The Analysis of Malignancy by Cell Fusion: The Position in 1988

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The Suppression of Malignancy

Let us begin by putting some old controversies to sleep. There is now no doubt that, when a wide range of different malignant cells are fused with normal fibroblasts, the resulting hybrid cells, so long as they have not lost certain specific chromosomes derived from the normal parent cell, are unable to grow progressively in genetically appropriate hosts. In the majority of cases where this result has not been obtained, the probable explanation is that the relevant chromosomes derived from the normal cell have already been eliminated from the hybrid cells before their ability to grow in vivo is tested. [A good example of this is the claim by Croce et al. (1) concerning the dominance of malignancy in hybrids between HT1080 sarcoma cells and normal fibroblasts, and its refutation by Benedict et al. (2).]

Several recent articles have reviewed the range of experiments in which fusion of malignant cells with normal fibroblasts has resulted in the suppression of malignancy as defined by progressive growth in vivo (3–7).

There do, however, appear to be some genuine exceptions to this general rule. It has been shown, for example, that the ability of normal cells to suppress malignancy in hybrids is gene dosage dependent (8); it sometimes happens that, where multiple copies of a particular chromosome derived from the malignant parent cell are present, the contribution made to the hybrid cell by only one copy of the corresponding normal chromosome is insufficient to suppress progressive growth completely. Examples of this kind of gene dosage effect are seen in experiments that show suppression of malignancy in hybrids containing one normal and one malignant chromosome set, but no suppression in similar hybrids containing one normal and two malignant chromosome sets (2). Moreover, although there is decisive evidence for the suppression of malignancy when malignant cells are crossed with normal fibroblasts or normal keratinocytes (9, 10), crosses in which the normal parent cell is a lymphocyte remain to be fully explored. Crosses have been described in which fusion of malignant cells with normal lymphocytes has yielded hybrids that are unable to grow progressively in vivo (11), but in other crosses this result has not been obtained (12). In most of these experiments, the lymphocytes used in the cell fusion were resting (noncycling) cells, and the hybrids, where examined, already showed a substantial deficit of normal lymphocyte chromosomes as soon as they were isolated. However, no satisfactory karyological analysis of lymphocyte hybrids has yet been done.

Surprising as it may seem, this is also true for crosses between myelomas and normal lymphocytes (the so-called hybridomas). Superficial analysis reveals that the hybridomas consistently show variable chromosome losses, but their significance has not yet been investigated by modern methods. The myelomas commonly used to produce monoclonal antibodies have grossly disordered karyotypes, are subtetraploid, and may have multiple copies of many genes. Even so, many hybridomas grow in the peritoneal cavity of genetically compatible mice only after a considerable latent period; this suggests that the cells that grow progressively under these conditions are a selected subpopulation of the cells injected. Whether a normal lymphocyte can suppress malignancy in the same way as a normal fibroblast or a normal keratinocyte is not a trivial question. If lymphocytes turned out in general to be unable to do so, then the suppression of malignancy in hybrid cells would have to be a function of a particular differentiated state in the normal cell and not a general property of a normal chromosome set. This question is clearly central to any analysis of the mechanism of suppression. It is discussed further at a later stage and is currently being investigated in a systematic way in two laboratories.

How Many Chromosomes Are Involved in the Suppression, and Which?

When, in 1969, it became apparent that chromosome loss could determine the reappearance of malignancy in hybrids in which it was initially suppressed, the suggestion was made that the loss of a single chromosome might be enough to induce this reappearance (13). It has taken several years of careful cytogenetic analysis and the development of new methods to establish that this is indeed the case, at least in some crosses. When a wide range of different malignant mouse tumors are crossed with normal mouse fibroblasts, suppression of malignancy in the hybrid cells requires the retention of the chromosome 4 derived from the normal cell (14). If both copies of this chromosome are retained, the hybrid cells are unable to produce any tumors. If a single copy is retained, some tumors are produced, but the take incidence is low, and examination of the tumors themselves reveals that there is further selection in vivo in favor of cells that have eliminated both copies. The analysis of hybrids in which the normal chromosome 4 was involved in a translocation permitted localization of the critical region to the lower part of the upper half of this chromosome. These experiments suggested that the genes on chromosome 4 responsible for the suppression of malignancy acted in a dose-dependent manner.

This was confirmed by the analysis of intraspecific hybrids in which the chromosomes 4 derived from the normal parent cell could be clearly distinguished from those derived from the malignant parent cell (8). It transpired that the number of normal chromosomes 4 required to suppress malignancy was sensitive to the number of malignant chromosomes 4 present in the hybrid cell. This finding provides some basis for elucidating the observation described above that a normal fibroblast may suppress malignancy when the hybrid cell contains only one malignant chromosome set, but not when it contains two. These observations do not correspond in any way to the "chromosome balance" model proposed by Hitotsumachi et al. (15). In the latter, it was proposed that malignancy was determined by the balance within the cell between one set of chromosomes that collectively favored the emergence of malignancy and an...
other different set that collectively favored its suppression. The interpretation given here to the role of the normal chromosome 4 in the suppression of malignancy is, on the other hand, formally equivalent to the interaction in a diploid cell between a mutated gene and its wild-type allele.

The results obtained with mouse cell hybrids have exact equivalents in hybrids between human tumor cells and normal human fibroblasts. In crosses between human fibrosarcoma cells (HT1080) and normal human fibroblasts, Benedict et al. (2) found that the reappearance of malignancy in hybrids in which it was initially suppressed was systematically associated with the loss of chromosome 1 from the normal parent cell; and a similar finding was made by Stoler and Bouck (16) in crosses between transformed hamster cells and normal human fibroblasts. Since there is an extensive region of homology between chromosome 4 in the mouse and chromosome 1 in humans (17), it seems probable that the suppressive effect was achieved by functionally equivalent genes in the two species.

A more detailed analysis has been made of the role of human chromosome 11 in suppressing malignancy. In crosses between human uterine carcinoma cells (D98AH2) and normal human fibroblasts, it has been shown that the loss of a single chromosome 11 derived from the normal fibroblast can induce the reappearance of malignancy in hybrids in which it was initially suppressed (18, 19). Moreover, gene dosage effects similar to those found for mouse chromosome 4 have been demonstrated for human chromosome 11 (20, 21). Hybrids containing two copies of the normal human chromosome 11 show more stable suppression of malignancy than do those containing only one copy. Direct evidence has been obtained that the normal chromosome 11 can suppress malignancy even in the absence of any other chromosome derived from the normal parent cell. When a single chromosome 11, originating in a normal human fibroblast, is introduced by microcell transfer into cells derived from a uterine carcinoma or from a Wilms' tumor, the ability of these cells to induce progressive tumors in nude mice is suppressed (22, 23). No other chromosome has yet been shown to suppress malignancy in isolation; the extensive study by Klinger and Kaelbling (21) of crosses between the malignant D98AH2 cell line and normal fibroblasts has provided evidence that, if other chromosomes are involved in the suppression, they act only in cooperation with chromosome 11.

How Many Different Genes?

The available evidence suggests that, in any one cross, very few genes, and perhaps even only one, might be responsible for the suppression of malignancy. We do not yet know whether the suppressive effect of a single normal chromosome involves the action of one gene or of a group of syntenic genes, but if, for purposes of discussion, one defines the active region as a locus, then it is remarkable that, in crosses with normal fibroblasts, one locus will suppress the malignant phenotype in a wide range of different tumors. This sets important restrictions on models of how, in biochemical terms, the suppressive effect might be brought about. However, as mentioned above, we still do not know that the same locus will suppress malignancy when the suppressive chromosome is derived from a normal cell of a differentiation lineage other than that of the fibroblast. If, for example, it was found that chromosome 11, when derived from a normal human lymphocyte, failed to suppress malignancy on insertion into a Wilms' tumor cell, or that some other chromosome, when derived from a normal human keratinocyte, determined this suppression, then two conclusions of extreme importance could be drawn. The first would be that chromosome 11 in a fibroblast is genetically different from chromosome 11 in a keratinocyte or lymphocyte, i.e., that the process of differentiation produces stable, heritable changes in the chromosomes themselves. The second conclusion would be that suppression of malignancy must, in some cases, be achieved by a mechanism other than the simple complementation of a genetic defect by the introduction of an unaffected allele. In the case of the mouse cell hybrids that have been studied, the normal mouse chromosome 4 determines the suppression of malignancy in a very wide range of different tumors, different not only in their histological type but also in their etiology. If the suppressive chromosome acted merely by complementing a genetic defect at an allelic locus, then the results obtained with the mouse cell hybrids would imply that the same locus is defective in a very wide range of different tumors. Whether or not this idea is plausible will be considered later.

The Mechanism of Suppression

In molecular terms, we have only fragmentary information about the mechanism by which the suppression of malignancy is achieved. The most interesting insight derives from a morphological observation first made by Stanbridge and Ceredig (24). They found that, when crosses between D98AH2 carcinoma cells and normal human fibroblasts were injected into nude mice, hybrids in which malignancy was suppressed behaved quite differently in vivo from segregants of these hybrids in which malignancy had reappeared. Malignant segregants grew progressively as undifferentiated epithelial tumors; but the cells in which malignancy was suppressed assumed an increasingly fibrocytic morphology and gradually ceased to multiply. This observation was explored more deeply in a range of different crosses between malignant mouse tumors and normal mouse fibroblasts (25). In all cases where malignancy was suppressed, the hybrid cells produced a copious collagenous extracellular matrix, assumed a progressively more fibrocytic morphology, and eventually ceased to multiply. The final lesion resembled the alignment of resting fibrocytes that one sees in an immature scar. However, where chromosome loss had permitted the reappearance of malignancy, the segregant cells failed to produce the characteristic collagenous extracellular matrix, did not assume a fibrocytic morphology, and continued to multiply. It thus appeared that the suppression of malignancy in the hybrid was associated with the execution of the pattern of terminal differentiation characteristic of the fibroblastic parent cell. When, as a consequence of chromosome elimination, the hybrid cell lost the ability to complete this program of differentiation, the malignant phenotype reappeared.

An essentially similar pattern was seen in crosses between D98AH2 carcinoma cells and normal human keratinocytes (9). In this case, suppression of malignancy in the hybrid cell was associated with typical squamous differentiation in vivo, the terminal program of the keratinocyte; and reappearance of malignancy was associated with loss of the ability to execute this program. In the case of keratinocyte crosses, some reduction of the phenomenon to molecular terms has been achieved. It has been shown that hybrids in which malignancy is suppressed continue to produce large amounts of involucrin, the key molecular marker of terminal differentiation in the keratinocyte; but malignant segregants produced no involucrin or very little (10).

It is clear that the execution of two quite disparate terminal differentiation programs (that of the fibroblast and that of the...
keratinocyte) can bring progressive cell multiplication in vivo to a stop. It therefore seems reasonable to infer that at least one functional defect in the malignant cell with which the fibroblast or keratinocyte is fused is that it has lost the ability to complete its own program of terminal differentiation. This idea is simply a reformulation of the general proposition that the progressive multiplication of malignant cells is a consequence of their having undergone some genetically stable impairment of their ability to complete their normal program of differentiation. The arguments in favor of this proposition have been considered elsewhere (5, 25). If it is true, then one can envisage that malignancy might be suppressed in hybrid cells in two ways: either by the imposition of the terminal differentiation program of the normal parent cell or by complementation of the genetic defect that impedes differentiation in the malignant parent cell. Where malignancy is suppressed by the introduction into the malignant cell of a single normal chromosome, as in the experiments of Saxon et al. (22), it is perhaps unlikely that the complete differentiation program of the normal parent cell would be imposed; it is known, for example, that different components of the extracellular matrix laid down by fibroblasts are specified by genes that map to different chromosomes. It seems more probable that, in this case, the introduction of the single normal chromosome does actually complement a specific gene defect in the malignant cell. In analyzing the phenotype of hybrid or reconstituted cells of this type, it is therefore of importance to examine the parameters of terminal differentiation not only of the normal parent cell, but also of the malignant parent cell. Regrettably, in most cases, malignant parent cells have been chosen whose terminal differentiation program has so far not been seriously explored from the biochemical point of view. It would be worthwhile repeating these experiments in which malignancy has been suppressed by the introduction of a single normal chromosome, but with malignant cells whose pattern of terminal differentiation has been better characterized in molecular terms.

Dominance, Recessivity, Gene Dosage, and Complementation

Since the word “dominant” has now become decadent through misusage, it will perhaps not give offense if I make the point that “dominance” and “recessivity” in Mendelian terms refer to the interaction between two alleles in a diploid organism; where there are only two alleles, one of them is said to be dominant if the phenotype it specifies is detectable in the organism and recessive if it is not. In hybrid cells we have, in principle, four alleles. In the context of the suppression of malignancy, we can conclude from the gene dosage effects that have already been described that the normal parent cell does contribute two functionally equivalent alleles. But in the case of the malignant parent cell, the position is usually obscure and has only rarely been examined. It is known that malignant cells are often hemizygous or homozygous at many loci (26, 27), and they may contain more than two copies of some genes, either as tandem duplications or as multiple copies of chromosomes. There have been very few studies on the suppression of malignancy in which any systematic attempt has been made to take into account the contribution, in terms of gene dosage, of specific loci or even specific chromosomes derived from the malignant parent cell. Nonetheless, with the reservations that have been made, the overall picture is not obscure; hybrids containing one chromosome set derived from the malignant parent cell and one chromosome set from a normal fibroblast are not malignant, as defined by their ability to grow progressively in vivo, provided that, in the case of mouse crosses, they retain both copies of the normal chromosome 4 and, in the case of human crosses, both copies of the normal chromosome 11.

Although there are a few adequately studied cases in which fusion of one malignant cell with a malignant cell of a different kind has yielded hybrids that are not malignant, most crosses between one malignant cell and another yield hybrids that are malignant. If the genetic determinants of malignancy are indeed recessive, then failure of two different malignant cells to complement each other indicates that they share a mutation at the same locus. When this suggestion was first made, it was greeted with incredulity, but in 1988 it does not seem so categorically implausible. As discussed in a more general context in the following section, a homozygous loss of alleles in the same chromosome region (11p) is found in a range of different tumors that currently includes Wilms’ tumor (28-30), hepatoblastoma, rhabdomyosarcoma (31), and adult tumors of the kidney (32), bladder (33), liver (34), and breast (35). Similarly, a homozygous loss of alleles in the 14q segment of chromosome 13 is found in retinoblastoma (36-39), osteosarcoma (40), leukemia, and other hematological abnormalities (41). It is therefore not at all impossible that, in the small range of malignant tumor cells that have so far been tested for complementation by cell fusion, the two cells that were fused together might have sustained a homozygous loss of alleles at the same locus.

However, there is perhaps a less fortuitous explanation for noncomplementation between different kinds of malignant cells. If the essential genetic lesion determining the malignant phenotype is a loss of the ability to complete a program of terminal differentiation, then two cells, each committed to a different program, might not complement each other even when the loss of alleles affects different loci. The great “Exclusion Principle” of cell biology is that a cell committed to one program of differentiation cannot simultaneously execute another; in a keratinocyte, for example, one must assume that the genes that determine hepatocyte differentiation are silent, and vice versa. If a keratinocyte that had sustained a genetic lesion that impaired the execution of its normal program of terminal differentiation were fused with a hepatocyte that had similarly sustained a lesion that impaired its program of terminal differentiation, each cell would contribute to the hybrid the alleles that were in principle capable of complementing the function that was impaired in the other partner; but in both cases these wild-type alleles would be inactive and might remain so. This model is anything but farfetched, for it has in fact been experimentally demonstrated that the genetic imprints that determine major lineages of differentiation are stably maintained in hybrid cells (42). In any case, whether noncomplementation in crosses between different malignant cells represents loss of identical alleles, or whether it represents a more complex interaction involving the genetic stability of differentiation patterns, the phenomenon should not now occasion the surprise that originally accompanied its discovery.

A Current, but Probably Transient, Difference of Opinion

The proposition that the genetic determinants of malignancy are recessive has found dramatic confirmation over the last few years in the now substantial, and rapidly growing, body of evidence that a cardinal event in the genesis of the malignant...
phenotype is the unmasking of recessive mutations by mechanisms that confer homozygosity. Although homozygous loss of alleles was first demonstrated in childhood tumors with a clear hereditary component (retinoblastoma, Wilms' tumor), it is now clear that a similar mechanism also operates in malignant tumors of the adult, including major malignancies such as cancer of the lung (43, 44), breast (35), bowel (45), and bladder (33). Since the search for homozygous recessives is still in its infancy, there seems little doubt that other examples will be found. Indeed, it would not be rash to predict that the great majority of malignant human tumors will be found to have sustained a homozygous loss of alleles.

There is, however, a loosely formulated doctrine that asserts, on the contrary, that there is a large family of genes (oncogenes) that determine malignancy in a genetically dominant manner. The root of the problem here is a semantic one: an insecure use of the word "dominant." Many of these oncogenes have been detected by an assay that involves the introduction of DNA into certain specialized cell lines, notably NIH 3T3, and the subsequent selection of clones that show morphological transformation. Since the interpolated oncogene may produce this transformation against the genetic background of a cell that does not initially show it, it is often said that the oncogene acts in a "dominant" manner or that it is "dominantly selectable." "Dominance" in this context is, of course, not at all equivalent to dominance in the classical genetic sense. This question has been discussed in detail elsewhere (5), but the following points bear repetition.

1. It has been shown that the introduction of an oncogene into NIH 3T3 cells or other untransformed cell lines of this type, all of which are aneuploid, produces multiple stable changes in the genome of the recipient cell (Refs. 46 and 47; Footnote 1). Against this complex background of genetic changes, no conclusion concerning the dominance or recessivity of the mode of action of the interpolated oncogene is possible, even when the parameter being studied is no more than morphological transformation in vitro.

2. The great majority of morphologically transformed cells are not malignant in the sense that they are capable of progressive growth in vivo. When the morphologically transformed cells are injected into an appropriate host, it can easily be shown by karyological analysis that the cells capable of progressive growth in vivo (those that generate the tumor) are a highly selected subpopulation.

3. Once malignant cells have been so selected, continued expression of the interpolated oncogene is not required for maintenance of the malignant phenotype.1

4. In the few cases where the question has been specifically examined in genuine tumors, it has been found that mutated oncogenes are frequently present in the hemizygous condition (48, 49).

5. When malignant cells containing known oncogenes that are actively expressed are fused with diploid fibroblasts, malignancy is suppressed whether or not the oncogene remains active in the hybrid cell (50–52).

If, by the term "dominance," one means dominance in the classical Mendelian sense, there is no evidence, as far as I can see, that any oncogene acts to produce malignancy in a genetically dominant fashion.

What Now Needs to Be Done?

1. As mentioned above, it is of critical importance that the range of normal diploid cells used in cell fusion experiments should be extended, so that the role of terminal differentiation in the suppression of malignancy can be more precisely delineated. There are no serious technical impediments to this being done. The experimental requirements are patience and expert cytogenetics.

2. Complementation experiments should now be done with human malignant tumors in which the location of a homozygous loss of alleles has been established. It would be of importance to cross, say, retinoblastoma cells, where the homozygous deletion is on chromosome 13, with Wilms' tumor cells, where it is on chromosome 11, and, conversely, to cross two different tumors having the homozygous loss of alleles at the same locus, for example, retinoblastoma and osteosarcoma. If complementation and noncomplementation occurred as expected on formal genetic grounds, then comparatively simple explanations for the genesis of malignancy could be advanced. If, however, the results were not explicable in formal genetic terms, explanations involving genetically imprinted patterns of differentiation would have to be envisaged. All that is required for these experiments is the introduction of appropriate selectable markers into the tumor cells.

3. The genes determining suppression of malignancy should be identified. This may or may not be very informative from the biological point of view, but it must be done. The outcome may be disappointing because, as in the case of the putative retinoblastoma (Rb) gene, the nucleotide sequence when determined may resemble no other and may contain no features that provide a clue to function. On the other hand, one might be lucky. The greatest piece of luck, in my view, would be to find that the gene that suppressed malignancy was one that played a key role in determining a pattern of terminal differentiation. That would tie malignancy and differentiation together at the molecular level. The main obstacle to progress in identifying the suppressive gene or genes has been the absence of a practical method for selecting nonmalignant cells from a malignant cell population. Several authors have approached this problem by transfecting DNA from normal cells into malignant cells and isolating colonies with less transformed morphology in vitro. However, I am not aware that any of these experiments have yet provided decisive evidence that a specific suppressor gene has been identified. Another approach to the problem would be the now conventional search for a restriction fragment length polymorphism that was consistently associated with the suppression of malignancy and, if one were found, to walk (or, more aptly, trudge) to the gene. But there is, of course, no assurance of success in this kind of approach, and most investigators would find it unattractive without the availability of slave labor. It could be that the identification of the gene or genes that suppress malignancy may yet require a sizeable injection of ingenuity.

Envoi

It is now almost 20 years since the suppression of malignancy by cell fusion was first described (53). The first decade was largely devoted to convincing a skeptical public that the phenomenon existed. The last few years have been much more interesting. However, there are crucial questions about the mechanism of suppression that remain unanswered. It is my optimistic view that, when these questions are answered, we will learn something fundamental not only about malignancy, but also about differentiation.
References

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