Tumor Cell Instability, Diversification, and Progression to the Metastatic Phenotype: From Oncogene to Oncofetal Expression

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The viewpoint that "no unitary concept can give a satisfactory explanation of the intimate nature of cancer" (1) remains quite valid today. We seem to be closer, however, at least to defining the wide range of characteristics that exemplify different cancer cells and their host environments, particularly those properties that contribute to the malignant phenotypes of tumor cells, clearly an important problem (2-5). Assembling these data into an explanation of the intimate nature of cancer remains quite suggestive of future approaches to others. An attempt will be made to meld recent information concerning the malignant phenotype from such interrelated but often insular disciplines as cancer molecular (4, 6, 7), developmental (1, 8, 9), and cell biology with recent views on mechanisms of tumor progression (11-15). Inherent in such a task is the frightening possibility that there exist so many variables in the multifaceted, dynamic process of cancer that it is doubtful that they could be understood in the context of the whole organism without a radical change in our theoretical framework (9). With this in mind, I will attempt to analyze some of the more challenging properties of malignant neoplasms, such as their cellular instability, diversification, and progression, in terms of genetic and epigenetic events that are modulated by host and tumor cell interactions.

Tumor Heterogeneity Revisited

Neoplasms, and in particular malignant neoplasms, are known to be composed of diverse cell populations that are heterogeneous for a wide variety of characteristics (2-5, 16-20), including many of those once thought to be homogeneously expressed in neoplastic cells (21). Tumor cell heterogeneity was first noted histologically (for a discussion see Ref. 22), and such morphological observations of heterogeneity have been extended to include a wide variety of genetic, biochemical, enzymatic, immunological, biological, and other properties (16-20). The cell-to-cell heterogeneous nature of such properties is generally thought to be transmittable, at least for several generations, although microenvironmental effects, among others, can modulate the properties of individual cells within a tumor (15, 20).

An interesting aspect of tumor heterogeneity is the ability of one tumor cell subpopulation to influence the properties of other cell subpopulations (23). Such tumor cell-cell interactions can modify cellular growth (24), immunological (25) and metastatic (26-28) properties, sensitivity to drugs (29), and expression of cell surface glycoproteins (28). Not all tumor cells, however, appear to be capable of modulating the properties of other tumor cells; thus this characteristic is also heterogeneous when expressed among neoplastic cells.

The cellular heterogeneity found in tumors is not unique to neoplastic cells. Normal cells and tissues are also heterogeneous for a variety of characteristics, such as their contents of cell surface antigens (30, 31) and enzymes (32, 33). In malignant neoplasms, however, cellular heterogeneity is usually more pronounced than in counterpart benign or normal tissues. When Peterson et al. (31) examined the variability in single cell expression of a M, ~400,000 cell surface epithelial antigen on normal breast epithelial and carcinoma cells, they found greater cellular heterogeneity of expression in the malignant tissues. A portion of such heterogeneity is thought to be due to differences in cell cycle fraction and states of differentiation of cells within tissues or tumors (34, 35).

Tumor heterogeneity, which has evoked considerable interest in recent years, is thus a normal, not a tumor, cell characteristic. The distinguishing feature of tumor heterogeneity may be its greater range of expression (increased quantitative variability) among cells.

Evolution of Malignant Cells

It is now well established that naturally occurring and induced neoplasms generally develop from single transformed cells (36). Even in tumors in which the progeny of a single cell have diversified to heterogeneous cell phenotypes, evidence of such clonal origin still exists (37, 38). The heterogeneous nature of tumors and their ability to change or evolve with time was studied extensively by Foulds (39), who described this phenomenon using a series of spontaneous mammary tumors. He noted that the mammary tumors changed gradually and independently and eventually gained autonomy from host controls, such as hormone regulation of cell growth and tissue control of cellular differentiation. Foulds (11) concluded that tumor evolution (progression) is characterized by a series of permanent, irreversible changes that occur individually in each tumor. The apparent reversibility of such changes (to be discussed) was not seen at the tissue level in Fould's studies, probably because of the long time intervals in his experiments.

Tumor progression probably starts when tumor cells diversify in their unique microenvironments, and variants with altered properties arise in the tumor cell population. To survive and proliferate in a competitive environment, such variants must have selective growth properties and other competitive advantages over other cells (13-15). In a tumor, these variant cells may rapidly become dominant, although it is almost as likely that they will not due to cellular and noncellular interactions that can stabilize cells, leading to changes in tumor cellular composition. In time, however, the gradual evolution of more stable variants may result in the eventual dominance of particular subpopulations of tumor cells with divergent properties and enhanced survival, growth, autonomy, and malignant characteristics (15). Virtually any characteristic of neoplastic cells should be subject to independent variation, selection, and evolution, leading eventually to tumors that are increasingly un-
linked to host regulatory and surveillance mechanisms (11–15, 17–19).

Tumor cells exist in a competitive microenvironment with other neoplastic cells as well as with normal surrounding and tumor-infiltrating cells. The ability of neoplastic cells to compete for necessary survival and growth factors and evade host nonimmune and immune responses and environmental signals probably determines, to a great degree, whether they become increasingly dominant tumor cell subpopulations. Changes within tumors do not always result in progression of tumor cells to more malignant phenotypes. Some of these changes may be unrelated to tumor progression and others may result in tumor regression (15, 40). Although rare, tumor regression has been documented in certain malignancies (41).

The rates at which tumor progression and phenotypic diversification occur appear to be quite variable. In the hypothetical scheme shown in Fig. 1, benign and malignant tumors are shown diversifying at differing rates and times, resulting in dynamic changes in the cellular compositions of tumors. One of the important events in such a scheme is the appearance of a significant number of metastatic cells that possess unique characteristics for their dissemination, implantation, invasion, survival, and growth at other sites (2–4, 14, 15, 19–21). It is expected that changes in the metastatic cell subpopulations determine one of the most important biological characteristics of tumors.

Although each tumor is expected to be unique in its progression and phenotypic diversification characteristics, tissue-associated gene expression programs probably modulate to varying degrees the phenotypic differences between tumors of the same histological class. As such tumors become more advanced, some of these tissue-associated gene programs may become obscured, producing “more undifferentiated” tumors. Obviously, the metastatic cell subpopulations within tumors need not be the only ones that display a more undifferentiated morphological pattern, since malignant properties are not the same as morphological properties. The histological patterns of tumors, however, are often symptomatic of phenotypic diversification and altered cellular behavior (11, 22).

Fig. 1. Hypothetical examples of tumor phenotypic diversification. A single tumor cell (T) may proliferate and diversify slowly with time, yielding a benign lesion (A) or low-grade malignancy (B). Transformation may not initially result in a malignant cell population, but diversification eventually yields malignant cell populations (C, D). Occasionally, a malignant tumor may revert to a more benign phenotype with the loss of metastatic subpopulations (F). In highly malignant neoplasms, diversification occurs at rapid rates (E, G), but in some malignancies, “interactions” between malignant cells limit diversification and the rate at which new metastatic variants are generated (E).

Genotypic Instability and Tumor Progression

During the progression of tumors, neoplastic cells are thought to accumulate increasing genetic alterations that are generated by random, somatic mutational events (13, 14). In concert with such “genetic instability” of progressing tumors, host selection pressures that are both nonimmune and immune in nature tend to enrich emergent tumors with cell subpopulations that are more adept at survival and growth (12–15, 40).

That neoplasms undergoing progressive changes display enhanced genetic instability has support from cyogenetic and genetic data. Examination of tumor cells for gross chromosomal alterations, mitotic errors, and spontaneous mutation rates indicates that advanced malignancies contain neoplastic cells with progressive changes symptomatic of increasing genomic instability (14, 42). Chromosomal abnormalities or alterations in the numbers, morphologies, or banding patterns of specific chromosomes can become progressively more pronounced in such tumors as they advance to more malignant phenotypes (42–44). In Rous sarcoma virus-transformed fibroblasts, consistent chromosome changes have been associated with tumor progression. One of the first changes found by Mitelman (44) was the appearance of an extra chromosome 7, followed by the acquisition of an additional chromosome 13, and finally an extra chromosome 12. Although a commonly found change during tumor progression is an increase in chromosome number (42, 45), some highly malignant tumors are strictly diploid. Our examination of malignant rat mammary tumor cells isolated from primary, regional lymph node, and spontaneous lung metastatic lesions revealed that although tumor cells from the primary site were aneuploid, the cells from metastases were diploid. In this system, the metastatic cells frequently possessed a particular set of Giemsa-stained chromosome markers while sharing others with nonmetastatic cells (46). Herlyn et al. (47) examined human malignant melanoma cells from primary and secondary metastatic lesions of the same patient. The cells from the primary tumor were characterized cyogenetically by the presence of abnormal markers, and the cells from metastases had these same chromosomal alterations plus additional changes characteristic of the metastases. Comparing cells from primary tumor regions of low invasiveness (radial growth phase) with deeply invasive regions (vertical growth phase) of the same lesion, they found that cells from the vertical growth phase more closely resembled cells isolated from metastases than cells isolated from the radial growth phase. The alterations involved chromosomes 1 and 6 in cells from the primary tumor, and cells from metastases had additional copies of chromosome 7 (47). Further alterations in the chromosomes of metastatic cells compared to primary tumor cells have also been documented in neuroblastomas (48).

A classic example of chromosome alterations associated with tumor progression is chronic granulocytic leukemia where the Ph1 minute chromosome was discovered (13, 14). Advanced disease is characterized by the original 9;22 chromosome translocation that produces the Ph1 chromosome and additional cytogenetic changes. As Burkitt’s lymphoma progresses, almost all cells contain a characteristic 8;14 translocation, which results in enhanced c-myc expression. This change is associated with the expansion of a highly malignant Burkitt’s cell subpopulation that possesses the specific translocation (7).

In addition to the gross chromosomal changes considered briefly above, genetic instability has also been studied by examining rates of spontaneous mutation. Although Elmore et al. (49) could not demonstrate differences in the spontaneous rates
of mutation of untransformed and chemically transformed human skin fibroblasts, Cifone and Fidler (50) estimated that the spontaneous rates of mutation to drug resistance in a series of murine melanoma and fibrosarcoma cells of different metastatic potentials were 6–7 times higher in cells with high metastatic potential. Correlations between metastatic potential and rates of spontaneous mutation were not observed, however, in a mouse mammary tumor system by Yamashina and Heppner (51). They did find that the frequency of induced mutation by ethyl methanesulfonate correlated with metastasis. The measurement of spontaneous mutation rates is difficult and fraught with technical limitations; thus, further research will be needed to demonstrate a correlation, although it is unlikely to exist, between spontaneous mutation rates and metastatic potentials.

A variety of different possible mechanisms for generating increasing genotypic instability have been discussed by Nowell (52) and Chorazy (53). In addition to the chromosome alterations described above, these include inherited defects in DNA repair, replication and maintenance genes, chromosome amplification regions, mutagenic agents, and nutritional changes. Such mechanisms are listed in Table 1 with additional intracellular and extracellular events that could alter tumor cell stability and modify diversification mechanisms.

The possible mechanisms listed in Table 1 for generating cellular instability are not of equal importance, and only a few of those listed may be relevant in a given tumor. Several involve epigenetic phenomena and will be discussed in a subsequent section. Although genotypic alterations are extremely important in modifying the behavior and stability of tumor cells, the frequency of their appearance is often inconsistent with the rapid phenotypic changes found in malignant cells.

### Oncogenes and Tumor Progression

Cellular oncogenes (c-onc) or protooncogenes and their viral counterparts, viral oncogenes (v-onc), first discovered as the transforming genes of retroviruses, have been established as critical elements in the neoplastic transformation of mamalian cells (7, 10, 54, 55). In transformed cells, oncogenes encode proteins that function abnormally, inappropriately, or at improper concentrations, resulting in circumvention of the normal cellular controls that regulate cell division and differentiation. Retroviruses can deliver to susceptible cells dominant transforming v-onc genes that can transform cells in essentially one step (7, 55). Although such rapid, one-step transformations are interesting and important models for viral carcinogenesis, they are probably not very relevant to spontaneous cancers that arise by slow, multiple steps (56, 57). Retroviruses and their nontumorigenic v-onc genes are often deleted under strong selection pressures to yield rare recombinational events that are probably encountered infrequently in the wild (58).

In spontaneous cancers, one or more c-onc genes appear to be involved in transformation processes (7, 10, 54, 55). Rare mutations in the coding regions of c-onc genes can cause functional alterations in the c-onc-encoded products, or the v-onc gene products can cooperate to induce the transformed state. These changes appear to be quite specific, since c-onc genes are regularly expressed in normal cells at various stages of differentiation, indicating the importance of their functions in normal cells (7, 55–58).

Insertion of normal numbers of unmutated c-onc genes into normal diploid cells usually does not result in neoplastic transformation, unless the recipient cells or the transferred c-onc genes have undergone further genetic change (58). Although mutations in c-onc genes have been found in chemically transformed cells (59), this does not appear to be a generic feature of spontaneous tumors (60–62). A more common finding is the increased expression of c-onc genes due to changes such as gene amplification (63–65) or chromosome translocation (66, 67); however, such alterations in c-onc expression are far from universal (62).

Changes in the amplifications of c-onc genes have attracted considerable attention (7, 68–71) and have been proposed as important determinants in tumor progression. Little et al. (72) found that c-myc was amplified 20 to 76 times in 5 of 8 human small cell lung cancer lines. The variant forms of small cell lung cancer are characterized by their increased malignant potential, and all of the variant lines examined showed amplification of the c-myc gene. The majority of these lines also showed changes in double minute chromosomes and HSRs and deletions in chromosome 3. Since amplified c-myc genes have been associated with HSRs in other tumors, such as colon carcinomas (65), it is likely that they will be found in the HSRs of the more malignant cells. Indeed, amplification of c-N-myc in multiple HSRs on different chromosomes has been cited as evidence that interchromosomal transpositions occur during

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2 The abbreviations used are: HSR, homogeneous staining region; 5-azaazacytidine; p180, M, 180,000 protein (other proteins similarly designated).
amplification events in neuroblastomas (73, 74).

In addition to c-N-myc, other chromosome fragments have been found amplified in advanced human neuroblastomas. Shilo et al. (75) examined the gene sets contained in the chromosome 2p HSR of neuroblastomas. They postulated a “neuroblastoma amlicon” in which c-N-myc is the central gene with other sequences increasingly peripherally located. Since novel DNA fragments were also contained in the HSRs of different tumor specimens, extensive DNA rearrangements must have occurred during the evolution of the amlicons.

In human neuroblastoma, Brodeur et al. (76) examined cell lines established from 63 primary tumors and found amplification of the c-N-myc gene in 23 of these. In 5 patients with stage I or II disease, none had c-N-myc amplifications, whereas 24 of 48 patients with the more advanced stage III or IV disease had c-N-myc amplifications. Similarly, a high proportion of malignant primary retinoblastomas also display c-N-myc amplification (77). If c-N-myc amplification is causative for neuroblastoma progression (78), then it is not clear why all of the patients with advanced disease did not have dramatic c-N-myc amplifications. Although c-onc gene amplifications have been documented in other malignant tumor systems, such as c-K-ras in a case of ovarian adenocarcinoma (79), c-myb in a case of acute myeloblastic leukemia (80), and c-myc in a case of chronic granulocytic leukemia (81), these are by and large uncommon events for most tumors. Considering the number of possible genomic alterations that could contribute to changes in oncogene expression, this is probably not surprising (Table 2).

In concert with most of the data on c-onc gene amplification, it appears that few human malignancies express activated (mutated) c-onc genes (58, 62, 82, 83). Most of the activated oncogenes that have been found are of the c-ras gene family, probably because of their identification using the NIH/3T3 transfection assay where activated ras genes are easily detected (58). Some studies have used fresh tumor samples as opposed to established cell lines for analysis of c-onc gene alterations, but for the most part, c-onc genes were detected in cultured cells established from tumors.

There are only a few examples where multiple oncogenes have been examined in several tumors of the same histological type. In one of these studies, Yokota et al. (84) examined 104 fresh human tumors of various histological classes for alterations in c-myel, c-H-ras, c-K-ras, c-myb, c-fos, c-N-ras, c-fes, c-abl, and c-mos expression. They were also able to evaluate the normal tissues from 72 of these patients, and in 64 cases these were of the same tissue type as the cancers. They found changes only in the c-myel, c-myb, c-K-ras, and c-H-ras oncogenes, such as amplifications of c-myc (10% of cases) and c-K-ras (1% of cases) and deletion of one allele of c-myc (11% of cases) and c-H-ras (18% of cases). Modifications in c-onc genes were not found in benign tissues, although only 3 were analyzed. In some patients, both primary tumors and metastases were examined. For example, amplification of c-myc occurred in 11% (7 of 64) of primary tumors (3- to 5-fold amplification) and in 19% (3 of 16) of metastases (5- to 8-fold amplification), and deletions of one c-H-ras allele occurred in 15% (4 of 26) of primary tumors and 29% (2 of 7) of metastases (84). Although the low number of some samples makes an overall evaluation of these results difficult, it is apparent that various c-onc gene changes can and do occur, but the frequencies of such changes indicate that none of these are absolutely required for progression to the metastatic phenotype.

Recent studies on animal cell lines also suggest a complex relationship between c-onc expression and progression. Leibovitch et al. (85) examined the differential expression of 15 c-onc genes in nontumorogenic and low and high malignant potential rat myogenic cells. When the nontumorogenic cells were induced to differentiate in low serum medium to myotubes, the levels of c-abl, c-myb, and c-H-ras transcripts remained unchanged, the level of c-N-ras increased, and the level of c-erbB decreased, concomitant with differentiation. Other c-onc genes (c-erbA, c-sis, c-src, c-fms, c-fos, c-myc, c-K-ras, and c-fgr) were not detectable. When the three cell types were proli-
erating at similar rates, c-mos was not expressed in the nontumorogenicic cells, c-fos and c-erbB were not expressed in the low malignant cells, and c-erbB, c-sis, and c-src were not expressed in the highly malignant subline. In contrast, c-K-ras was increased in expression in the highly malignant cells, and one of the transcripts, a 3.8 kilobase pair mRNA, was barely (low malignant cells) or not (nontumorogenicic cells) detectable in the other cell lines (85).

These data indicate that tumor progression can be associated with decreased as well as increased expression of c-onc genes, but a direct relationship between onc gene expression and progression cannot be established with any certainty. This is perhaps not astonishing, since oncogenes were discovered and defined largely in terms of their roles in cellular proliferation, not tumor progression. Further changes in onc gene expression are apparently not obligatory for tumor cells to progress to more malignant states, although in some tumor systems onc gene expression may have an indirect effect on or contribute to tumor progression (68, 78).

Oncogenes and Metastasis

In animal tumor systems, progression to the metastatic phenotype has also been examined with respect to increases and decreases in oncogene expression. Vousden and Marshall (86) noted that mouse lymphoma cells were tumorogenicic but nonmetastatic until they expressed an activated c-K-ras gene, which occurred in a spontaneous variant cell type. In other examples, loss of specific oncogene expression occurred. Abelson virus-transformed lymphomas lost expression of the v-abl gene and its encoded product, p160, with progression during ascites growth (87). In an extension of such studies, Rotter et al. (88) examined murine lymphoma lines of different metastatic potential for expression of the v-abl oncogene and the amounts and activities of the p160 protein kinase. There were no differences in the expression of v-abl or in the amounts or enzymatic activity of its encoded product. This tumor system also expresses the p53 oncogene, but similar to abl there was no difference in p53 gene expression or in the amounts of its encoded product, the p53 protein (89). The expression of four other oncogenes was examined, but again there was no difference in expression, if it occurred (88). In other metastatic murine tumor systems, such as melanoma and fibrosarcoma, onc expression was also unrelated to the metastatic phenotype (90).

Although the studies of Yokota et al. (84) on the expression of several c-onc genes in human cancers and Albino et al. (61) on c-H-ras expression in primary and metastatic human melanoma showed that some alterations in oncogenes or their expression can occur in metastases, these data are not overwhelming in suggesting a mechanistic relationship between such changes and progression to the metastatic phenotype. Indeed, in some human tumors, it is difficult to detect expression of oncogenes in metastases, although primary tumors from the same patients showed high amounts of this component. Regions of deeply invading tumors possessed greater levels of staining, however, compared to superficially invasive carcinomas, and there were undetectable levels of p21⁰ in surrounding normal colonic or benign tissues (92). Ohuchi et al. (93) used anti-p21⁰ antibodies to evaluate the expression of this oncogene product in benign and malignant breast tumors. Heterogeneous expression of p21⁰ was found, although the malignant tissues showed, in general, higher percentages of positive cells and greater tumor-to-tumor variability in expression than the benign tissues. Some of the metastases showed very low or undetectable levels of p21⁰ expression. They concluded that enhanced p21⁰ expression may be involved in the earlier rather than the later stages of mammary tumor carcinogenesis and progression (93).

Variations in the expression of c-onc genes have also been found in chemically induced metastatic animal tumors. Sukumar et al. (94) have demonstrated that about one-third of N-methyl-N-nitrosourea-induced rat carcinomas express an activated c-H-ras gene and an altered p21⁰ product. N-Methyl-N-nitrosourea was used by Thorgeirsson et al. (95) to produce metastatic adenocarcinomas, and the DNA was isolated and analyzed from primary tumors and their metastases. Southern blot analyses revealed that the primary tumors had 10 times higher DNA levels of the c-H-ras gene. When multiple metastases from the same rat were examined for c-H-ras gene contents and mRNA expression, lesion-to-lesion variability was noted. Some metastases had higher levels of c-H-ras DNA content and mRNA expression, while others were lower than the original primary tumor. After serial transplantation of one of these tumors, however, the differences in c-H-ras contents and expression were less than 2-fold (95). Thus it is unlikely that onc gene amplification or increased expression of c-onc genes, per se, are direct determinants of the metastatic phenotype.

Although modifications in oncogenes or their expression are associated with the metastasis of some tumors, it is difficult to conclude that such changes are required for progression to or maintenance of the metastatic phenotype. It is more likely that such oncogene alterations contribute to but do not solely determine states of tumor progression (15, 40).

Transfected Oncogenes and Metastasis

Under certain circumstances, direct insertion of oncogenes into cellular DNA by transfection can result in a cell's acquiring the metastatic phenotype. Use of the NIH/3T3 transfection system has evolved from experiments designed to identify activated oncogenes in transformed animal cells and human tumors (54, 55, 58). As mentioned, most of the oncogenes discovered with this technique belong to the activated ras family. NIH/3T3 cells are preneoplastic aneuploid cells that spontaneously transform easily, especially when cell cultures are allowed to reach overconfluency. More recent studies have used normal diploid embryo fibroblasts for transfection, but in some cases, this may require two cooperating oncogenes for full transformation (96), an event rarely seen in human tumors (62, 84). The rapid transformation found in transfection experiments is also unlike the usual slow, sequential changes that characterize spontaneous transformation and progression events that occur in vivo (11, 39, 56–58). The use of embryo cells for transfection assays can also be questioned because these cells possess characteristics, such as cell surface and growth properties, similar to those found in transformed cells (69, 70). Although human cell transfection recipients for human
DNA, such as the PA-1 teratocarcinoma system (97), overcome the difficulty of crossing species barriers (58), these recipient cells are not normal.

Induction of the metastatic phenotype in NIH/3T3 cells has been accomplished by transfection of activated c-ras oncogenes (95, 98–101). Thorgeirsson et al. (95, 98) found that c-H-ras but not c-N-ras transfection resulted in spontaneously metastasizing 3T3 cells that were also invasive and expressed high levels of collagenase IV activity. Using a variety of oncogenes and recipient cell types, Muschel et al. (101) studied the transformation and induction of the metastatic phenotype. After transfection and i.v. or s.c. injection of 0.5–5 × 10^6 transformed cells into nude mice, the animals were examined 4–8 weeks later for lung tumor nodules. Although activated c-H-ras transfection was sufficient to induce the metastatic phenotype in NIH/3T3 cells, multiple copies of the corresponding c-onc gene were not. Diploid fibroblasts transfected with activated c-H-ras genes were also spontaneously metastatic; however, ras-transfected C127 cells, derived originally from a murine mammary tumor, were not metastatic. Even though C127 cells could be productively transformed by an activated c-H-ras gene into tumorigenic cells expressing high levels of p21wt, they remained nonmetastatic in the two assays used (101). When Vosudden et al. (102) transfected low metastatic potential mammary tumor cells with c-H-ras genes, they became more metastatic, but one metastasis did not express the oncogene.

The studies above illustrate the importance of the recipient cell in transfection experiments. Heterogeneity in the recipient cell population may be an essential characteristic in successful transfection to induce the metastatic phenotype. This suggests that changes other than those involving oncogenes are critical in inducing malignant behavior.

The difficulty in concluding that transfected oncogenes, per se, are responsible for acquisition of the invasive and metastatic properties of cells is exemplified in the studies of Van Roy et al. (103). They used various rat fibroblast lines for transfection experiments and were able to produce invasive cells with essentially any of the constructs (including polyoma large T, polyoma middle T, and activated v-myc), but only the activated c-H-ras or c-H-ras + v-myc constructs were capable of producing transfectants that were metastatic in syngeneic rats. Some of the untransfected rat cells were, however, also invasive and metastatic, and the authors could not conclude definitively (with the possible exception of activated ras) that oncogenes were directly involved in producing the invasive/metastatic phenotype.

There are several possible explanations for the ability of transfected, activated ras genes to induce the metastatic phenotype (40, 101). (a) Recipient cells may express all of the genes necessary for the metastatic phenotype, except that they need additional growth potential in vivo. This possibility is usually dismissed, because tumorigenic transfectants expressing high levels of a ras-gene are often nonmetastatic. (b) Transfection induces genetic instability, and metastatic variants are generated at high rates in the cell population. This was proposed previously (15, 40) to explain the fact that Grieg et al. (100), Eccles et al. (104), and Kerbel et al. (105) eventually detected metastatic properties in NIH/3T3 cells transfected with a control pSV2Neo vector alone. However, if significant numbers of metastatic variants arise in a transfected cell population, this event must occur rapidly, because recent experiments indicate that the metastatic phenotype is acquired within a few cell generations after transfection (102, 106). In addition, gross cyogenetic abnormalities were not seen in activated c-ras-transfected diploid cells (107). Whether such dramatic chromosomal changes are necessary, however, for producing genomic instability and the metastatic phenotype is dubious. (c) Transfection of activated ras genes induces the expression of a cascade of genes normally associated with embryogenesis and required for metastasis. This explanation is favored by Muschel et al. (107), but it may be valid only for the activated ras gene family. In this explanation virtually all of the ras-transfected cells should have their gene expression programs modified soon after transfection. Alternatively, transfection with activated ras constructs may grossly accelerate the rate of alterations in the expression of genes needed for the metastatic phenotype of fibroblastic cells.

A major problem with the gene transfection systems is that multiple copies, often in tandem, of the transfected genes with their strong promoter/enhancer constructs are incorporated at several sites in the DNA of recipient cells. Also, the transcription rate of inserted genes may be elevated over the same gene in its normal environment by foreign promoter/enhancer elements (108). Since most of the cell types successfully transfected to obtain metastatic properties are embryo cells or they are already transformed, many of the gene products necessary for metastasis may be constitutively present in these cells. The fact that some diploid cells can be successfully transfected to metastatic cells, but others cannot, might explain these results.

An even more disturbing result is that the transfection technique itself may result in genomic instability and alterations in the tumorigenic and metastatic properties of cells. Kerbel et al. (105) found that the calcium phosphate treatment used in transfection experiments can modify the heritable tumorigenic and metastatic properties of benign mammary cells at high frequencies. They also observed changes in the expression of the H-2Dk antigen upon calcium phosphate treatment, suggesting that this procedure alone can alter gene expression and phenotypic properties.

Another interesting finding is the apparent suppression of the metastatic phenotype by transfection of two oncogenes, c-H-ras plus the adenovirus E1a gene (109). In their studies on the effects of one and two oncogenes on the acquisition of metastatic properties, Pozzatti et al. (109) found that transfection of c-H-ras alone into rat diploid embryo cells resulted in tumors that were metastatic, although some were poorly metastatic. Transfection of the E1a gene alone did not result in transformed cells, and all of the double oncogene-transfected (c-H-ras + E1a) cells were nonmetastatic, although they grew faster at s.c. sites than the c-H-ras-transfected cells. It was proposed that the low metastatic potential of the c-H-ras + E1a transfectants was due to an increased susceptibility to lysis by host effector cells, or different subpopulations of rat cells were transformed by the unique transfection constructs (109). Another possibility is that related but dissimilar sites of integration of the two constructs in the rat embryo DNA of different cells could be important in determining subsequent host gene expression programs. Since the embryo cells used for such transfections are generally mixed cell populations, it is likely that cellular heterogeneity plays an important part in the transfection/transformation process. If slightly different sites of integration, reading frame alterations, or effects on neighboring genes in the appropriate host cell occur, then such alterations might trigger rapid, multiple changes leading to progression at rates that are not found during the natural progression of tumors in vivo (Table 2; Ref. 70).

It is difficult to conclude from the studies above that incorporation of an oncogene into normal adult cell DNA will in itself produce a metastatic cell. Problems with the technique,
the number of multiple gene copies incorporated, the genomic locations, the nature of the recipient cells, and alterations in cellular gene expression programs upon transfection render such studies inconclusive.

Similarly, the use of transfection techniques to identify "metastasis genes" may also be misleading. If a heterogeneous cell population is destabilized by transfection or if such cells are constitutively expressing most of the gene products necessary for the malignant phenotype before transfection, only slight alterations in gene expression might lead to the metastatic phenotype. Thus the transfection assay as currently performed is of questionable value in analyzing the induction or control of a complex, multistep phenomenon such as metastasis.

Genotypic Suppression

In addition to the apparently dominant-acting oncogenes, there exists another class of genes that can affect malignant cell properties. These genes have been termed tumor suppressor genes (108, 110) or antioncogenes (111). These putative suppressor genes were originally proposed to account for the suppression of tumorigenicity when many types of normal and malignant cells are fused to yield nontumorigenic cell hybrids (108, 110). Tumorigenic segregants can arise from the apparently recessive nontumorigenic cell hybrids that show loss of specific chromosomes, and it has been proposed that these chromosomes contain the dominantly transacting genes that can control tumorigenic characteristics (112, 113).

The dominant role of oncogenes in transforming cells and the recessiveness of cell hybrids between oncogene-transformed and normal cells have been examined by Geiser et al. (114). They fused human EJ bladder carcinoma cells with normal human fibroblasts to form cell hybrids that behaved like transformed cells in culture but failed to form tumors in nude mice. When tumorigenic segregants of these hybrids arose, they were examined for expression of the EJ-activated c-H-ras gene and its encoded p21\textsuperscript{*}. The expression of c-H-ras and p21\textsuperscript{*} was similar in the EJ cells, the nontumorigenic hybrids, and the tumorigenic segregants. To increase the level of c-H-ras expression in the nontumorigenic hybrid cells, they were transfected with activated c-H-ras genes. One of the transfected cell clones showed a 2-fold increase in c-H-ras expression and p21\textsuperscript{*} content over the nontransfected hybrid cells, but they were still nontumorigenic, suggesting that the expression of an activated oncogene is insufficient for the tumorigenic phenotype (114).

The metastatic phenotype in at least certain types of cells can also be suppressed by cell fusion. Using rat mammary carcinoma or mouse melanoma cells, the fusion of metastatic with nonmetastatic (115) or with normal (116) cells yielded suppression of the experimental (115) or spontaneous (116) metastatic phenotype. In lymphoid cell systems, however, this does not seem to be the case (to be discussed below). Layton and Franks (117) produced cell hybrids from mouse metastatic lung carcinoma cells and tumorigenic but nonmetastatic mouse L-cells or human EJ bladder carcinoma cells. All of the hybrid cells were tumorigenic in nude mice, but 13 of 14 of the mouse-mouse hybrids were nonmetastatic in a spontaneous metastasis assay. The mouse-human cells were metastatic, but they had lost some human chromosomes and isozyme markers. These results have provided strong indirect evidence for the existence of tumor suppressor genes, and perhaps metastasis suppressor genes.

The identification of tumor suppressor genes and their mechanism of action have not yet been elucidated (108, 110). They could antagonize certain activities of oncogenes, but it is difficult to see how they could act on so many different onc gene products. Alternatively, they might act at the level of the genome to suppress onc gene expression. This is unlikely since Geiser et al. (114) found normal or even elevated expression of c-H-ras in nontumorigenic cell hybrids. Perhaps they act close enough to the final biochemical steps required for expression of the tumorigenic or metastatic phenotype, activities that may depend on the expression of multiple genes, or they could act on critical steps in the process, such as cell growth, angiogenesis, or immunogenicity (108).

Phenotypic Instability and Progression

In addition to genotypic instability of tumor cells and host selection mechanisms, there exists another cellular phenomenon that can contribute to normal and tumor cell diversification and heterogeneity, and this has been termed phenotypic instability (15, 40). This property includes all epigenetic and microenvironmental changes that can result in tumor cell diversity (Table 1). Genotypic instability and selection alone cannot explain the rapid rates of tumor cell phenotypic variation that are often found in vitro and in vivo, and they cannot explain the rapid, apparently reversible changes seen in the cellular properties of tumor cells (15, 40). Among the highest rates of spontaneous gene mutation are those found by Cifone and Fidler (50) for highly metastatic mouse cells (~$7 \times 10^{-5}$ mutations/cell/generation), but these are orders of magnitude lower than the rates of variant formation in biochemical, immunological, enzymatic, and biological properties found in malignant cells. Phenotypic variation appears to be governed by quantitative, not qualitative, changes in cells, so it is reasonable that the rates of phenotypic diversification are orders of magnitude higher than the rates of genotypic diversification, unless the latter types of qualitative changes are in gene regulatory sequences that affect the widespread expression of many genes (40).

The rates of appearance of quantitative variants with significant differences in cellular properties have been calculated for a number of normal and tumor cell systems by examining cell clones, lines, and tissues. For example, rapid phenotypic shifts, termed phenotypic drift (118), have been documented in the biochemical and biological properties of mammary tumor cells as well as in their sensitivities to radiation, drugs, and hyperthermia (118-123).

Quantitative phenotypic drift in the amounts of specific proteins, enzymes, and antigens in normal and tumor cells have been found by examining cell-to-cell variability in isolated cells and in tissues (for review see Ref. 124). For example, the rate of appearance of quantitative variants in albumin production in rat hepatoma cells was $~10^{-5}$ variants/cell/generation. Similar rates were found for a variety of hepatoma enzymes. Peterson et al. (31) examined the rates of significant quantitative variation in the single cell expression of a high molecular weight surface glycoprotein on normal breast epithelial cells and on breast carcinoma cells. The rates of quantitative variation were $~6$ times higher (mean, $~2.2 \times 10^{-2}$ variants/cell/generation) on malignant breast cells than on normal breast epithelial cells (mean, $~0.36 \times 10^{-2}$ variants/cell/generation). Peterson (124) found that the cell-to-cell variability in enzyme activities and other components conforms approximately to a geometric series, in which the consecutive values vary by a factor of $\sqrt{2}$. To explain this series he postulated that variations in transcriptional control occur because of changes in chromosome number,
gene amplification, or alterations in some regulatory genes. However, these changes may not occur at rates high enough to explain the rapid rates of formation of unstable tumor cell phenotypic variants. There are a variety of other possible mechanisms (listed in Table 1) for generating cellular diversity; some will be discussed briefly below.

Phenotypic drift and the rapid production of variants has allowed the isolation of phenotypic revertants from highly malignant cell populations, such as the phenotypic revertants obtained from cloned tumor cells growing in semisolid agar (125, 126). Kerbel (127) isolated and examined several low metastatic potential variants of a mouse carcinoma. The rapid phenotypic drift of such low metastatic variant cells back to the metastatic phenotype was assessed by Dennis et al. (128) using a wheat germ agglutinin-resistant subclone. Cells that quickly reverted to the metastatic phenotype in vivo were also changed in their lectin sensitivities. This phenotypic shift appeared to occur at a rate of $\sim 2 \times 10^{-5}$ revertants/cell/generation (129).

Rapid phenotypic drift of tumor cells has been documented in a number of systems (130–133). Bosslet and Shirrmacher (134) examined the rapid phenotypic changes in vivo of the ESb murine lymphoma. They estimated that these tumor cells became resistant to host cytotoxic T-cells at a frequency of $\sim 10^{-3}$ variants/cell/generation. "Phenotypic drift" (118) and "dynamic heterogeneity" (135) models were proposed to explain such rapid shifts in metastatic behaviors of malignant cells. These models are basically similar in that they attempt to explain the rapid rates of generation of cell variants and their even more rapid reversion rates. Young and Hill (136) examined the rates of phenotypic variation of murine sarcoma and melanoma cell lines from nonmetastatic to metastatic and from metastatic back to nonmetastatic cells and estimated rates as high as $10^{-3}$ to $10^{-4}$ and $10^{-2}$ to $10^{-1}$ variants/cell/generation, respectively, for these processes. They also found that soon after lung colonization the metastatic nodules contained higher frequencies of metastatic cells, but further growth resulted in a rapid decline in the average metastatic efficiency of individual cells. Metastatic cells proliferating at secondary sites rapidly diversify, creating heterogeneous subpopulations that include nonmetastatic cells (137), and with time, these lesions may stabilize in their rates of metastatic variant formation and evolve with decreasing fractions of highly metastatic cells. Not all metastatic cell systems appear to be, however, as unstable as the animal tumor systems described above (138–140).

It should be emphasized that phenotypic variability in cellular populations is a normal cell attribute that is probably necessary for ensuring some cellular diversity and tissue adaptability. In tumors, however, such variability may be accentuated beyond that required for normal tissue adaptation.

Modulation of Phenotypic Instability

Tumor cell phenotypic drift and diversification can be modulated by a variety of different mechanisms. One of the most interesting, described briefly above, involves modulation by cell-cell interactions within tumors (23). Poste et al. (27) found that by mixing several clonal populations of murine melanoma cells, stabilization in their metastatic properties occurred, and their normally rapid rate of phenotypic (metastatic) diversification was decreased. Similarly, Miner et al. (28), using a highly metastatic brain meninges-colonizing melanoma line, found that clonal isolates of this line were unstable and quickly lost their brain-metastatic properties, unless the clones (at least three) were mixed and allowed to grow together as a triclonal population. In contrast, mixing three of the same clonal cell subpopulations just before injection into animals failed to stabilize their brain colonization properties. Similar results were obtained when the expression of a $M_1$, $\sim 90,000$ cell surface glycoprotein was monitored in the same way. The polyclonal mixture stabilized the expression of this component in parallel with the biological properties of the cells (28). The $M_1$, $\sim 90,000$ glycoprotein expression is interesting because this component appears to be similar or identical to a growth factor receptor (transferrin receptor) found at high concentrations on brain-metastasizing melanoma cells (141). The expression of this receptor may allow the melanoma cells to respond to organ microenvironmental conditions. The nature of the polyclonal stabilization mechanism remains unclear, although it has been suggested that cell-to-cell signals may be exchanged in the polyclonal cell populations (142).

Cellular interactions are important in regulating development and in stabilizing normal tissues. These include the formation of cell junctions and contacts and interactions with extracellular matrix and tissue stroma (Table 1). When cells are transformed, contact-mediated cell communication systems are altered (9, 143, 144). Similar alterations have been reversibly induced by treatment with tumor promoters (144), agents that can also stimulate diversification of lymphoma cells (145). Recently we found that highly metastatic mammary epithelial cells fail to communicate via cell junctions, while counterpart benign or normal epithelial cells are tightly coupled, unless they have been treated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. In other studies the role of the src oncogene in modifying cell-cell communication in v-src-transfected NIH/3T3 cells indicates that the src gene product increases protein kinase C activity and decreases gap junction-mediated cell communication (146). These results suggest that cell-cell junctional communication may be one of the processes involved in stabilizing the generation of cellular diversity, and they could explain the high rates of phenotypic diversification seen when cells are dispersed and allowed to proliferate as separate cell clones (9, 125, 126, 147). Rubin (9) has reviewed the effects of disrupting normal cell spatial arrangements and their interactions on cellular diversification. He concludes that tumor as well as normal cell heterogeneity can arise from perturbations in the spatial organizational controls that maintain normal tissue architecture and cellular diversity.

Modulation of phenotypic stability can also occur when tumor cells fuse with normal cells (148). Although thought to be relatively rare, such events can lead to tumor suppression or to progression and the emergence of metastatic cells. As an example of the progressive effects of cell fusion, DeBaetselier et al. (149) found that hybridization of nonmetastatic mouse mammary adenocarcinoma cells with normal mouse B cells resulted in cell hybrids that were highly metastatic to liver and spleen. The emergence of highly metastatic cells in a population of lectin-resistant low metastatic cells during in vivo growth was proposed by Legarde et al. (150) to be due to cell fusion followed by extensive chromosome segregation. In addition, Larizza and Shirrmacher (151) have proposed that the progression of the Eb murine lymphoma to highly metastatic cells may be due to the fusion of an Eb cell with a macrophage and then by extensive segregation and loss of chromosomes. Although such phenotypic changes probably require genotypic alterations, when they occur in vivo, they are probably initiated by events in the tumor cell microenvironment.

*G. L. Nicolson, K. Dulske, and J. E. Trosko manuscript in preparation.*
Another mechanism for rapidly generating phenotypic change is via nonmutational DNA modifications that alter gene expression (152, 153). For example, DNA hypomethylation can result in activation of specific genes (154-156), and these expression changes can persist for several cell generations before reversion is complete (152, 153). Using the nucleoside analogue 5-aza-C, Jones and Taylor (155) demonstrated that hypomethylation of cytosine residues can cause rapid phenotypic changes in cells, but the new phenotypes were unstable and eventually reverted in the absence of the drug. Kerbel et al. (153) used 5-aza-C to modulate the tumorigenic and metastatic properties of several murine tumor cell lines, and Olsson and Forschhammer (157) found that 5-aza-C induced the metastatic phenotype concomitant with the expression of a cell surface antigen associated with metastasis. The DNA methylation levels of murine melanoma cells treated with 5-aza-C have been correlated with acquisition and loss of experimental but not spontaneous metastatic properties (158). It is argued that such drugs are nonmutagenic; however, under certain conditions 5-aza-C might act by genetic rather than epigenetic mechanisms. Such phenotypic changes can also be induced in vivo. Liteplo et al. (159) induced enzyme activity in an enzyme-deficient tumor cell line by administration of 5-aza-2'-deoxycytidine to tumor-bearing mice.

Comparisons of the average methylcytosine contents of DNA of cells from benign and malignant or primary and secondary tumors have been inconclusive. Although examination of the methylation state of selected genes of colon carcinoma cells revealed that the malignant cells contained less methylated DNA than benign cells (160), this is not always found (161). When Omerod et al. (162) examined the relationship of DNA methylation levels to the metastatic capacity of a series of murine and human melanoma cell lines, no correlation was detected.

In addition to DNA modifications, there exists a variety of postgenetic processes that could be important in regulating the synthesis and activity of gene products. Among these are regulation by transcriptional, posttranscriptional, translational, and posttranslational controlling mechanisms (15, 40). Unfortunately, little is known about the possible role of these mechanisms in generating phenotypic instability and diversification.

It should be obvious that there are several possible mechanisms by which tumor cell phenotypic instability can be altered or modulated. Unfortunately, it is virtually impossible to predict with any degree of certainty the underlying source of the instability of an individual tumor cell.

Differential gene expression has been examined in tumor cells by hybridization of expressed mRNA to gene libraries. For example, Shiosaka and Saunders (163) used nonfetal complementary DNA clones to study differential gene expression between chronic lymphocytic leukemia and normal leukocytes. They found a series of genes that were differentially expressed in the leukemia cells. Similarly, Mars et al. (164) described sequences that were preferentially expressed in chronic myelogenous leukemia. Differential gene expression has also been correlated with the metastatic phenotype in large cell lymphoma (165) and colorectal cancer (166).

When cancers are treated therapeutically, the treatments themselves can cause dramatic phenotypic changes in the surviving tumor cell population. In addition to the known gene mutagenic and amplification effects of many therapeutic agents (71, 153), they may also stimulate phenotypic diversification by epigenetic mechanisms. Cytotoxic drugs can deplete polyclonal cell populations of drug-sensitive cells to yield surviving cells that are highly unstable phenotypically. These unstable cells can quickly diversify to yield heterogeneous cell populations, and some but certainly not all of these rapidly diversifying cells may be restabilized by polyclonal cellular interactions (27, 28). DNA methylation may play a role in this process, because it is known that low concentrations of carcinogens and chemotherapeutic agents can cause hypomethylation of cellular DNA (153, 167). Therapeutic agents may also cause other changes in tumor cells (some of which are listed in Table 1) that result in modulation of cellular phenotype and diversification mechanisms. That surviving tumor cells might be more unstable phenotypically and diversify at rapid rates adds to the complex problems facing clinical oncologists (16, 19, 168-170).

Effects of the Microenvironment

The microenvironments of tumor cells are important in determining their phenotypic stabilities (15, 40, 171, 172). Slight differences in microenvironment may induce events that lead to zonal differences within tumors. In addition to some of the cellular interactions described above, differences in tissue innervation, stroma, nutrients, growth factors, oxygen, hormones, enzymes, inducers, ions, and other possible tumor regulators may play important roles in destabilizing tumor cells and determining their susceptibilities to genetic and epigenetic changes (Fig. 2).

One of the more important types of interactions that occurs in tissues is the interaction of cells with extracellular matrix and stroma. The extracellular microenvironment of tumors is determined by the matrix synthesized by normal and tumor cells, as well as host stromal components secreted by surrounding or infiltrating host fibroblasts and other normal cells. Modifications in the extracellular matrix and stroma can lead to alterations in gene expression and cell phenotype (173-175). The differing influences of extracellular elements synthesized by parenchymal cells, endothelial cells, mesothelial cells, fibroblasts, and other host cells capable of nonimmune "host reactions" on tumor phenotype may be important in modulating states of "differentiation" in tumors (40, 175, 176). Although neoplastic cells are often altered in their response to extracellular matrix and stroma, they can be partially or differentially responsive to such microenvironmental constituents.

The tumor microenvironment is also accessible to soluble differentiation inducers or modulators, hormones, and growth factors. Although differences in tumor cell responsiveness to and intratumoral concentrations of such molecules could lead to heterogeneous cellular responses to such agents, tumor cells are thought to be at least partially responsive to these environmental agents. In fact, certain malignant cells can be reverted to more benign or normal phenotypes by soluble molecules and cell fields of syngeneic embryos. After implantation of mouse embryonal carcinoma cells into mouse blastocysts, they can develop into phenotypically normal cells and tissues (177, 178). The regulation of embryonal carcinoma malignancy was tissue specific and dependent on the close correspondence of the malignant cell with the proper embryonic field (179). Subsequently, it was determined that malignant cell contact with the trophectoderm and the presence of blastocoele fluid were both required (180). Not all malignant cells, however, can be controlled by the embryonic environment. A notable exception was mouse melanoma cells, which could not be regulated by the blastocyst environment and continued to grow into melanoma lesions (178). Gerschensen et al. (181) have now been able to decrease significantly the formation of melanoma tumors in...
mouse embryos, but only if they are injected into the embryonic skin at precisely the time (10 days) when premelanocytes migrate into the skin. Injection of the melanoma cells at later times was much less effective. When conditioned medium from embryonic skin cultures was incubated with the melanoma cells, they were growth inhibited, appeared morphologically more differentiated, and failed to reinitiate growth in melanoma growth medium. In the leukemias it has been proposed that changes in malignant phenotype can be induced by environmental signals that cause differentiation to more normal phenotypes (182). Sachs (183) has proposed that as tumors progress, they shift from inducible pathways of gene expression to constitutive pathways. This could explain the increasing nonresponsiveness of progressing malignant cells to their microenvironments.

In addition to the microenvironmental signals received by tumor cells at primary sites, there is also evidence that such signals may differ at secondary sites. Among these are tissue-associated soluble growth factors that could be important in the differential proliferation of metastatic cells at specific organ sites (184–186).

Tumors are often extensively infiltrated by normal host cells, such as lymphocytes, granulocytes, mast cells, and macrophages. When these normal cells are in proximity to neoplastic cells, they can have profound effects on tumor cell growth and other properties. Under certain conditions, infiltrating host cells may kill or inhibit tumor growth, but under other conditions, they can stimulate growth (2, 5, 21). The effects of normal infiltrating cells on mammary tumor cells have been examined by Yamashina et al. (187). They found that activated macrophages released mutagenic substances, such as peroxides, that quickly induced the formation of drug-resistant variants in a tumor cell population. Similar events in situ may enhance tumor diversification by genetic and epigenetic mechanisms.

It is expected that tumor microenvironment is extremely important in modulating gene expression and phenotypic properties of cells. Slight differences in microenvironment could have profound effects on cellular phenotypic diversity, especially if tumor cells are differentially responsive to their microenvironments. Although advanced tumors may lose their responsiveness to a variety of microenvironmental signals, this probably does not occur equivalently or simultaneously among tumor cells, resulting in further cellular diversity.

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Fig. 2. Intratumoral microenvironmental influences on malignant cell phenotype. Soluble components, extracellular matrix, tumor and host cells, and other factors can differentially influence tumor cell phenotypic properties [modified from Nicolson (40)]. LAK, lymphokine-activated killer; NK, natural killer.
Models for Tumor Diversification

The underlying causes in the generation of phenotypic diversification of malignant neoplasms may be unique to each tumor and its host environment. However, there are certain similarities with the somatic generation of diversity seen in normal cells during development. One interesting model for this process is the diversification of normal resting lymphocytes in response to foreign antigens (15, 40). The antigen-stimulated diversification of lymphocyte stem cells can result in a diverse set of mature immunoglobulin-producing B-cells, each synthesizing a unique immunoglobulin molecule (188). Secreted immunoglobulin molecules, as well as probably many other cell surface molecules, are encoded by multigene families that are separated from each other and must undergo rearrangements to form single, contiguous genes before they can be translated (189). The generation of immunoglobulin molecular diversity occurs at the genetic level where hypermutable regions occur in the immunoglobulin genes and at the combinational level where different joining mechanisms are used to put together the genes that code for various immunoglobulin homology units. This combination of hypermutation and different joining mechanisms allows for rapid molecular diversification of the immunoglobulin molecules during an immune response (189). Hood et al. (188) have speculated that the generation of diversity of cell surface developmental and recognition molecules (called “area-code” molecules) can be explained by this type of diversification model. Indeed, such a model has also been proposed to explain one of the possible mechanisms of diversification of tumor cell phenotypes (15, 40).

During B-cell diversification, the activation of specific variable gene segments occurs when they are rearranged near a transcriptional enhancer element that is located upstream from the constant region gene segments. This model provides an interesting mechanism for the activation of normally quiescent genes. In tumor cells, the relationship of enhancer elements to gene rearrangements and transcriptional activation has been studied in virus-transformed systems in which the integration of a transcriptional promoter or enhancer element adjacent to a c-onc gene results in enhanced oncogene transcription and maintenance of the transformed phenotype (190). The activation of c-onc genes could also occur when these are translocated into a locus of active area code genes, such as in human non-Hodgkin’s lymphomas in which the c-myc gene is recombined into the immunoglobulin gene locus (191). Alternatively, enhanced expression of cellular oncogenes could occur by accumulation of mutations in regulatory regions not associated with genetic translocations, or they could regulate their own transcription after translocation by escaping gene repression mechanisms (192).

Although the use of developmentally regulated gene rearrangements to create diverse sets of complete genes from a limited number of inherited gene segments could be important in the generation of tumor phenotypic diversification, this is probably only one of several possible mechanisms responsible for tumor diversification and progression. In the example cited above, cellular oncogene insertion or translocation near enhancer elements in the host genome could result in the misuse of developmentally regulated, cellular enhancer elements, resulting ultimately in the activation of multigene families that could control phenotypic diversification. Such activation would not then require enhanced expression of oncogenes during malignant progression, consistent with recent results (87–91). Alternatively, the changes associated with progression and malignancy may not require dramatic modifications in oncogenes, area code genes, or their regulation. Acquisition of the metastatic phenotype, for example, may depend only on “turning up,” not “turning on,” of the specific genes required for this process (165, 193). Tumor progression then would be characterized mainly by quantitative, not qualitative, changes in gene expression, and the role of qualitative changes, such as genotypic alterations, might be to circumvent normal growth and differentiation controls and enhance developmental diversification mechanisms that lead to quantitative differences in gene expression among tumor cells. This would explain the dynamic heterogeneity or rapid phenotypic drift seen in metastatic cell populations, an event that occurs much too quickly to be explained by genotypic changes in a minor cell subpopulation.

Most, if not all, malignancy-associated gene products are probably also important during certain normal stages of development (1, 8, 9). Thus, virtually all of the cellular characteristics of malignant neoplasms may be encoded by normal, not cancer, genes (193). The reappearance of fetal characteristics and gene products (1) may also be a reflection of quantitative changes in gene expression, if these gene programs were previously operating at basal levels in rapidly proliferating tumor or even normal adult cells. Since the genes that control embryonic cellular growth, diversification, differentiation, and migration may be intimately linked in the genome, their quantitative variation in expression may ultimately ensure that cells will arise with the proper “embryonic” characteristics for their malignant behavior.

Summary

It is proposed that tumor cell instability and the expression of cellular diversification mechanisms ensure that malignant neoplasms contain heterogeneous, phenotypically diverse tumor cell subpopulations. In such potentially unstable cellular mixtures of tumor cell phenotypes, some malignant cells may ultimately evolve with the most favorable properties for their progression to metastatic cells. Rates of cellular phenotypic instability and phenotypic diversification as well as their underlying causes appear to vary greatly among different tumor cells, and they are probably modulated by further genetic and chromosome changes and more frequently by intra- and extracellular epigenetic events that also differ, depending on the nature of the tumor cells and their cellular and microenvironmental interactions. Diversified malignant cells are characterized by quantitative and perhaps a few qualitative differences in gene expression, which may explain their abilities to undergo rapid changes in phenotypic properties. As tumor diversification and selection proceed uniquely in vivo, highly malignant cell subpopulations may eventually become dominant and gradually and independently lose their cellular and microenvironmental responsiveness. Tumor cell diversification mechanisms may be similar or identical to normal developmentally regulated diversification mechanisms that are used during embryonic and postembryonic cell diversification and development.

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References


Tumor Cell Instability, Diversification, and Progression to the Metastatic Phenotype: From Oncogene to Oncofetal Expression

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