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Tumor heterogeneity: biological implications and therapeutic consequences

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Summary

It is now appreciated that cancers can be composed of multiple clonal subpopulations of cancer cells which differ among themselves in many properties, including karyotype, growth rate, ability to metastasize, immunological characteristics, production and expression of markers, and sensitivity to therapeutic modalities. Such tumor heterogeneity has been demonstrated in a wide variety of animal tumors of differing etiology, tissue and cellular origin, and species. It has been shown in autochthonous, as well as transplanted, tumors. Similar results have been reported for human cancers, although much of the evidence that heterogeneity of human cancers, also reflects, at least in part, the existence of clonal subpopulations, is still indirect. Heterogeneity is not a unique property of malignancy. Preneoplastic tumors, as well as normal tissues, are also composed of cellular subpopulations.

Proposed mechanisms for the origin of tumor heterogeneity include coalescence of multiple foci of cancer clones and the generation of diverse subpopulations from a single clone. This latter process could be due to genetic errors arising from classical genetic mechanisms or to the production of cellular variants as in normal tissue differentiation. Indeed, certain tumor subpopulations have been shown to produce variants at high frequency. In some cases this frequency can be modified by environmental circumstances. Nontumor cells may also contribute to production of cancer cell variants, perhaps, in the case of infiltrating phagocytic cells, by producing mutagens or by somatic hybridization with cancer cells. Production of tumor cell variants is a dynamic process which can occur at any time.

Although tumors are mixed populations of cells, knowledge of the characteristics of individual components is not sufficient to predict the behavior of the whole. Individual cancer subpopulations can interact to affect each other's growth, immunogenicity, ability to metastasize, sensitivity to drugs, and clonal stability. The existence of multiple, interactive subpopulations provides a basis for the well-known phenomenon of 'tumor progression' in which tumors undergo qualitative changes in characteristics over the course of time. Selection of subpopulations better able to survive changing environmental circumstances allows for such changes as autonomy in regard to endogenous growth regulation, more 'malignant' behavior, and loss of response to therapy. Tumor subpopulation interactions may play a regulatory role in this process.

Tumor heterogeneity has obvious consequences to the design of effective therapy. It provides one rationale for combination therapies and suggests that initial treatment should be early and comprehensive. The continuing emergence of new clones suggests that treatment which is unsuccessful at one point might be

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effective later. Assays to predict effective therapy for individual patients need to address the multiplicity of tumor subpopulations and the ability of these subpopulations to influence each other. Subpopulation interactions may also be useful in therapy design, as may be efforts to control the extent of tumor heterogeneity by agents which effect cellular differentiation. Thus, tumor heterogeneity presents both problems and, perhaps, new solutions for control of cancer.

Introduction

The idea that tumors are not uniform populations of 'cancer cells' has gained new strength in the past few years. Attention is now focused on the many ways by which cancers differ and on the basis for these differences. This has led to the rediscovery of concepts of tumor biology which were known to cancer researchers in the past but which had become lost during the euphoria of the revolution in molecular biology. The purposes of this review are to document the increasing evidence for one such concept – tumor heterogeneity – and to speculate on its implications to tumor biology and consequences to cancer therapy.

Definition of tumor heterogeneity

Tumors are 'heterogeneous' in several ways. There is the heterogeneity among cancers in different individuals who nominally have the same type of disease. It is this heterogeneity which fuels the search for prognostic indicators and for methods to individualize therapy. A second type of heterogeneity is that seen within the same patient over the course of time. The biological, as well as the clinical, characteristics of an 'early', preinvasive tumor are not the same as those exhibited by the same cancer when it has disseminated. This type of heterogeneity is acknowledged by Fould's concept of 'progression' (1).

Heterogeneity is also seen within a single tumor at any one time. Histological examination of tumor samples often reveals considerable differences in the morphology of cancer cells in different areas of the same lesion. Host infiltrating and connective tissue are not evenly distributed. Areas of necrosis may be present. Depending upon tumor size, marked disturbances in vasculature can occur, leading to focal differences in oxygen tension, pH, substrate supply, and waste drainage (2). Related in part to this structural heterogeneity is heterogeneity in growth compartments. The cells within a tumor may be cycling or noncycling, quiescent or reproductively dead (3). If cycling, they may be at any stage in the cycle. Insofar as stage of cell cycle may influence cellular properties such as membrane biochemistry (4, 5), antigen expression (6–8), sensitivity to immune killing (9, 10), drug cytotoxicity (11), and ability to metastasize (12, 13), tumors will be heterogeneous in regard to those properties.

The type of heterogeneity which has received the most attention, and which is the subject of this review, is that due to the simultaneous existence of multiple clonal subpopulations within the same tumor. It is well to remember that such subpopulations are individually subject to all the other types of heterogeneity described above: as will be described, new subpopulations can arise during neoplastic progression. Furthermore, depending upon local conditions, structural and cell–cycle heterogeneity will be present within, as well as among, subpopulations. In addition, subpopulation heterogeneity imposes additional structural heterogeneity on the tumor as a whole. Cells in individual subpopulations may be located in distinct areas, or zones, of a tumor, rather than comingling (14–16). The zonal distribution of tumor subpopulations needs to be taken into account in devising methods of sampling tumors for various types of analysis. Investigators who serially transplant tumors in vivo with pieces of tumor, rather than cell suspensions, in reality may be transplanting only certain subpopulations.

Heterogeneity of experimental tumors

The coexistence of multiple subpopulations of tumor cells within single neoplasms has been re-

peatedly demonstrated in animal tumors of diverse etiology and histological type. These include melanoma (17–19) lymphoma–leukemia (20, 21), sarcoma (14, 22–26), and carcinoma (27–35). Heterogeneity in tumors induced by chemical agents (24, 32), physical agents (19, 25, 26), steroids (28), or viruses (20–22, 27, 30, 33–35) has been described. Long-term passaged tumors (18, 23, 24, 31), tumors of recent origin (19, 25, 26), and autochthonous tumors (20, 30) have been the source of multiple subpopulations. At this time it appears that no class of neoplasm is excluded from being heterogeneous, but quantitative differences among classes may be revealed by further experience.

Tumor heterogeneity is manifested by a variety of phenotypic differences. Differences in cellular morphology (30) and tumor histopathology (21, 29, 36, 37), as well as differences in growth rate, both in vivo and in vitro, have been seen (17, 19, 30, 31, 37). Tumor subpopulations can differ in expression or production of 'markers' of differentiation, including appropriate pigment (16, 17), receptors (38), cell products (21), and specialized biosynthetic enzymes (28). Phenotypic diversity has also been reported for immunological characteristics, including antigen expression, immunogenicity, and sensitivity to immune attack (14, 20, 30, 34, 39–44). (Immunological heterogeneity has been reviewed in depth elsewhere in this series (45).) Perhaps the most significant phenotype by which tumor subpopulations can differ is ability to metastasize. Following the lead of the classic experiment by Fidler and Kripke with the B16 mouse melanoma (18), the existence of tumor subpopulations that vary in ability to metastasize has been demonstrated in several experimental systems, including a recently isolated u.v.-induced melanoma (19), a variety of sarcomas (23, 25, 26), and mouse mammary tumors (31, 46, 47).

Primary tumors contain subpopulations that can metastasize to specific organ sites at high, medium, or low frequency, relative to the parent tumor (23). On the other hand, subpopulations that are unable to metastasize (at least by themselves), and may even be unable to produce tumors except at high innocula and after prolonged latency periods (30, 37, 48, 49), can be isolated from highly tumorigenic parent neoplasms. As will be discussed below, the simultaneous existence within a single tumor of subpopulations that differ, when tested independently, in degree of tumorigenicity suggests that within the parent tumor there are interactive mechanisms among the subpopulations that regulate growth and dissemination.

In addition to differences among tumor cell subpopulations, nonmalignant tissue within neoplasms may also be heterogeneous. Recent results from our laboratory suggest that normal cell heterogeneity may be related to tumor cell heterogeneity. Infiltrating lymphocytes have been isolated from solid mammary tumors produced by a series of cell lines which were originally derived from a single strain BALB/cfC_3H mouse mammary tumor. Not only did the percentage of lymphocytes isolated vary among the lines, but the type of lymphocyte also differed. In particular, the relative proportion of T cells belonging to the helper class versus those identified as members of the killer-suppressor class was characteristic for different tumor subpopulations (50). Tumor-infiltrating cells independently isolated from two different subpopulations growing on the same mice belonged to the T cell type characteristic for the individual subpopulations. Thus, the type of T lymphocyte response was a characteristic of the tumor, not the host, and was associated with specific tumor cell subpopulations. Whether tumor cell heterogeneity similarly influences other host components of tumors remains to be determined.

The wide range of phenotypic differences among tumor cell subpopulations suggests the existence of genotypic differences. Indeed, numerous investigators have described karyotypic differences (22, 30, 37, 51–55), as well as the presence of different marker chromosomes in different tumor subpopulations (37, 56). Using murine mammary tumor virus (MuMTV) DNA as a probe, cellular heterogeneity in the location and copy number of a specific gene has been demonstrated in strain GR mouse mammary tumors (33, 35). This is in accordance with the heterogeneity in expression of MuMTV-coded antigens within individual mammary tumors (34). Studies on the differential re-

sponse of BALB/cfC$_3$H mammary tumor subpopulations to inducers of MuMTV gene expression suggest that differences in regulation of MuMTV genes also correlate with tumor subpopulation heterogeneity (57).

Heterogeneity of human cancers

There is considerable indirect, and increasing direct, evidence that human cancers, like their animal counterparts, are composed of heterogeneous subpopulations. Heterogeneity in histological pattern may be seen in multiple samples of breast carcinoma (58, 59) and in small cell anaplastic carcinoma of the lung (60). Histological and ultrastructural heterogeneity of tumor cells from bronchial carcinoid has been described (61). Intratumor heterogeneity in tumor cell DNA content has been observed in colon carcinoma (62) and small cell carcinoma of the lung (63). Expression of tumor-associated antigens has been shown to be nonuniform among cells from single neoplasms, such as osteosarcoma (64), and pancreatic (65), and breast carcinoma (66). Other markers of tumor cell differentiation have likewise been shown to be distributed heterogeneously within tumors, for example, B_2-microglobulin (67) and estrogen receptors (68–70) in breast cancer. Tumor cell heterogeneity for calcitonin has been described in virulent medullary carcinoma (71). This is especially interesting in that it was shown that heterogeneity for calcitonin staining was seen in medullary carcinomas with a high likelihood of metastatic spread, whereas uniform staining was seen in tumors with a small chance of recurrence.

Additional evidence that human cancers contain tumor cell subpopulations comes from comparison between primary tumors and metastases. Here again one may see divergence in histological type (59). Differences in levels of histaminase and L-DOPA decarboxylase have been reported between primary small cell carcinoma of the lung and hepatic metastases (72). Different hepatic metastases from the same patient likewise vary in L-DOPA decarboxylase activity. Differences in sensitivity in vitro to antineoplastic drugs between cells from primary ovarian carcinomas and their metastases have also been seen (73). Furthermore, estrogen receptor content can vary between primary breast cancers and their metastases and among multiple metastases of the same patient (70).

As with animal tumors, formal proof of the existence of tumor subpopulations requires their isolation and characterization. This has now been accomplished with a growing list of human tumors. Tumor lines that differ in drug sensitivity (74, 75), antigenicity (76, 77), or tumorigenicity in nude mice (78) have been isolated from single melanomas, both from primary lesions (74, 75, 78) and multiple metastases of the same patient (77, 78). Tumor subpopulations have also been isolated from primary human colon carcinomas (79, 80). Certain of these subpopulations differ in karyotype (80), in vitro growth properties (79, 80) tumorigenicity (79) and histology of tumors in nude mice (79–80). Similar isolations of tumor subpopulations have also been reported for lung (81), ovarian (82), and pancreatic (83) cancer.

Isolations of tumor subpopulations from human cancers have frequently been accomplished using cell cultures which had been maintained and passaged in vitro for fairly long periods of time prior to cloning. Only rarely have the subpopulations been obtained directly from the patient (77, 82). This raises the possibility that the production of heterogeneous variants is a consequence of the in vitro environment and occurs sometime after removal of the tumor from the patient. In this regard the elegant study of Shapiro et al. (84) needs emphasis. These investigators karyotyped tumor cells from fresh samples of human gliomas within six to 72 h after surgery. An array of unique karyotypes was found in each tumor. Simultaneously, dissociated tumor cells were cloned by dilution plating and the clones were karyotyped. By matching karyotypes of the clones with those in the fresh sample, it was possible to show that the clones were present at the time of resection. Each of eight gliomas was found in this way to have from three to 21 subpopulations – a minimal estimate since different subpopulations can have similar karyotypes. Different clones from the same tumor differed in morphology and growth kinetics. Antigenic heterogeneity has also been

reported in clones derived from a single human glioma (84).

The work of Shapiro et al. (81), as well as work done with animal tumors (18, 30), suggests that heterogeneity is not induced by culture in vitro. On the other hand, it is often assumed that long-term cell lines are not heterogeneous, or minimally so, due to selection in vitro. That this is not so is shown by the ability of investigators to isolate subpopulations from lines such as murine L1210 (40, 41) and human tumor lines, including HT29 colon carcinoma (86), MOLT-3 malignant T-lymphoblasts (87), MCF-7 breast carcinoma (88), and other established lines (76, 80, 81, 83, 84).

Origin of tumor heterogeneity

A point of confusion in understanding tumor heterogeneity is reconciling its existence with the large body of evidence pointing to a single cell origin for many, if not most, neoplasms (89). Strong as this evidence is, it must be remembered that it is not universal. Some tumors, such as 'venereal' warts in man (90) and fibrosarcoma induced by relatively high doses (91) of methylcholanthrene in mice have been shown to arise from more than one clone (92). Furthermore, some human cancers are characterized by numerous foci of neoplastic change. Multiple lesions of hyperplastic, in situ, and intraductal neoplasia can often be demonstrated in breasts of women presenting with invasive breast carcinoma (59). Thus, a developing malignancy could incorporate elements from other lesions, and hence become 'heterogeneous'.

Even if all cancers were truly of single cell origin, the opportunity for heterogeneity to develop occurs as soon as that single cell divides. As will be discussed, both structural and regulatory alterations in genetic function may contribute to cellular variation. After all, most multicellular organisms begin as single cells. Even among cells from grossly homogeneous tissues, biochemical and functional heterogeneity is apparent. The heterogeneity among hemopoietic cells, ultimately derived from clonal stem cells (93), and the multiple cell types within the lymphocyte family (94) are obvious examples. Even quite similar cells, such as thymocytes (87) or mammary epithelial cells (95) are heterogeneous in regard to enzymatic activity or antigen expression. Griffin et al. (96) have demonstrated that normal cells can be cloned into heterogeneous subpopulations; different clones of genital skin fibroblasts display a wide range of activity of 5α-reductase, the enzyme that catalyzes the conversion of testosterone to dihydrotestosterone.

If normal tissues exhibit cellular heterogeneity, it is not surprising that minimally transformed or preneoplastic tissues would do so also. Heterogeneity in expression of a battery of marker enzymes within foci of hyperplastic, preneoplastic hepatocytes has been demonstrated at the earliest time of recognition of such lesions (97). Intranodule heterogeneity in expression of MuMTV antigens was seen in mammary hyperplastic alveolar nodules of MuMTV-infected mice (98). Similarly, chromosomal analysis of tumors produced by subcutaneous implantation of $C_3H/10T\frac{1}{2}$ cells attached to plastic suggests that they arose from minor subpopulations within the original culture (99), indicating a heterogeneity within that line in regard to induction of tumorigenicity. That such heterogeneity can have a genetic basis was shown for susceptibility to ultraviolet light-induced transformation by cloning differentially susceptible variants from BALB/3T3 cells (100). Thus, cellular heterogeneity is present before, as well as after tumor production, and is itself a factor in tumorigenesis. Clearly such heterogeneity is not unique to cancers, and tumor heterogeneity does not *necessarily* require any special explanation.

Numerous mechanisms have been proposed for the production of diverse subpopulations within a developing tumor. The most pervasive ideas are those of Nowell (55) who theorized that concomitant with the initiation of neoplasia within a single cell is the acquisition of genetic instability beyond that seen in normal cells and not due only to loss of growth restraints. Nowell cited studies showing a higher frequency of genetic errors in neoplastic than in normal cells and futher suggested that genetic instability becomes greater as a neoplasm evolves. Direct evidence for this latter hypothesis has recently been presented by Cifone and

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