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Tumor Stem Cells and Malignant Cells, One and the Same

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ABSTRACT

Cancer is a proliferative, invasive, and metastatic disease often caused by repeated tissue insults resulting in accumulation of genetic abnormalities that rarely produce malignant cells. The survival of mouse L1210 leukemia was determined for inoculations of 1 cell up to 10^6 cells. The survival times varied in a log-linear manner with the inoculum cell number from 19 days with 1 cell to 7 days with 10^6 cells implanted. In preclinical tumor models or in patients, tumor nodules of 10^8 – 10^9 cells are advanced cancer. Malignant cells frequently secrete growth modulatory substances that regulate their growth and alter growth of normal cells. Whether the metastatic malignant cell is the same or significantly different from the primary lesion malignant cell remains a topic of active investigation. Reaching a detectable lesion takes 10 years. Genetic instability produces variants in the primary tumor and metastases that are more heterogeneous than the early disease. The argument that cancer arises only from the tissue stem cell populations and that cancer stem cells comprise perhaps 1 in 100,000 or 1 in 10,000 cells within the tumor leads to the notion that agents that selectively kill cancer stem cells will not decrease the tumor mass. The cells that initiate, sustain, and populate cancers are malignant cells. Cancer stem cell notion is useful if it leads to important research questions and to better therapeutics.

Key Words: Colony forming units, Malignant cells, Genetic instability, Metastasis, L1210 leukemia

INTRODUCTION

Cancer is a proliferative, invasive, and metastatic disease that is frequently caused by repeated insults to a tissue resulting in accumulation of genetic abnormalities that, by rare chance, produce a malignant cell. Cancer cells are genetically aberrant and instable. Some cancers begin as a single clone (and a few remain clonal) and other arise from a field of repeatedly damaged cells. The search for an understanding of cancer and for the key as to how to control and ablate malignant disease often returns to the remarkable processes of normal tissue/embryo development and normal tissue repair. The “well-behaved” proliferative and self-limiting biology of wound repair, gut lining replacement, liver regeneration, skin renewal, and bone marrow generation of hematopoietic cells has taught us that cell proliferation and differentiation are a constant process in complex organisms and are well-controlled under normal circumstances.

The concept of a stem cell was put forth by Till and McCulloch to describe the ability of a single mouse bone marrow cell to produce a colony of cells in the mouse spleen and later to describe the

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ability of similar single bone marrow cells to give to colonies of varied types in cell culture (1, 2). A colony-forming unit (CFU) is an individual cell that is able to clone itself into a colony of identical cells. A CFU is a measure of viable bacterial numbers or a measure of viable mammalian malignant cells in a culture. In reconstituting the immune system of lethally irradiated mice, bone marrow cells from syngeneic donors are intravenously injected into the recipient animals and colonies form in the spleen. Each colony is the progeny of a pluripotent stem cell; therefore, the number of colonies is a measure of the number of stem cells. These findings led to the notion that cancer can arise from multiply insulted cells that by rare chance have aberrantly turned on genes that normally are expressed only by normal tissue “stem” cells. Thus, cancer cells have aberrantly reverted to a dedifferentiated proliferative state. These malignant cells are trying to build a tissue but they are abnormal and lethal. Indeed, an area of therapeutic investigation has a goal to discover agents that can terminally differentiate malignant cells to a quiescent nonproliferative state.

EARLY OBSERVATIONS

An interesting aspect of the current cancer stem cell debate regards the number of human tumor cells required to initiate the growth of a subcutaneous nodule in immunodeficient mice. A very large number of variables would need to be optimized to achieve reliable data from such observations. A historical perspective looking at syngeneic mouse tumors may help. The L1210 and P388 mouse leukemias were developed in 1948 and 1955, respectively (3–5). L1210 and P388 leukemias were both chemically induced in a DBA/2 mouse by painting the skin with methylcholanthrene. The leukemias have been propagated in DBA/2 mice by implanting intraperitoneally 0.1 mL of a diluted ascetic fluid containing either 10^5 L1210 cells or 10^6 P388 cells. These mouse leukemias were the first tumors used for large-scale drug discovery screening programs by the national drug development program instituted in 1954 by Congress, which directed the National Cancer Institute to start a program. The Cancer Chemotherapy National Service Center (CCNSC) screen consisted of three mouse tumors: L1201 leukemia, SA-180 sarcoma, and mammary adenocarcinoma 755 (6). Over the years, the primary screen varied from the original three tumors to L1210 plus two arbitrarily selected tumors to L1210 plus Walker 256 carcinosarcoma to L1210 plus P388 leukemia to L1210 plus B16 melanoma or Lewis lung carcinoma. In 1976, a change occurred in the NCI primary screen. The new screen included a panel of colon, breast, and lung tumor models (mouse and human); however, compounds were initially screened in P388 leukemia (7).

Skipper and Schabel and colleagues explored the growth characteristics of the L1210 and P388 leukemias in mice (8, 9). The testing was conducted in a hybrid of DBA/2 hosts. Tumor cell implant sites were intraperitoneal injection, subcutaneous implant, intravenous injection, and intracranial injection. For L1210 leukemia with an inoculum of 10^5 cells, the mean days of survival and tumor cell doubling times for these implant sites were 8.8, 9.9, 6.4, and 7.0 days and 0.34, 0.46, 0.45, and 0.37 days, respectively (Fig. 1). The mean survival times of mice implanted with L1210 cells by these various routes was determined for inoculations of 1 cell up to 10^6 cells. The survival times varied in a log-linear manner with the inoculum cell number. Thus, when the mice were implanted with 1 L1210 cell by intraperitoneal injection, they survived 19 days and when the mice were implanted with 10^6 L1210 cells intraperitoneally, they survived 7 days (Fig. 1). Similar studies were conducted with P388 leukemia. For P388 leukemia (10^6 cells), the mean days of survival and the tumor doubling times for the same implant sites were 10.3, 13.0, 8.0, and 8.0 days and 0.44, 0.52, 0.68, and 0.63 days, respectively. From these studies, it must be concluded that every L1210 and P388 cell is a cancer stem cell.

Skipper and Schabel applied similar analyses to solid tumors especially the mouse Ridgway osteogenic sarcoma (10). In preclinical tumor models or in patients, tumor nodules of 10^8 – 10^9 cells are

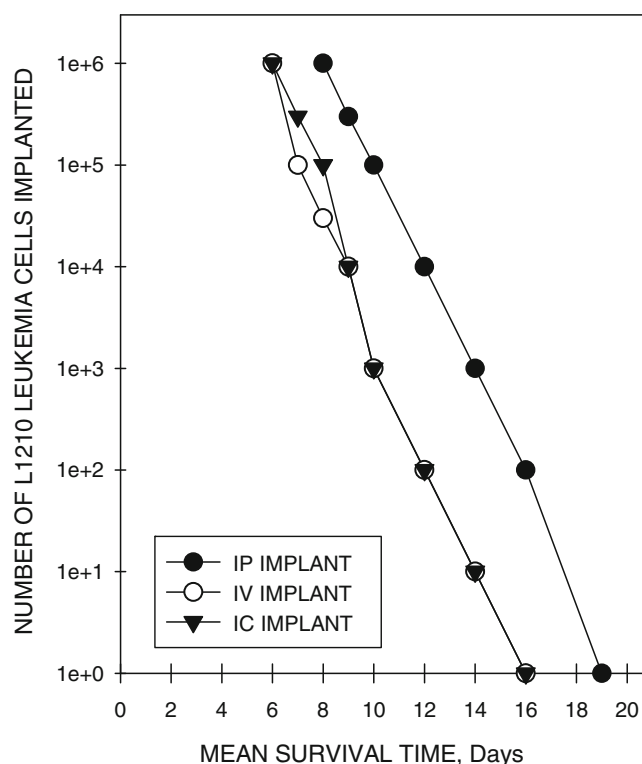


Fig. 1. Mean survival times of mice inoculated with various numbers of murine L1210 leukemia cells injected intraperitoneally, intravenously, or intracranially. These data form the basis for the in vivo bioassay method for determining the number of L1210 cells surviving after treatment of L1210 tumor-bearing mice with therapy. From these survival curves, it was determined that from: (1) intraperitoneal inoculation of L1210 cell-generation time = 0.55 days; the lethal number of L1210 cells = 1.5×10^9 ; (2) intravenous inoculation the L1210 cell-generation time = 0.43 days; and (3) intracranial inoculation the L1210 cell-generation time = 0.46 days (8,9).

advanced cancer (Fig. 2) (10, 11). One source of variability in the response of drug-sensitive tumor cells to a drug is the heterogeneity of the blood supply such that the drug does not reach the tumor cells distal from the blood supply in sufficient concentration to be lethal. Thus, the pharmacokinetics and concentration of a drug required to kill tumor cells distal from vasculature should be documented. In addition, the physiologic heterogeneity of tumor masses as a source of varied treatment response, Skipper and Schabel considered the heterogeneity of tumor stem cells, defined as cells capable of unlimited proliferative thrust, caused by the inherent genetic instability of malignant cells to be a source of variable treatment response. Skipper and Schabel considered various types of tumor stem cells that might account for fluctuation in response to chemotherapy in similarly treated individuals bearing a specific cancer, and classifications of cancers by chemotherapeutic effect. Fluctuating ratios of treatment responsive to treatment resistant stem cells, as predicted by the mutation theory, could account for one patient responding to a drug and the next not responding. Differences in tumor growth fraction and differences in tumor distribution into pharmacologic sanctuaries could also strongly influence a patient's response to therapy. Treatment resistant stem cells are primarily responsible for the failure of the best available chemotherapy to cure responsive, refractory, and very refractory experimental neoplasms. These data examined suggest that differences in the resistant to responsive stem cell ratios in different types of cancer may account for their being classified as responsive, refractory, or very refractory (12).

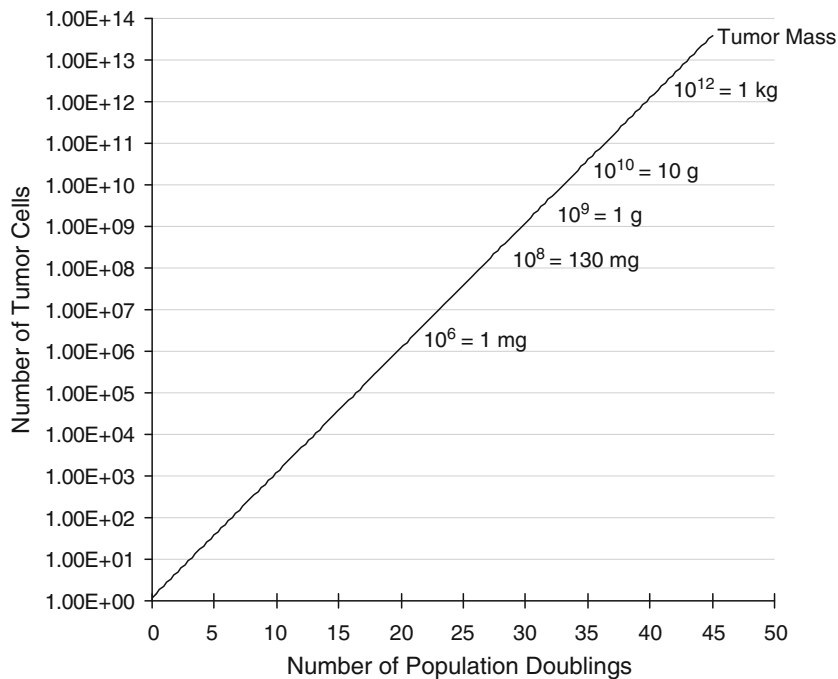


Fig. 2. Tumor cell numbers and weight of the tumor mass are shown. In patients, tumors are advanced at first presentation or at recurrence after initial noncurative therapy (10,11).

TUMOR CELL HETEROGENEITY

Experience with the heterogeneous response of well-controlled preclinical tumor models grown in inbred strain of mice led investigators in the mid-1980s to believe that the assumption that there should be a common pattern of cellular heterogeneity for histologically identical types of cancer was not warranted (13). Although malignant disease may develop from a single transformed cell, even in tumors where the single cell has diversified to heterogeneous cell phenotypes, evidence of a clonal origin still exists (14). Although Foulds concluded that tumor evolution (progression) is characterized by permanent, irreversible changes, we recognize today that cells remain very plastic and adaptable and can often modulate their biology to changes in the microenvironment (15). During molecular progression of tumors, neoplastic cells accumulate increasing genetic alterations that are generated by mutational events, genetic instability (16, 17). Tumor cell genetic instability ensures that malignant disease contains heterogeneous, phenotypically diverse tumor subpopulations (14). Tumor cell diversification mechanisms may be similar or identical to normal development during embryonic and postembryonic diversification and development. Tumor cell subpopulations can influence the properties of other subpopulations in the tumor including proliferation, sensitivity to drugs, immunogenicity, and metastatic potential (14, 18–20).

Understanding the biology of malignant cells (cancer stem cells) that allows them to escape the constraints that normally regulate cell growth and differentiate is critical. Malignant cells frequently secrete growth modulatory substances that regulate their own growth (autocrine) and/or alter the growth of normal cells (paracrine) in the vicinity of the malignancy (Fig. 3) (21). A malignant tumor whose growth depends upon the release of autocrine and paracrine growth factors may be vulnerable to treatment with specific receptor antagonists or growth factor neutralizing antibodies.

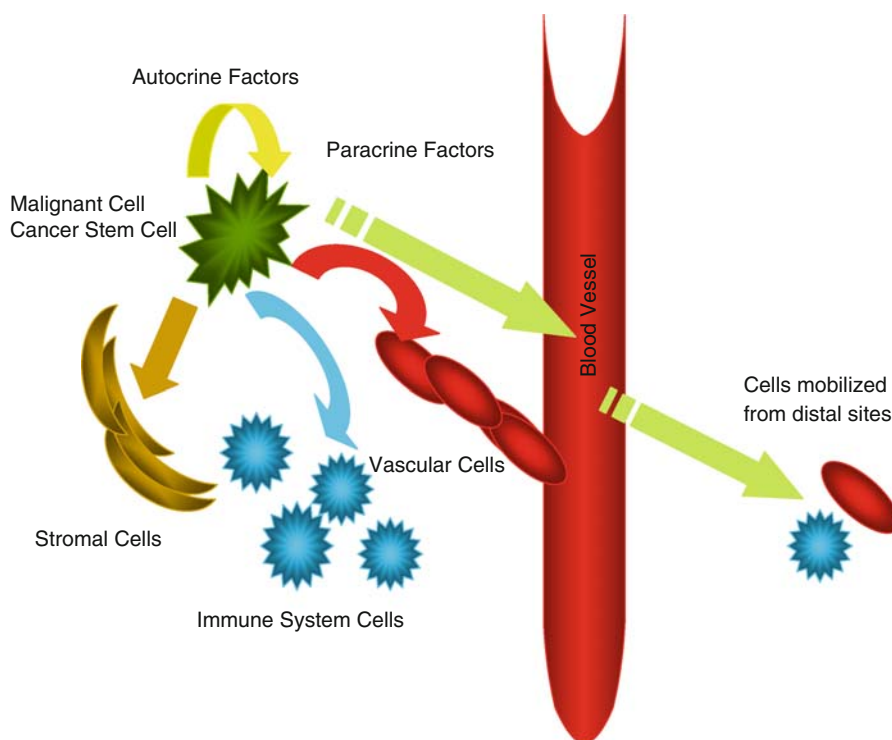


Fig. 3. Malignant cells can have abscopal effects on the host through secretion of paracrine factors and can produce autocrine factors that can sustain proliferation of the malignancy.

HEMATOPOIESIS AS A MODEL

Analogies for the development of malignancy have been sought in the processes of normal aging and in the differentiation of cells in the hematopoietic system (22, 23). The incidence of many cancers increase with age because of increased probability of DNA changes that may allow occurrence of a malignant cell and because some of the alterations associated with normal aging increase the susceptibility of cells to carcinogenic events. In normal aging, there is a decrease in DNA repair capacity and a decline in cellular immune reactivity that could contribute to permitting malignant growth (22). Normal hematopoiesis, the formation of the many cell types in blood, is a process of development, self-renewal through mitosis, and differentiation of hematopoietic stem cells, the source cell of all blood cell lineages (24). Because most blood cells have relatively short lifespans, hematopoietic stem cells continuously replicate themselves through self-renewal to prevent depletion of the stem cell pool while simultaneously differentiating into multiple lineages of the varied blood cell types. The fate choice of hematopoietic cells to either self-renew or differentiate is controlled by intrinsic mechanisms and extrinsic signals from the environment or the stem cell niche (25). In adults, the hematopoietic stem cell number is relatively constant under normal conditions. Bone marrow hematopoietic stem cells appear quiescent; however, the majority divide regularly as shown by their slow constant incorporation of radio-labeled nucleotides (26, 27). There are two proposed mechanisms by which asymmetric cell division may be achieved called divisional asymmetry and environmental asymmetry. In divisional asymmetry, specific cell fate determinants in the genome, RNA, and proteins are distributed unequally during cell division. After cell division, only one daughter cell receives the determinants, thus retaining the hematopoietic stem cell fate while the other daughter differentiates.

In environmental asymmetry, one hematopoietic stem cell niche and retains the stem cell identity, while the other enters a different environment favoring its differentiation (24).

The first human leukemia-lymphoma cell lines were Burkitt's lymphoma lines developed in 1963 (28). The most widely used of these lines is the Raji Burkitt's lymphoma (29). The term "cell line" indicates that the population of cells grew continuously in culture (to this day); therefore, the cells were "immortal" or capable of continuous self-renewal. Some the leukemia-lymphoma cell lines were presumed to be monoclonal that is derived from one malignant cell and that the current multiclonal lines emerged during extended culture. The differentiation of the cells arrested at a discrete stage during maturation of the lineage. The early cell lines were grown autonomously in basic nutrient media supplemented with fetal calf (or other serum) serum independent of external growth factors, although, in some cases, exogenously added growth factors could stimulate proliferation. Later cell lines were selected to be growth factor dependent (30). Nearly all of the established leukemia and lymphoma cell lines are genetically abnormal. Of 429 lines analyzed only two had normal karyotypes (31). In addition to gross cytogenetic alterations, many of the lines harbor point mutations deletions and amplifications of specific genes (32). However, despite the evidence of genetic instability and abnormality, these cell lines have remained "stable" in long-term culture for nearly 50 years.

An interesting case is the impact of stem cell "dose" on hematopoietic recovery in autologous blood stem cell recipients (23). Hematopoietic cells collected from blood and reinfused into patients following high-dose chemotherapy are generally termed "stem cells," even though the population of cells contains true stem cells and differentiated progenitor cells (33, 34). Mobilized blood stem cell quantity, identified by expression of CD34, may be the strongest predictor of days to hematopoietic recovery (i.e., platelets and neutrophils) in autologous blood stem cell recipients (35). The majority of patients will recover if a stem cell dose of $\geq 5 \times 10^6/\text{kg}$ stem cells are infused, which corresponds to 3.5×10^8 cells (36). Bone marrow engraftment is the result of early undifferentiated stem and progenitor cell self-renewal and differentiation. The rate of engraftment is dependent, in part, on the number and type of stem cells infused. Using a genetically engineered model cell to represent human leukemia stem cells, Hope et al. tracked these human leukemia stem cells in SCID mice and found that the leukemia stem cells were not functionally homogeneous (37). Like normal hematopoietic stem cells, some human leukemia stem cells divided rarely and underwent self-renewal rather than commitment after cell division and others demonstrated heterogeneity in self-renewal potential.

METASTASIS

The detection of circulating tumor cells in the blood and lymph system and micrometastases has been investigated through multiple eras of cancer research because of the potential importance of these cells to prognosis and therapeutic approaches (38). The first publication of circulating tumor cells was in 1869 when Ashworth reported cancer cells in the blood of a patient at autopsy (39). In the decade 1955–1965, 5,000 cancer patients were tested for circulating tumor cells using 20 different cytological methods; however, it was finally realized that the tests used were not sufficiently discriminatory (40). When immunohistochemical detection methods were developed and more recently with the development of PCR techniques, interest in documenting a connection between circulating tumor cell numbers and stage of disease and in detecting occult metastases renewed (41–43). Although the accuracy of methods for detecting tumor cells in circulation has markedly improved over the years, the application of these data to determination of the most appropriate treatment regimen for individual patients has yet to occur. It is not clear whether circulating tumor cells are enriched for cancer "stem cells," malignant cells that can initiate metastasis or whether circulating tumor cells reflect the quality of the primary lesion vasculature or other properties of the malignant disease (44, 45). There is no doubt that malignant cells must be plastic and adaptive to altering environments and stress. The capacity

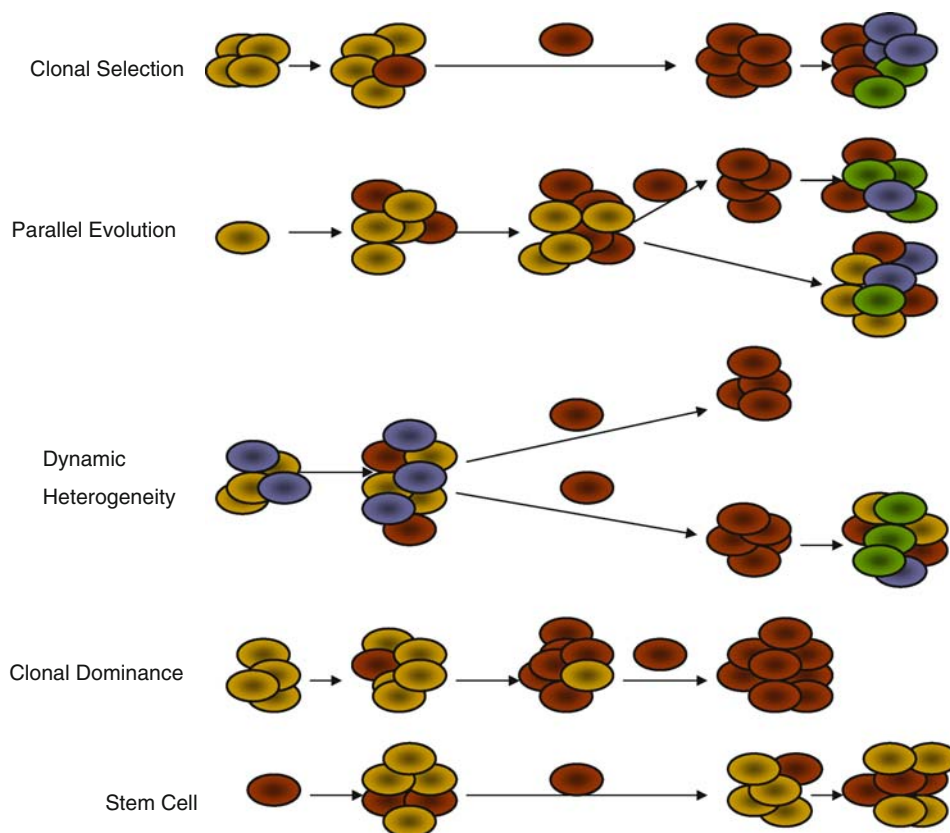


Fig. 4. The various patterns of cell division that can lead to the cellular heterogeneity found in most malignant disease.

of some malignant cells to make epithelial to mesenchymal transition and then return to an epithelial phenotype is an example of the plasticity of malignant cells (44). During wound healing processes normal cells can undergo these changes as well.

Metastasis is a critical characteristic of malignant disease (45) (Fig. 4). The placement of a cancer stem cell in the metastatic process and understanding whether the metastatic malignant cell is the same or significantly different from the malignant cell of the primary lesion remains a topic of active investigation. The clonal selection hypothesis is that cell populations with metastatic capacity are subpopulations within the primary lesion. The parallel evolution model theorizes that metastasis occurs early in malignant disease and evolves along a different path than the primary lesion. The dynamic heterogeneity hypothesis indicates that metastatic variants arise within the primary tumor, and these variants spread and evolve further at secondary sites. The notion of clonal dominance is that more virulent metastatic subclones occur within the primary tumor and outgrow the original malignant cells and eventually dominate the primary lesion and metastases. The stem cell model indicates that only cancer stem cells and not the majority population of the tumor have the capacity to metastasize and establish distant lesions (46–48).

Gatenby and Gillies proposed a model for the somatic evolution of invasive cancer as overcoming a series of barriers to proliferation (49). Tumor development and the genotypic and phenotypic heterogeneity of cancer cell populations is described using an equivalence principle such that multiple alterations in the cell may allow to successfully adapt to and overcome a critical barrier moving toward malignancy. During tumor initiation, progression and metastasis, approximately 30 generation

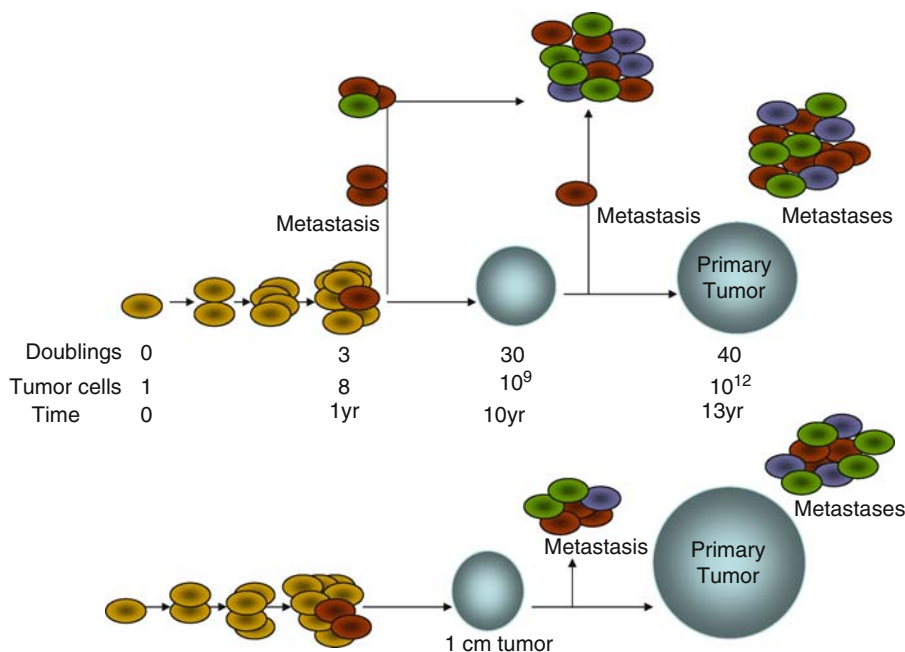


Fig. 5. Two possible patterns of malignant tumor growth and spread of metastatic disease over time and malignant cell doublings is shown.

times or about 10 years are required for a single malignant cell to produce a palpable mass of about 1 cm³ composed of about 10⁹ tumor cells (45) (Fig. 5). Over the course of these 10 years to reach a clinically detectable lesion, genetic instability produces variants in the primary tumor and metastases that are more heterogeneous than the early disease. A lethal tumor burden of 10¹² tumor cells could be expected after 10 cell doublings or 3 years from detection of the disease without treatment.

CARCINOGENESIS

Chemical carcinogen-induced rat mammary tumors provided an *in vivo* model for the early cancer stem cell hypothesis (50). In the rat mammary gland, epithelial stem cells insulted by a chemical targeting DNA can give rise to more-rapidly proliferating benign stem cells, which retain some response to normal growth-controlling factors. These altered but benign stem cells can also differentiate to a degree. The genetic instability of these cells results in the generation of malignant cells in subsequent generations with loss of response to growth-controlling factors, decreased capacity for differentiation and escape from immune surveillance. The cells which then compose the malignant disease have departed in critical ways from the normal epithelial stem cells of origin and continue to suffer from genetic instability and to evolve.

Colony formation is a demonstration of self-renewal capacity. It was noted early that colony-forming human epidermal cells were heterogeneous in capacity for sustained growth and that the potential for continued growth from a single cell could be predicted from the phenotype of the colony produced (51). Three types of colonies were described: (1) holocolonies characterized as large with a smooth perimeter containing mainly small cells that may be considered the most undifferentiated population; (2) paracolony characterized as being small with a highly irregular perimeter containing cells that are large and flattened and appear terminally differentiated; and (3) merocolonies characterized

by medium to large size and a wrinkled perimeter with heterogeneous cells. In the case of normal cells, the transitions from holoclone (stem-like) to meroclone to paraclone (terminally differentiated) are thought to be unidirectional and result in progressively restricted growth potential. However, malignant cells have increased plasticity and may dedifferentiate toward holoclone states or abnormally differentiate forming giant multinucleated cells that no longer proliferate. Locke et al. studied seven well-established human cancer cell lines from varied histology and found that cell lines generated from carcinomas produced cell culture colony patterns similar to those produced by the stem and amplifying cells of normal epithelia as described earlier (52). The cancer cell lines appeared to maintain a subpopulation of “stem cells” during passage of the line. The heterogeneity of the colonies produced from the cancer cell lines indicates that the stem cell property of asymmetrical division persists in cancer cell lines but is shifted toward stem cell self-renewal. Thus, malignant cells attempt to recapitulate the behavior of normal tissue cells; however, their behavior is aberrant and detrimental. Malignant cells are damaged cells that have dedifferentiated, returned to the behavior of embryonic cells rather than dying or being recognized as abnormal by the immune system.

The skin epidermis is a normal tissue that is continuously confronted with physiochemical traumas from the environment (53). The skin undergoes continual rejuvenation through homeostasis and is primed to undergo wound repair in response to injury. The epidermis has tissue “stem cells,” which both self-perpetuate and give rise to the differentiating cells that constitute the tissue. The TGF β pathway is involved in the control of proliferation of many cells. The loss of response to TGF β proliferation control is a necessary step in malignant transformation (54–56). In the absence of TGF β , proliferation control, squamous cell carcinomas spontaneously develop in mice (57). These tumors have many of the characteristics of invasive squamous cell carcinomas.

Although some may argue that the embryological, stem-like properties of tumors and tumor cell lines indicate that the malignant cell originates only from tissue stem cells, this argument seems unnecessary to explain the dedifferentiated properties of the malignant cell (58). The argument that cancer arises only from the stem cell populations of tumor that comprise perhaps 1 in 100,000 or 1 in 10,000 cells within the tumor leads to the notion that treatment agents that selectively kill the cancer stem cell will not result in decrease in the tumor mass. Classic studies and recent studies show that with some tumor cell lines inoculation of mice with few cells from 1 to 10 can produce tumors that are lethal to the host (59). Most transplantable tumor models are carried out with the implant for 10^5 – 10^7 tumor cells primarily due to the impatience of the experimentalist, who would like to see a palpable nodule in 1 or 2 weeks and not in several months or years. Generally, if a tumor nodule is not palpable in 2 or 3 weeks, the tumor is considered a no take. The timeline for these studies is that of the experimentalist not reflective of the nature of cell proliferation. Although it appears that only a minute proportion of human tumor cells can grow readily and rapidly in mice, syngeneic tumor models indicate that inoculums of many fewer cells that divide quite rapidly in the murine host yield lethal malignant disease in a short time (60).

CONCLUSIONS

In most incarnations, the cancer stem cell is very similar to a normal tissue stem cell. The cancer stem hypothesis has been advanced to describe how a cancer originates, how it is sustained, and what makes it drug resistant. Among the properties of tissue stem cells in the adult are the relative quiescence manifested by infrequent cell divisions, resistance to drugs/toxins mediated by ABC transporters, active DNA repair pathways and resistance to apoptosis. The cancer stem cell has been defined as a cell involved in malignant disease process that possess the capacity to self-renew and give rise to the heterogeneous lineages of cancer cells that comprise the tumor. Whether the malignant cells with

long term self-renewal capacity that sustain the cancer arose from tissue stem cells that acquired mutations leading to a differentiation block and malignancy or whether more differentiated cells acquired mutations that dedifferentiated them and resulted in a malignant cell capable of self-renewal has not been determined. Thus, the use of the term “stem cell” may not be accurate. The cells that initiate, sustain, and populate cancers are malignant cells (61, 62). The notion of the cancer stem cell is useful, if it leads important research questions being addressed and to better therapeutics being developed. The self-renewal, pluripotency, and drug resistance of malignant cells is a product of their abnormality and genetic instability.

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