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FOR DEBATE

Heterogeneity, biodiversity and bioperversity in solid neoplasms

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Heterogeneity of biological structure and function is an impediment to the analysis and treatment of human solid tumours. Its importance is frequently underestimated in clinico-pathological research. This article reviews the many facets of heterogeneity in tumour systems, and its importance to the interpretation of tumour biology.

Key words: **heterogeneity; neoplasia; histochemistry; flow cytometry.**

Introduction

Heterogeneity may be broadly defined as the state of diversity, or of composition by diverse elements. It is a fundamental feature of biological systems and is a major impediment to the understanding of the behaviour of solid neoplasms at the genetic, cellular, tissue, tumour and organ levels. Heterogeneity has been recognized as a feature of human tumours for many years, but it is rarely addressed in systematic fashion. It may be classified as true or artefactual, morphological, genetic, phenotypic, structured or organized, and unstructured or random. It has two important consequences for surgical oncologists. Firstly, heterogeneity complicates the understanding of tumour biology and the interpretation of clinico-pathological studies. Secondly, it conditions the response of tumours to adjuvant treatment.

The measurement of the expression of biological markers is a common feature of modern clinico-pathological research. A variety of technologies, including histochemistry and flow cytometry, may be used to generate quantitative data to be correlated with indices of clinical behaviour such as time to recurrence; response to therapy; or survival. This commentary addresses some of the key features and problems of heterogeneity, which may make this elusive phenomenon the great invalidator of much clinico-pathological research.

Heterogeneity of structure and function

Genetic heterogeneity

Genetic heterogeneity, whether caused by chromosome shuffling at meiosis or by somatic mutation, is the foundation of Darwinian evolutionary competition, of species diversity, and of malignant change. Patients with cancer display genetic differences between the parent genotype and the tumour. and those differences vary with time, with growth and with site within the tumour. The characteristics of tumour stem cells were once held to be replicated in the malignant phenotype. It is now clear that the abnormality is not a single switch or event converting the normal to the malignant state, but a randomly staged and triggered progression of genetic and functional changes in the cell. These may be accelerated by environmental factors such as background radiation, and by hereditary genetic changes. Hereditary genetic defects seem able to bypass some of the regulatory stages in carcinogenesis, thus promoting the development of malignancy early in life. The existence of the stages has been demonstrated most convincingly in colorectal cancer progression and in the polyposis coli phenotype.[1]

Single genes display considerable mutational heterogeneity within populations, but many of the mutations may be functionally neutral. For example, more than a thousand point mutant variants of the p53 gene have been detected.[2] Nevertheless, cumulative point mutations in DNA lead to increasing genetic instability. At the chromosomal level, genetic disorder may arise through a number of different mechanisms. These include gene loss, gene amplification, and DNA methylation. This disorder gives rise to pluripotentiality of biological function and biomarker expression. One morphological manifestation of this genetic heterogeneity is the state of aneuploidy which is found in a large proportion of solid tumours.[3]

It now appears that there is no one point at which a tumour can be considered to have achieved its definitive genotype. The genetic instability which arises from the malignant condition may lead to many different cell lines, or clones, which may have better or worse survival characteristics. It can be demonstrated that genetic diversification continues over the life of a tumour. For

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example, Shackney and colleagues[4,5] haave shown that patterns of ploidy and oncoprotein expression change with time in individual breast tumours followed from primary resection to clinical recurrence. Progessive heterogeneity of DNA ploidy and tumour proliferation can also be demonstrated using flow cytometry in prostate cancer.[6]

Structural heterogeneity

The observer can readily appreciate spatial and structural heterogeneity in tumours at the two-dimensional, microscopic level in the stained tissue section. Most epithelial tumours display considerable variation in cellular morphology and tissue architecture from one microscope field to the next, for example in the proportions of live and dead cells, of quiescent and proliferative cells, of tumour cells, stromal cells and supportive elements of various lineages.

The three-dimensional architecture of tumours is also heterogeneous. In many tumours, this manifests as a proliferative face or edge, with hypoxic or necrotic areas elsewhere. This can be clearly seen macroscopically in a large liver metastasis or colorectal tumour. Thus, the site sampled may be of considerable importance if representative data are to be obtained. If a proliferative marker is being assessed, it will clearly be of profound significance to know whether a tissue section under study derives from the proliferative face or the necrotic centre. Intratumour variation for such markers may exceed the intertumour variation, thus rendering population-based prognostic studies of tumour markers meaningless.

Heterogeneity of malignant potential

Histological appearances may disguise the heterogeneity of potential for biological aggressiveness. In the absence of a complete understanding of the determinants of tumour morphology, invasiveness and metastasis, it is not possible to determine which cells or microscopic fields in a tumour are representative of the greatest proliferative, apoptotic, invasive or metastatic potential. Fields of features selected for reporting in a clinico-pathological series may well not be representative of biological capability. The occult potential of individual clones may be more important in determining clinical outcome than the median behavioural traits of the whole population.

Mathematical descriptions of heterogeneity

There are no satisfactory models or benchmarks to predict, describe or analyse heterogeneity in tumour biology. For example, in the histological study of a biomarker in fixed or fresh tumour material, it is not certain how many tissue blocks should be studied from any one tumour to ensure true representation of that marker. From what sites within the tumour (e.g. growth face, centre of tumour) should measurements be obtained, and in what proportions? Within each tissue block, how many sections should be studied, and how many microscope fields within each section? Will the parameters vary between an anaplastic tumour with little visible variation, and a well-differentiated, visibly heterogeneous tumour? Mathematical models or protocols are required which impose on researchers a consistent approach to recording the heterogeneity inherent in the expression of any marker under study in any one locus of a tumour. The absence of consistent protocols should not deter observers from attempts to address the problem of heterogeneity both in the methodology of measurement and in the discussion of each and every clinico-pathological series.

Heterogeneity may be truly random in its development and manifestations. Conversely, it may be a mathematically predictable expression of biological order.[7] A novel proposal is that Chaos Theory may help to explain tumour heterogeneity.[8] 'Chaos' is the mathematics of the seemingly complex but non-random and highly ordered behaviour which may be shown by simple, non-linear systems. The rules which govern the behaviour of the system may not be readily apparent to the outside observer. Thus, 'chaotic' (i.e. predetermined) rules and patterns may regulate the evolution of the genotype and phenotype in individual tumours, and in turn may reflect simple disturbances in the underlying molecular processes. However, Chaos Theory has yet to be validated in complex biological systems such as tumours.

Temporal heterogeneity

All biological systems change with time. Time-dependent events cannot usually be deduced from static techniques of biological measurement. Biological processes change over short and long periods of time. In the former case, many biomarkers are key enzymes or functional proteins (growth factor receptors, oncoproteins) which act dynamically and which can turn over rapidly (second, minutes or hours) within the cell. Thus, inspection of a tumour fixed at one time point may not be at all representative of its immediate past history or future potential.

Over longer time periods, (days to weeks), the physical and functional characteristics of a tumour change significantly. Tumour growth is the product of an imbalance between cell production and cell loss, which varies considerably with time and changing environmental factors. Tannock[9] has described this infinitely varying status of the composition and behaviour of a tumour as the 'heterogeneity of heterogeneity', thus recognizing that the heterogeneous characteristics of any tumour may change continuously both in place and time.

The passage of time also confers heterogeneity upon cell poulations through the process of ageing. Cell replication confers age upon the cell during mitosis, through the loss of repetitive DNA from the telomeres at the ends of chromosomes each time a cell divides. This mechanism is believed to impose a limit on the number of divisions that a cell can make before the replicative mechanism is terminally damaged. In the presence of the enzyme telomerase, stem and malignant cells may be protected from DNA loss and become effectively 'immortalized', as in testicular germ line cells. A tumour population may possess considerable heterogeneity of replicative potential, in part due to differences between cells in telomere length and expression of the telomerase enzyme.[10]

We cannot be certain that heterogeneity in space and time for any one parameter within a tumour is any less or greater than inter-tumour variation for the same parameter. Take, for example, a hypothetical series of archival tumours in which the S-phase fraction, an indirect measure of proliferation, is being correlated with outcome. Proliferative potential and growth fraction change with time, size, vascularity and certainly with location in a tumour such that the proliferative biology in one part of small, unconstrained, well-vascularized early invasive tumour A may bear no biological comparison with any part of large, hypoxic, ageing tumour B. Thus, hypothetically, a clinico-pathological series of tumours of varying biological ages may display considerable heterogeneity for the S-phase fraction marker in the crude data, but this variation may simply be an index of tumour age rather than series heterogeneity.

Heterogeneity of critical cell functions

Heterogeneity is found in the biology of critical cell functions. Such processes include proliferation, apoptosis, intra- and intercellular signalling, cell adhesion, angiogenesis, and metastatic behaviour, and are usually under the control of regulatory genes. Many specified genes and their protein products have been implicated in the sequence of events leading from normal cell behaviour to malignancy, including c-myc[11] and p53.[12,13]

It might be expected that key functional enzymes, regulatory proteins and membrane receptors should be *de facto* expressed in all living cells, albeit at variable levels. In clinico-pathological studies, it is common to fail to detect key biomarkers in a significant fraction of the cell population. This raises the question of whether observers are reporting true or artefactual (*vide infra*) heterogeneity. For example, the expression of the biomarker may be below the levels of detection of the assay in use. Key regulatory molecules are often rapidly synthesized and degraded in cells. Failure to detect a target molecule or process in a static assay may give a quite misleading indication of the significance of the marker. Conversely, point mutations in proteins such as p53 may considerably alter their stability and biodegradability, thus changing the apparent rate of expression of the marker. An outstanding example of this problem is provided by the fundamental process of apoptosis, which proceeds so rapidly in tissues as to have evaded recognition completely until the 1970s.[14-16]

Cell cycle asynchrony and apparent heterogeneity

Cell cycle asynchrony in complex cell populations such as tumours imposes apparent heterogeneity of biomarker expression on a population of otherwise identical cells. Tumour cell populations are a complex mixture of proliferating, quiescent, non-cycling and apoptotic cells. Levels of most proteins vary between quiescent and proliferating cells, and fluctuate through the cell cycle with DNA synthesis and cell division. For example, less than 10% of cells may be in the S-phase at any one time in an asynchronous but otherwise homogeneously proliferating tumour cell population. Single, 'static' measurements of a proliferation marker such as the S-phase fraction will yield a labelling index which does not identify the entire population of proliferative cells. The integrated measurement of temporal accretion of a chosen marker will yield very different results to a static 'snap shot' analysis. This can be illustrated in the case of cell proliferation, by continuous infusion of a tumour with a halogenated pyrimidine thymidine analogue such as bromodeoxyuridine. This labels all cells as they come into cycle, and identifies a much larger proliferative population, the growth fraction, than does flash or pulse labelling.

Heterogeneity and metastasis

Metastasis is the process which above all determines the biological aggressiveness of the tumour and the clinical outcome. There may be considerable heterogeneity of genetic content, of form, function and composition between primary tumours and their metastases, which cannot necessarily be regarded as simple clones of their parent primary tumours.[17] Metastases develop in a very different immunological or nutritional environment, such as in a lymph node, liver or bone marrow, which influences their form and structural heterogeneity. For example, a liver metastasis is not spatially confined to the same growth surface as its parent primary intestinal tumour.

Much clinico-pathological research is undertaken on samples from the primary tumour. However, once this has been resected, it can no longer influence the clinical progress which is determined by the metastases. Thus, correlative studies of biomarkers in primary tumours and clinical outcome are underpinned by an assumption which may be fundamentally flawed. It cannot be assumed that there is consistency of biological structure and function between all cells in the primary tumour and in the metastasis. This problem is frequently overlooked, and there is a considerable requirement for detailed comparative studies of the biology of primary tumours and their metastases.

Artefactual heterogeneity

Heterogeneity may be perceived where it does not in fact exist as a result of experimental artefact. In immunohistochemical studies, variation in staining sensitivity, antibody specificity and preparation artefacts are common. Fixation is a particular problem, and it is surprisingly uncommon for studies on an archival series to be unsupported by parallel studies on freshly obtained material to control for fixation artefact, and sample degradation with time. Such problems have also been reported in relation to flow cytometric studies.[18] Exhaustive controls appropriate to the experimental technique are thus essential.

Heterogeneity and therapy

All human cell lineages possess subtle, complex and reliable systems to guarantee the fidelity of reproduction, function and survival over evolutionary timescales. They possess considerable redundancy in their molecular engineering to

guarantee against environmental damage. This makes cell lineages extraordinarily resilient to deliberate damage by medical intervention. Over aeons, cells have adapted to lifelong background and cosmic radiation, which in turn has prepared their damage repair mechanisms for modern drugs and radiotherapy.

Structural heterogeneity and resistance to therapy

The anatomical heterogeneity of solid tumours creates natural resistance mechanisms to adjuvant therapy. Most solid tumours proliferate at the periphery, leaving the centre or core relatively undervascularized. As tumours enlarge, so internal blood flow and diffusion of gases and nutrients becomes less and less uniform. This prevents the even perfusion of drugs throughout the substance of the tumour. The resulting central hypoxia, acidity and raised interstitial pressure can be shown to impair drug uptake and biological activity, and hence increase resistance to therapy.[19-21]

Heterogeneity of chemotherapeutic resistance mechanisms

Heterogeneity of a variety of cell functions contributes to the evolution of resistance to therapy. Genetic heterogeneity produces phenotypic changes in protein and enzyme expression and function which affect the uptake and response to cytotoxic drugs or to DNA damage. Resistance to treatment is conferred *de facto* by the existence of a genetically diverse and pluripotential cell population. Competition for survival within the complex environment of a single tumour may be intense. As some cells are destroyed by therapy or necrosis, so the biological 'fitness' of others may manifest as changes in proliferation, drug resistance, greater capacity for DNA repair, reduced rates of apoptosis, or a greater capacity to metastasize. A natural selection process is thus at work within the growing tumour among multipotential cells, and environmental forces such as cytotoxic therapy will effect different selection pressures on different clones.

DNA repair is a particularly important and heterogeneous function of cells. One component of the repair system is the Topoisomerase family of DNA conformational enzymes.[22,23] DNA damage may arise spontaneously in the course of normal molecular processes, from natural or therapeutic radiation, or through chemotherapy. DNA repair imparts reproductive continuity and fidelity upon cells, and may protect against a much higher incidence of cancer in the natural world. To put this capability in context, it has been estimated that the DNA of each cell, comprising 4×10^9 bases, temporarily loses 10,000 nucleotide bases each day from spontaneous DNA damage alone. There is a predicted efficiency of only three mistakes per 3 billion base pairs during DNA copying, in each of the 10^{14} cells in the human body, and in up to 10^{16} cell generations per human lifespan in stem cell lines (such as the haematopoeitic system). Not only is the existence of such a DNA repair capability remarkable, but it highlights a profound problem in cancer therapy, because such an evolutionarily stable biological process is likely to possess considerable redundancy against DNA damage induced by clinical therapeutic manoeuvres.[24-27]

Cellular detoxification mechanisms also contribute to drug resistance.[28] Early success in chemotherapy is often followed by relapse and subsequent failure. Mutant, drug-resistant cell lines may be selected during the course of treatment and may supercede the drug-sensitive cells.

Heterogeneity of multi-drug resistance (MDR) expression is likely to be an important factor in favour of therapeutic selection of the MDR phenotype. The MDR glycoproteins are a family of membrane-bound ATP-dependent glycoproteins of which p170 has been well characterized.[29-33] They pump out of cells lipophilic drugs such as colchicine, adriamycin, vinca alkaloids, and dauno-rubicin. An increase in drug efflux may confer a degree of cytotoxic drug resistance upon the cell. MDR-p170 gene or protein expression can be found in most tumour classes in which it is sought. Heterogeneity p170 glycoprotein expression imparts flexibility to the capacity for response of the tumour cells to therapy.

Radiotherapy and heterogeneity

Tumour radiosensitivity is a function of factors which include the proliferative behaviour and the cell cycle stage of the tumour cells during therapy. These factors are heterogeneous within tumours.[34,35] Quiescent, non-cycling cells may be less sensitive to radiation damage. Tissue hypoxia such as is commonly found in the centre regions of solid tumours imparts radioresistance, as do intracellular damage limitation processes, such as the presence of thiol compounds to mop up superoxide radicals, and the activity of DNA repair mechanisms. Cell populations respond to radiotherapy with the processes of repair, redistribution, reoxygenation and repopulation. Cells tolerate and overcome sublethal radiation doses to varying degrees as a result of the complex interactions of the many heterogeneous processes, and resistance clones commonly come to be selected during treatment.

Conclusions

Heterogeneity of tumour biology poses major problems for researchers and therapists in surgical oncology. Failure to pay due regard to the problem of heterogeneity devalues much of this work and handicaps the interpretation of many clinical research studies. The recognition of heterogeneity at all levels in biological systems is but a step in the direction of the formulation of better treatment for solid tumours. To quote Rennie,[10] perhaps we should view cancer cells as 'like Dorian Gray: internally corrupt and destructive but miraculously blessed with eternal vigour.' Biological heterogeneity, the bringer and sustainer of life, may also be the key to the perverse corruption, the destruction and the vigour of the surgical tumour which life has spawned.

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