-ras 1 mutations were found in small patches of mammary epithelium in young untreated Fisher female rats (82). Treatment with N-nitroso-N-methylurea resulted in mammary tumors, >90% of which carried the Ha-ras 1 mutation. This mutation was rarely seen in mammary tumors induced by other carcinogens. The implication is that N-nitroso-N-methylurea specifically selected for growth of cells bearing the preexisting ras mutation. The selective multiplication of ras-activated cells would increase the opportunity for additional mutations and drive progression to malignancy.

There are a number of examples in experimental animals in which chronic endocrine imbalance stimulates persistent cell multiplication in specific tissue, leading eventually to tumor formation (65). The hormone imbalance is not directly mutagenic but presumably fosters the selective growth of cells with a growth advantage in that imbalance. In some of these endocrine disturbances, correction of the hormone imbalance results in disappearance of the tumors, including metastases that developed during the imbalance. This indicates that persistence of the selective environment is necessary for continued neoplastic expression of the altered cells.

Homeostasis of epithelial tissues, such as epidermis and intestinal mucosa, is maintained by topographical features of cell multiplication, differentiation, and apoptosis. Continuous multiplication occurs in the basal layer of the epidermis. Cells that move into a higher layer lose the capacity to multiply. Similarly, stem cell multiplication occurs in the lower epithelial layers of colorectal crypts and continues in transit cells ascending toward the lumen (83). The cells stop multiplying in the upper one-third of the crypt and are extruded into the lumen. In both tissues, treatment with carcinogen produces diffuse, persistent hyperplasia in which cells multiply in previously restricted sites (84, 85). The first sign of true neoplasia is the appearance of nodular growth in the form of papillomas of the skin and polyps of the colon. Selective growth in human colorectal carcinogenesis is usually driven by mutations in both alleles of the APC gene, which results in dysplastic polyps known as adenomas (1, 86). Colorectal carcinomas can also arise, albeit less frequently, by alteration of the microenvironment that results from excessive proliferation of the underlying stroma in juvenile polyposis syndrome (87). Disruption of the normal architecture of the tissue in both these cases produces a microenvironment that interferes with the orderly regulation of growth. The increased multiplication facilitates genetic changes that drive progression to carcinoma.

Another indication that breakdown in tissue structure provides a selective environment for tumor development comes from experiments in which cells were physically dissociated from one another. Mammary epithelium was removed from young C3H mice, dissociated enzymatically, and reimplanted into the cleared fat pad of young syngeneic mice (88). This procedure greatly accelerated the development of hyperplastic alveolar nodules, which are a precursor to carcinomas and only appear after a protracted period of time in undisturbed mammary glands. If cells of the extirpated epithelium were not dissociated from one another before reinoculation, there was much less acceleration of tumor development (89). Hence, the normal architecture of the mammary gland is a restraint against neoplastic growth.

Tissue microenvironment is also affected by age. Inoculation of rat hepatocarcinoma cells into the liver of syngeneic rats produces a tumor much more readily in old rats than in young rats (90). Multiplication of normal crypt cells of the intestine slows down in aging mice, and the time of onset of DNA synthesis in the cells becomes more heterogeneous (91, 92), suggesting the accumulation of damage
to DNA. It is not unlikely that this decreases the capacity of the mucosa to regulate the growth of rogue clones and increases the likelihood of tumor formation. Radiation damage to the lungs of mice increases the number of metastatic lesions produced there by i.v. inoculated tumor cells (93, 94). Preirradiation of the cleared mammary fat pad of mice increases the frequency of tumor formation by neoplastic epithelium grafted into that site (95).

The implication of the mammary grafting experiment is that the state of stromal tissue plays an important role in epithelial tumor development. It has, in fact, been known for some time that epithelial-mesenchymal interactions have a strong influence on tumor development (96). The unit responding neoplastically to polyoma virus infection was the complex of epithelium and mesenchyme, with neither responding alone. Many related findings made in recent years were recently summarized in a report that fibroblasts associated with prostatic carcinomas direct neoplastic progression of initiated but nontumorigenic prostatic epithelium (97). Normal prostatic fibroblasts did not have this effect. Therefore, in some cases, normal fibroblasts are required for tumor growth (96), and in others, they hold it in check (97), but the importance of tissue interactions in tumorigenesis is inescapable.

Evidence for the importance of the local environment in tumor development is not restricted to experimental animals. Metastases were found in the cervical lymph nodes in patients with no primary tumors in the mucosa of the upper aerodigestive tract (98). However, identical genetic lesions were found in the lymph node metastases and in defined areas of the histologically normal mucosa that were common sites of primary tumors. Some of those normal sites eventually did develop into carcinomas. The results indicate that the normal mucosa was able to regulate the growth of preneoplastic epithelial cells that were already forming metastases in the microenvironment of the cervical lymph nodes.

The foregoing discussion emphasizes the role of altered tissue topography and microenvironment in selecting rogue clones for tumor growth. The APC mutations of the colorectal epithelium are apparently sufficient to allow selective growth of the altered cells into early tumors without additional mutations or microenvironmental changes (99). Analysis of mouse aggregation chimeras shows that the action of the APC mutations and that of modifier genes are localized within the cell lineage that gives rise to intestinal tumors (100, 101). This does not rule out a role for microenvironmental effects in determining the growth of intestinal tumors because the stem cells of the human small intestine multiply even faster than colorectal stem cells (102) and are therefore likely to have at least as many APC mutations, but they very rarely form tumors. This disparity may be related to the difference in the distribution of dividing cells in the crypts (102) and in the architecture of the two tissues: the epithelial crypt cells of the small intestine ascend into elongated villi that extend into the lumen, whereas those of the large intestine are extruded directly from the crypts into the lumen (103). Whatever the explanation, it seems that selective growth can arise (a) in a cellularly autonomous fashion by mutation in the prospective cancer cell, (b) by differences in the regulatory microenvironment, or (c) by some combination of both.

**Mutation Frequency in Normal Tissues**

It is axiomatic that the effectiveness of selection for tumor formation depends on the frequency of mutation in tissues. Simpson (5) has surveyed the literature on the frequency of somatic mutations in solid tissues of humans and concluded that it can account for multistep carcinogenesis. For example, the frequency of somatic mutations in the HPRT gene of normal kidney cells of men rises from $5 \times 10^{-5}$ in the first decade of life to $2.5 \times 10^{-4}$ beyond the age of 70 years (34).

The APC tumor suppressor gene is considerably larger than the HPRT gene, and the estimated frequency of inactivating mutations of this gene is $1.7 \times 10^{-3}$ per cell (5). Given the square of this figure for the frequency of biallelic APC mutations, there may be many colorectal cells in the colon of aging people that carry biallelic mutations in this tumor suppressor gene and therefore have the potential for tumor growth. In this scenario, there is no need to invoke higher mutation frequencies to account for multistep human carcinogenesis. Indeed, there is the implicit suggestion that many such mutated cells are held in check in normal tissues. In fact, it was recently reported that there are about 11,000 mutations of inter-simple sequence repeats in polyps and carcinomas of the human colon (104). This suggests there may be many mutations of this type in normal crypts of the colon, but these would be hard to detect because of their occurrence in single, expanded crypts surrounded by a large excess of crypts without the same mutations in the normal tissue sample.

Continuing along this line, clones have been isolated from histologically normal human breast by dissecting ducts and terminal ductal-lobular units (105). Nine highly informative microsatellite markers were examined, half of which were at chromosomal regions involved in breast cancer. At least one genetic abnormality was found in half the women examined with a weighting toward mutations known to be involved in breast cancer, but there was no association with histological abnormality, nor did genetic abnormalities necessarily lead to cancer many years later (106). These considerations indicate the need for additional genetic changes in the lesion-bearing clones and/or microenvironmental changes that permit selective growth of the clones for neoplastic development to occur.

**General Considerations and Future Directions**

The prominence of selection in tumor development in a sense is an extension of findings in experimental embryology that began in earnest with Spemann’s discovery (107) of the role of the organizer in early frog development. An intriguing offshoot was the demonstration that differentiation depended on tissue fragments larger than a minimal size (108). Whereas the fate of large blocs of tissue may be strictly determined, that of individual cells in those blocs is not (109). Such observations have been formalized in the concept that there can be regularity in the large where there is heterogeneity in the small or “order above heterogeneity” (110). This concept was developed on the basis of heterogeneity of the atomic and molecular constituents of cells that are ordered by higher levels of organization from the cell to the organism. If genetic changes of DNA are inserted in this hierarchy, one might infer that great genetic diversity is generated in the growth and development of somatic cells but is usually not expressed in the integrated architecture of a tissue. A breakdown in the ordered state can come from the accumulation of multiple genetic changes in a prospective tumor cell or in the ordering capacity of its microenvironment or a combination of the two. Both aspects of control may be weakened by the accumulation of somatic mutations in the aging process. Most of the emphasis in contemporary cancer research has been on finding genetic changes in tumor cells, but this effort needs to be extended to clones in normal tissues to provide a broader foundation for understanding normal regulation and its breakdown in malignancy.

The normal tissue controls commonly used as a baseline for identifying genetic changes in tumors involve many clones. Random mutations in the DNA of any clone are swamped out by the DNA from all of the other clones in the tissue sample. Techniques have to be applied to isolate the DNA from clones or otherwise identify clonal mutations, i.e., immunocytochemistry, clonal selection, and so forth. The continuous improvement of methods for characterizing small
amounts of DNA, e.g., PCR, should facilitate the task. It should then be possible to evaluate the relative contributions to tumor growth of mutation in a potential cancer cell and the regulatory capacity of its microenvironment.

Addendum

Haddow (111) first added a role for selection in tumor development from the contrast between the inhibition of spontaneous tumor growth by carcinogenic polycyclic aromatic hydrocarbons and their failure to inhibit the growth of tumors induced by the same and other compounds. Recently, evidence was presented for the thesis that selection is a critical factor in determining the mutations found in human cancer (112). Additional evidence indicates that physiological stresses aggravated by carcinogens in tobacco smoke select endogenous mutations to drive the development of lung cancer in humans (113). Loss of function mutations in the p53 tumor suppressor gene select, under stressful conditions, for cells which would otherwise be eliminated by apoptosis (114).

References

Selected Cell and Selective Microenvironment in Neoplastic Development

Harry Rubin


**Updated version**  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/61/3/799

**Cited articles**  This article cites 106 articles, 68 of which you can access for free at:  
http://cancerres.aacrjournals.org/content/61/3/799.full.html#ref-list-1

**Citing articles**  This article has been cited by 12 HighWire-hosted articles. Access the articles at:  
/content/61/3/799.full.html#related-urls

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.