A long view of fashions in cancer research

Henry Harris

Summary
Despite the spectacular contributions to knowledge made by molecular biology during the last half century, cancer research has not delivered an agreed explanation of how malignant tumours originate. The models assiduously investigated in molecular terms largely reflect waves of fashion, and time has revealed their inadequacy: cancer is (1) not caused by the direct action of oncogenes, (2) not fully explained by the impairment of tumour suppressor genes, (3) not set in motion by mutations controlling the cell cycle, (4) not governed by the dependence of malignant tumours on an adequate blood supply and (5) not triggered by a failure of programmed cell death. But there is now strong evidence that cancers may have their origin in mutations that block the execution of critical steps in the process of normal differentiation. Cancer, thus seen, is not initially a disease of cell multiplication, but a disease of differentiation. The evidence for this point of view should now be explored. ***BioEssays*** **27:833–838, 2005.**

Introduction

Driven by the increasing sophistication of molecular biology, cancer research in the last half century has been buffeted by successive waves of fashion. First, there wa a tidal wave of 'oncogenes', a term originally coined by Huebner and Todaro in 1969.[1] This, after a long delay, was eventually displaced by a comparable wave of tumour-suppressor genes,[2,3] although those who had made a heavy commitment to oncogenes[12] long adhered to implausible models that envisaged some form of 'balance' between oncogenes and tumour suppressor genes. When it was discovered that the genes governing the cell cycle in yeast had their homologues in man,[4] mutations affecting these genes rapidly became the subject of intensive investigation, an activity encouraged by the hope that tumorigenesis might be explained by aberrations in the control of the cell cycle. Then came 'angiogenesis' and the theory that the progressive growth of tumours was critically dependent upon the establishment of an adequate blood supply.[5,6] After that came 'apoptosis'.[7] Here the argument was that programmed cell death was an integral part of tumour development and that inappropriate cell multiplication occurred when the execution of this form of cell death was impeded. And currently we are witnessing a resurrection of interest in the role of aneuploidy in carcinogenesis, touched off by a claim that aneuploidy is merely a secondary consequence of tumour progression[8] and not a precondition or essential determinant of malignancy. Since none of these ideas has so far yielded a generally acceptable explanation of how malignant tumours are formed, it is perhaps worthwhile examining them with the acuity of hindsight.

Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK. E-mail: henry.harris@path.ox.ac.uk
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Oncogenes

Although it rapidly became apparent that the model proposed by Huebner and Todaro was erroneous, the term 'oncogene' proved irresistible and was quite generally applied to any gene, whether of cellular or viral origin, that produced a tumour on injection into the animal[9] or produced the morphological change known as 'transformation' in vitro.[10] The completely understandable assumption was that these oncogenes produced their effects in a genetically dominant fashion: it was held that, by introducing new genetic information into the cell, they induced it to become tumorigenic. So strongly entrenched were these views that at one point it was actually argued that a single amino acid substitution in the protein coded by the Harvey murine sarcoma virus (*Hras*) was not only enough to induce tumour formation, but that continued presence of the mutated oncogene was required to maintain the tumorigenicity.[11] These experiments were, however, soon called into question.[13] It was shown that the tumours produced by the injection of cells carrying the *Hras* oncogene resulted not from the unselected multiplication of the population of cells injected, but by the selective overgrowth of minority cell populations in which other genetic events had occurred. Moreover, the tumorigenic cells remain tumorigenic even after the *Hras* oncogene has been eliminated. The failure to appreciate the power of in vivo selection long remained a besetting sin of oncogene experiments of this sort.

Looking back on 20 years of controversy, it is possible to conclude that a wide variety of oncogenes with different functions in different parts of the cell can produce phenotypic changes, which we generically call transformation. But they do this by mechanisms that are more complex than is implied by the doctrine of genetic dominance. No single oncogene, and no combination of oncogenes can induce a cell to become tumorigenic unless some gene or genes that normally act to

suppress the various elements of the transformed phenotype are inactivated. Experiments with transgenic animals are unanimous in demonstrating that oncogenes do not form tumours directly but merely establish a predisposition to tumour formation. Tumorigenesis ultimately requires the intervention of other genetic changes and these occur in a stochastic fashion.[14] Whatever the nature of the oncogene it becomes dispensable once the tumour has been established.

Tumour suppressor genes

The assumption that oncogenes operated in a simple genetically dominant fashion met, or ought to have met, its death knell in 1969. In that year it was demonstrated by means of cell fusion that when a wide range of malignant mouse tumour cells, of different cell type and different aetiology, were fused with non-tumorigenic cells, tumorigenicity was systematically suppressed.[15] Some 25 years later, Edward Harlow, assessing the impact of this paper in the 'Landmarks' series of the Journal of N.I.H. Research[16] concluded that, in retrospect, the paper could clearly be seen as a landmark[17] in that it changed or ought to have changed our concepts of how tumour development occurs; but, according to Harlow, it was largely ignored because most people just couldn't understand it. I doubt whether incomprehension was the reason for the paper's failure to gain acceptance. The experimental design was simple and the result not difficult to assimilate. What impeded acceptance of these findings was not incomprehension, but inattention. Experimentalists immersed in their own technology tend to ignore reports that are not couched in their own language. This, coupled with a firm commitment to the genetic dominance model of oncogene action, resulted in the paper being essentially ignored, or disbelieved, for many years. And this despite a long series of subsequent cell fusion papers that established beyond reasonable doubt that normal diploid cells contained genes that had the ability to suppress the tumorigenicity of a wide range of malignant tumour cells;[18] and despite the fact that Stanbridge[19] and others, studying hybrid cells of great karyological stability, had in the meantime established that this was also true of normal human cells. It was clear to the few who cared to listen that malignancy was not, in simple Mendelian terms, genetically dominant, but was recessive to the wild type.

The tide began to turn with the publication of two theoretical epidemiological papers by Alfred Knudson[20] and by Knudson and Strong,[21] the first dealing with retinoblastoma, the second with Wilms' tumour. From one point of view, it seems surprising that two theoretical epidemiological papers should have attracted the interest of molecular biologists, whereas a series of experimental cell fusion papers had failed to do so. The critical difference between the two approaches was that the model proposed by Knudson drew attention to a specific gene, the retinoblastoma (*Rb*) gene. This was soon located to a precise chromosomal site by Franke,[22] and the region was then subjected to intense investigation by several groups[23] with increasingly sophisticated methods. The gene was finally cloned and sequenced by Lee et al.[24] and Fung et al.[25]

Knudson's argument was that both the familial and sporadic forms of retinoblastoma were determined by recessive mutations at the same locus, but that in the familial form one of the alleles was already mutated or deleted in the germ line. This proposal, which became known as the 'two-hit' model, appeared to fit the epidemiological data reasonably well, but so strongly held was the assumption that cancer was caused by dominant mutations that Knudson's proposal was also largely ignored for several years.

It is of interest to see how well Knudson's 'two-hit' model has survived after the passage of thirty years. As early as 1976 Bonaïti-Pellie et al.,[26] in an extensive epidemiological study of retinoblastoma in France, noted that their data more readily accommodated a 'three-hit' model than a 'two-hit' one. As pointed out by Hamel et al.[27] all retinoblastomas are aneuploid, which suggests that a third event must take place before the malignant tumour emerges. (The relationship between aneuploidy and malignancy will be discussed in greater detail at a later stage.) Although Cavanee[28] was able to list some 25 different tumours in which 'loss of heterozygosity' had been found at specific chromosomal loci, the evidence is now decisive that 'two-hits' are not enough to generate a malignant tumour. In several well-studied cases, loss of heterozygosity generates benign tumours in which a further genetic event, or events, must take place before malignancy emerges; malignant tumours may arise, and in a stochastic fashion, when only one of the alleles at a tumour suppressor locus is inactivated; the insertion of a normal *Rb* gene into retinoblastoma cells does not curb their tumorigenicity. Knudson now acknowledges the approximate nature of the 'two-hit' model. An article of his published in 2001 is entitled 'Two genetic hits (more or less) to cancer'.[29]

Nonetheless, despite prolonged indifference and some fierce opposition, it was finally conceded that normal cells did harbour genes that had the ability to suppress tumorigenicity, and that the genetic basis for the formation of a malignant tumour was a loss of normal function. This was formally acknowledged, 20 years after the appearance of the cell fusion paper, at a symposium held at Cold Spring Harbor. The resulting publication[2] was entitled 'Recessive oncogenes and Tumor Suppression', and much argument took place about what these normal suppressive genes should be called. In the event, the decision was taken to call them tumour-suppressor (*ts*) genes and this name was eventually universally adopted.

A torrent of papers describing tumour suppressor genes and analyzing their mode of action in molecular terms was thus unleashed, comparable to the earlier flood of papers dealing with oncogenes and, as had happened with oncogenes, the term tumour-suppressor came to be used more loosely than was originally intended. Only in a minority of cases where

recessive genes were classified at *ts* genes was it shown that these genes actually suppressed the growth of malignant tumours.

Mutations affecting the cell cycle

Studies on the cell cycle in the fission yeast, *Shizosaccharomyces pombé*, had been vigorously pursued since the 1950s,[30] but it was the discovery by Lee and Nurse[4] that the genes governing that process in the yeast were homologous to human genes engaged in the same process that aroused world-wide interest in the molecular analysis of the cell cycle. The hope was that malignancy might be determined by mutations in these genes, apparently critical for the multiplication of the cell. Although this idea is obviously attractive, there is only conjecture to support it, and it is not easy to see how it can be reconciled with the observations, discussed later, which show beyond reasonable doubt that karyological disorder precedes the appearance of the tumour. There are indeed robust and elaborate mechanisms that maintain the stability of the human karyotype,[31,32] and it is clear that once these break down, as occurs during the growth of a malignant tumour, large numbers of mutations, including cell cycle mutations, are accumulated. These no doubt contribute to the final phenotype of the tumour, but the event that precipitates karyological disorder is not the one that initiates tumorous growth. It is little short of special pleading to argue, in the absence of evidence, that cell cycle mutants are responsible not only for the subsequent elaboration of the tumour phenotype but also for the onset of tumour formation; this is especially so in the light of the strong evidence that now exists indicating that the onset of tumour formation is determined by quite different mechanisms.

Angiogenesis

In 1971 and 1972 the idea was launched that, if a solid tumour failed to establish an adequate blood supply, it would not grow beyond a very limited size.[5,6] (The initial experiments were done with an experimental tumour growing in a rabbit's eye.) This idea was widely accepted and, in a manner with which we are now familiar, inspired a flood of experimental papers in which attempts were made to inhibit the growth of malignant tumours by inhibiting their vascularization (anti-angiogenesis).[6] Since, after more than 30 years of concerted effort, no generally effective anti-tumour agent has emerged from this programme, it seems reasonable to ask whether the impediment lies in the synthesis or isolation of such compounds or whether perhaps the basic assumptions are questionable.

My own experience with experimental mouse tumours leads me to doubt whether it is generally the case that solid malignant tumours must become vascularized in order to grow. It is commonly found that many transplantable malignant mouse tumours are characterised by a necrotic centre and a rim of viable cells. This is so whether the tumour shows any morphological evidence of vascularization or whether it does not. The outer rim of the tumour, a few cells thick, survives without establishing a new blood supply, not even in the form of visible capillaries. That the outer rim of a tumour could be adequately supplied by diffusion alone is hardly surprising, but what is notable about such tumours is that they may grow to an enormous size, eventually kill the animal, and occasionally form secondary tumours. The malignant growth is supported by cell multiplication in the outer rim, and the necrotic centres of the tumours expand as the tumours grow. We do not know what determines the central necrosis. It may simply be the result of overcrowding, as is seen in many cellular structures in tissue culture where, in the absence of a blood supply, the formation of expanding necrotic centres does not arrest the growth of the cells in the surrounding rim. Whatever the cause or causes of the necrotic centres, it is clear that malignant tumours with that morphology can grow beyond any arbitrary limited size, and that they are lethal. It seems remotely improbable that attempts to limit their vascularization, if any exists, would much influence the outcome.

But there is a more fundamental question. A substantial body of meticulous work in the 1960s established the fact that tumour blood vessels are profoundly defective in many respects. They do not adequately oxygenate the tumour tissue. It is therefore not enough to show that in a malignant tumour new blood vessels are formed; one must also show that they are effective. In recent years, little attempt appears to have been made to see whether, in the tumours being studied, this is the case.

All this does not in the least detract from the importance of studying angiogenesis in its own right. The formation of new blood vessels is a fundamental characteristic of tissue development and repair, and a great deal of valuable information has certainly been obtained by studies on the formation of new blood vessels. Nor is it excluded that some particular malignant tumours may, at least in their initial stages, have a more intimate relationship with their internal vasculature. But in the light of the evidence that I have discussed and especially the observation that some malignant mouse tumours can grow and kill in the absence of perceptible vascularization, it is difficult to see the notion that malignant growth is controlled by blood supply as much more than an optimistic surmise.

Apoptosis

When, in 1972, Alistair Currie[7] asked the professor of Ancient Greek in Edinburgh to suggest an appropriate word to describe physiological, genetically determined forms of cell death, he came away with the word 'apoptosis' (literally, a falling away); the word 'necrosis' he retained for cell death that was adventitious. Currie and his colleagues had noted that these two categories of cell death could be distinguished by certain morphological features that were apparent in fixed sections of human tissue. Homer uses the term apoptosis to describe the

generations of men falling away like the leaves of a tree. The Homeric word is appropriate in the present connection in so far as it refers to a natural pre-ordained process; but it is less appropriate in that leaf-fall is a more or less synchronized process, and synchrony is in no way a defining feature of the many forms of physiological cell death that Currie subsumed under the term apoptosis.

During the 19th century, biologists came to realize that waves of cell death took place not only in plants, but also in the development of animal forms, and it is these synchronized events that eventually acquired the name programmed cell death. In its traditional sense, programmed cell death describes a genetically determined process that results in the more or less synchronous breakdown of a large number of cells at a particular site and thereby permits certain morphological specializations to take place. The classical example in man is the dissolution of the cells in the interdigital regions to form the clefts that separate the fingers of the hand. Programmed cell death did not originally refer to the many other forms of cell death that may be involved in determining the ultimate size and shape of all organs and organisms nor to the variable turnover of cells that takes place in most parts of the body. The terminological problems began when apoptosis and programmed cell death came to be used interchangeably. I have no desire to guide the evolution of language, but the progressive extension of what is meant by programmed cell death has certainly given rise to a great deal of semantic confusion and, along the way, the distinction between synchronous and asynchronous processes has been lost.

Perhaps surprisingly, the term apoptosis caught on, and the biochemistry of cell death soon attracted the interest of large numbers of molecular biologists. Once again, we were inundated with a torrent of papers that rapidly defined many of the genes involved in cell death and their modes of action. But, for all that, the theory that impairment of apoptosis provides a general explanation for the genesis of malignancy is, in my view, misleading. A glance at the histopathology of squamous skin carcinomas, for example, reveals that the cancer is capable of generating populations of cells that undergo terminal differentiation and are shed as lifeless keratinized squames. In these cells there can be no block to apoptosis, however it is defined. But it is generally agreed that malignant tumours are clonal or oligoclonal in origin. If this is so, the problem at once becomes more complex. First, one must determine how, in any particular case, a single cell produces descendants that undergo terminal differentiation and others that continue to reproduce themselves (the so-called stem cells). That, of course, is the classical dilemma at the heart of differentiation. And second, one must determine what has gone wrong with the normal balance between cell death and cell replacement. Regrettably, we do not yet have a clear idea of what, in molecular terms, the nature of that imbalance might be. The study of apoptosis has obviously made a major contribution to our understanding of the biochemistry of cell death, but it does not provide a plausible analysis of how malignant tumours originate.

Aneuploidy

Most cytogeneticists would take the view that the temporal relationship between the appearance of aneuploidy in a tissue and the emergence of a tumour had long ago been established. But a recent contention that aneuploidy is a secondary effect of tumour growth and not a condition that precedes the formation of the tumour has reopened the question. In support of this position, the claim has been made that euploid malignant tumours can be produced.[8] These experiments were at once challenged and the supposedly euploid tumours were found to be aneuploid.[33] An acerbic passage of arms followed and attracted a great deal of attention.[34] In support of the view that aneuploidy is a secondary effect, Hernando et al. have recently shown that inactivation of the *Rb* gene induces destabilization of the karyotype.[35] In the light of the almost universal experience, one might doubt whether euploid malignant tumours were indeed produced (even haematological malignancies turn out, on closer inspection, to be pseudodiploid, not euploid), and it is hardly surprising that a mutation in a gene such as *Rb* might induce destabilization of the karyotype. That finding does not resolve the question whether aneuploidy precedes or succeeds the formation of the tumours.

The matter, to my mind, was settled in the 1960s and 1970s. In 1959 Levan,[36] reviewing the experimental evidence, came to the conclusion that aneuploidy reflected continuous structural remodelling of the karyotype until eventually a variant was produced that could grow progressively in vivo. The meticulous karyological studies of Spriggs and his colleagues[37–39] and Stanley and Kirkland[40] on cancerous and pre-cancerous conditions of the human uterus established that: (1) aneuploidy was present in the epithelia long before any tumour appeared, and (2) when frank cancers did emerge, the mode of chromosome numbers narrowed and marker chromosomes appeared, supporting the view that the tumours were clonal outgrowths arising from a background of cells with disordered chromosome complements. The work of Spriggs et al. on carcinoma of the cervix uteri is of special interest in a clinical context. The variation in the size and shape of the cell nuclei seen in pre-cancerous epithelium is a reflection of the underlying aneuploidy. It is clear that karyological disorder may be present in the cervical epithelium whether a tumour is produced or not.

Is it not time to consider an alternative model?

It can only be a source of amazement that after so much effort, so much skill, such dramatic increases in our knowledge of genes and how they act, that there is still no agreement about

how malignant tumours are generated. One often hears the plea that the problem is so complex that massive frontal assaults on it have not been as enlightening as might have been hoped. But this seems an unconvincing explanation. Is it just possible that we have been looking in the wrong place? I am going to argue that this is so, and that a plausible alternative for the formation of malignant tumours is available, that this model has strong evidence to support it and that it is now time to explore it.

I have presented an outline of my position in a recent publication.[3] There I made the following suggestions. (1) The inherent steady state of all cells is not 'rest' but exponential multiplication.[1] As a consequence of evolution, all cells are so constituted that, given adequate nutrient and a clement environment, they will multiply exponentially without further stimulation. To look for stimuli to cell multiplication is thus supererogatory. (2) Leaving aside conceptually unproblematical factors such as external toxicity, there is only one natural process that restrains exponential multiplication of cells during the development of an organism and that is differentiation. (3) It then follows that, if cells do not require stimulation in order to multiply exponentially then the disordered cell multiplication seen in malignant tumours must be the result of an error in differentiation.

Let me now flesh out the argument and consider the evidence. The crucial evidence is provided by the work of Mechler and his colleagues on *Drosophila*.[41–43] By the use of genetic mosaics that permit the accurate timing of events, Mechler was able to show that, during the development of the organism, tumours arose at specific times and at specific sites when mutations occurred that blocked the execution of critical steps in the process of normal differentiation. The review published by Mechler and his colleagues in 1994[43] manifests the scope and cogency of the *Drosophila* experiments that had already been done at that time. Some 40 genes had been identified that gave rise to overgrowth of cells when the genes were put out of action by mutation. Of these, 11 had been cloned, the gene product identified and, in some cases, its biochemical function elucidated. These genes exerted their effects in a wide range of different tissues including the imaginal discs, brain, haemopoietic system, ovary and cuticle. By the use of direct molecular cloning techniques, the frequency of such genes in different parts of the chromosomes could be estimated and an assessment made of the total number in the whole *Drosophila* genome. Mechler's estimate was that, in all, there may be in excess of 100 such genes. They have the following characteristics. (1) The mutations that produce an overgrowth of cells engender a loss of normal function, not a gain of a new function. (2) The function lost is an essential part of the normal differentiation programme of the tissues affected. (3) Inactivation of these genes produces a spectrum of hyperplastic reactions that range from benign hyperplasias to frankly invasive tumours. (4) At sites where they do not generate cellular overgrowths, these same genes, when inactivated, may produce other developmental abnormalities.

These studies have permitted a more refined analysis of tumorigenesis than has so far been possible in any other organism, and it remains a puzzle to me why the cardinal importance of this work has not been widely appreciated by those involved in cancer research. My suspicion is that the work on *Drosophila* is not seen to be of immediate relevance to the cancer problem in mammalian somatic cells. It has been observed that tumorigenesis in *Drosophila* is limited to the embryo whereas the great majority of human cancers arise in mature, indeed aged tissues. Given that there is essentially no cell multiplication in the tissues of the adult insect, this observation is hardly surprising, but it does not militate against the relevance of the *Drosophila* experiments to man. There was indeed a time when there was great enthusiasm for the theory that sporadic human cancers arose from 'embryonale Reste' (embryonic remnants). What the *Drosophila* experiments show is that we are no longer dealing with the generalities of differentiation or its gross features, but with a manageable number of specific genes that play critical roles in linking differentiation to cell multiplication. Nothing of comparable precision has yet been achieved with mammalian cells, but what evidence there is is entirely consistent with Mechler's data. In hybrids formed by fusing a range of different malignant tumour cells with normal diploid fibroblasts or diploid keratinocytes, tumorigenicity is suppressed when the composite cell retains the ability to execute the differentiation programme of the fibroblast or the keratinocyte. But when the ability to execute these differentiation programmes is lost, tumorigenicity reappears.[44,45] The study of Bremner and his colleagues on retinoblastomas induced by the inactivation of the *Rb* gene shows, in what is the first analysis of differentiation in the mammalian retina in the absence of the *Rb* gene product, that an impediment to retinal differentiation is an essential element in the formation of the malignant tumour.[46]

Conclusion

My argument, then, is that the initiating event in the formation of a malignant tumour is a block to some critical step in the process of normal differentiation. The data at present available indicate that this is usually a mutation that inactivates a specific gene, but it is possible that other mechanisms may eventually emerge. Is it not reasonable, in the light of this body

[1] Historical note (requested by the editor). In the English language literature this view of cell multiplication seems to have been first set down in print in 1958 in a chapter on Cell Growth and Cell Multiplication written by Henry Harris for Florey's "General Pathology" (2nd edition) Lloyd-Luke, London, p. 439–440. It is reiterated in all subsequent editions of that text book (3rd edition 1962, 4th edition 1970). In recent years, this idea has been elaborated by Ana M. Soto and Carlos Sonnenschein, notably in their monograph "The Society of Cells" 1999, BioScientific Publishers, Oxford, UK.

of evidence, to ask that this model should now be explored with the massive molecular armamentarium at our disposal? Can preconceived notions be set aside for a moment and the proposition be considered that cancer originates as a disease of differentiation?

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