

Hormones, anchorage and oncogenic cell growth

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When objects on the exterior membrane press,
The alarm runs in most thro' each dark recess,
Impulsive strikes the corresponding springs,
And moves the accord of sympathetic strings...

Henry Brooke
Universal Beauty, iv, 41-44

ABSTRACT

Oncogenic transformation of cultured cells is thought to mimic the heritable loss of growth control that is the earliest step in the development of a neoplasm. While the mechanism of oncogenic transformation remains unknown, current phenomenology provides boundaries sufficient to permit the construction of a hypothesis. According to this hypothesis, the critical event in normal growth control is the restriction by cell anchorage of lateral movement of different hormone receptors in the cell membrane. This hypothesis generates a series of experimentally testable predictions for the roles of oncogenic viral gene products.

INTRODUCTION

The in vivo and in vitro endpoint assays applied to cells after exposure to a potential oncogenic transforming agent are cellular tumorigenicity and transformation. Tumors are a failure of in vivo growth control; transformations are failures of in vitro growth control. Many agents cause tumors in vivo, many agents transform normal cultured cells, and some agents do both. However, even when caused by a single agent, the in vivo and in vitro endpoint assays show only a partial

overlap. For example, with papovaviruses only some but not all virus-induced tumors will grow as transformed cells in culture, and only some but not all in vitro transformants will be tumorigenic upon injection into susceptible animals^{1,2}. Recently we have described a subset of in vitro phenotypic changes matching in vivo tumorigenicity for one agent capable of acting in both assays, the papovavirus SV40^{3,4}. I will detail this subset, and then present a hypothesis to link its parts as perturbations of a single mechanism of growth control operating in normal cultured cells.

SUBSET OF CELLULAR CHANGES YIELDING TUMORIGENIC TRANSFORMATION

1) Serum requirement reduced

Many tumor cells, and some normal cell lines, can be grown in defined media with mixtures of hormones, in lieu of serum^{5,6,7}. We will adopt Sato's term and say that all cells have a hormone "address", consisting of the sum of hormones necessary for cell proliferation. This address may be more or less complex, but all fibroblast lines studied to date, whether normal or transformed, need at least two hormones for growth in the absence of serum. Complete deprivation of any one of the hormones in the address is sufficient to prevent the growth of a normal cell.

2) Anchorage requirement reduced

A normal cell must spread out in order to proceed through the cycle^{8,9}. The anchorage assay deprives a cell of a substrate on which to spread, and thereby prevents cell division. The normal point of spreading is at the M/G1 boundary,¹⁰ close or identical to the point of blockade by hormone deprivation¹¹. Anchorage-transformed cells are almost always found to have acquired the ability to grow in low serum, but not vice versa^{12,13}. This suggests that a change in hormone address may be prerequisite to a loss of the need to spread in order to divide. Tumorigenicity and anchorage-independence are well correlated, both qualitatively^{1,2,14} and quantitatively^{3,15,16}. Tumorigenic cells by this correlation have the ability to proceed past the M/G1 boundary without spreading.

3) Saturation density increased

A population of normal cells can maintain a stable maximum cell density despite repeated feeding with fresh

nutrients¹⁷⁻²⁰. Both the serum and anchorage requirements contribute to this saturation of cell density⁸. The cells of any dense culture are actually less well spread than those in a sparse culture⁸. Since anchorage dependence means that cell thickness must be reduced to a threshold before normal cells can divide, saturation is likely to be the cell density for a given serum concentration at which the spreading that occurs cannot reduce cell thickness to this degree. Saturation densities of both normal and transformed cells increase with serum concentration¹⁷. This ability of increased serum to counter the requirement for spreading suggests that spreading may be a necessary part of a normal cells's mechanism for making efficient use of limiting amounts of serum growth hormones.

4) Fibronectin is decreased

In the normal cell, the secreted glycoprotein fibronectin covers regions of cell anchorage, both to substrate and to other cells^{21,22}. Transformation is sometimes accompanied by reduction of about 10 to 100-fold in surface associated FN²³. FN can be crosslinked by transglutaminases (eg clotting factor XIII) to an extracellular matrix that contains collagen and glycosaminoglycans²⁴. FN can be released from the cell surface by mild denaturation (in urea) or very low concentrations of certain proteases²⁵. The proteases do not cleave FN, but rather disrupt as yet undefined FN receptor proteins at the cell surface^{21,24}.

5) Cytoskeleton is disorganized

The major intracellular protein of both normal and transformed cells is actin^{26,27}. The actin of a normal spread cell is partitioned by cofactors into four major states^{26,28,29}. These states are a soluble complex that is unable to participate in the formation of long chains of F-actin,^{28,30} and three different macromolecular states that have double-helical F-actin as a core. The microfilament gel consists of poly-actin, an actin-binding protein such as filamin³¹ and the Ca binding protein calmodulin³². The gel is located just under the membrane²⁹. Microfilament cables contain poly-actin, myosin, α -actinin and tropomyosin³³. In normal cells, cables are located primarily at the adherent side of the cells just under the surface³⁴. Thus, they share a localization with that part of the microfilament gel which also lies under the adherent surface. Finally, an isotropic matrix of single microfilaments fills the cytoplasm, interacting with

microtubules³⁵, and intermediate filaments³⁶⁻³⁹. In tumorigenic anchorage-transformed cells, the actin is repartitioned among these four states. More of the actin is found in microfilament gel, and less of it in the cables^{40,41}. This last event is most dramatic because the cables, mostly in one plane of focus, are easily visualized by immunofluorescence microscopy^{39,42,43}.

6) Lateral mobility of trans-membrane proteins is increased

Freeze-fracture studies of normal cells show that trans-membrane proteins (TMPs) are evenly distributed in the cell surface^{30,44,45}. Binding of a number of agents to the extracellular portion of TMPs eventually leads to directed lateral migration and finally to clustering of the TMPs in patches on the cell surface. Lectins, antibodies and hormones are examples of patching agents⁴⁶⁻⁴⁸. Clustering and patching are very slow in normal, spread cells compared to the response in transformed cells^{48,49}. This difference underlies the classic observation that transformed cells are agglutinated by lectins more rapidly and efficiently than normal cells: in the presence of lectins, patches of lectin-receptor are observed at regions of cell-cell sticking⁴⁶.

7) Proteases are secreted

A normal, well spread fibroblast releases little if any proteolytic activity into the medium. Such cells also have little capacity to proteolyse a variety of substrates on which they can spread, including fibrin and FN, so it is also unlikely that most cells have appreciable proteolytic activity associated with their outer surfaces⁵⁰. However, for a brief period, as normal cells proceed through G2 to M, they do surround themselves with proteolytic activity⁵¹. Presumably this proteolysis is a necessary part of rounding. Most tumorigenic transformed cells synthesize copious amounts of neutral serine protease, plasminogen activator (PA)^{13,52}. Synthesis occurs throughout the cell cycle, not just at the G2/M interface^{53,54}. While the substrates for plasmin are limited, they include FN as well as fibrin. In the presence of plasminogen, a transformed cell making PA becomes bathed in plasmin⁵². Thus a normal cell in mitosis, and a transformed cell at all times, usually are surrounded by higher than normal amounts of the two proteases PA and plasmin.

8) Cell cycle transition probability increases

Normal cells, restricted by serum deprivation or by

suspension without anchorage, have an extremely low probability of progressing through mitosis into the S period. When this probability is low enough, it becomes experimentally indistinguishable from a synchronizing block at G1. Nevertheless under all degrees of restriction the probability of transition from M to S is random and shows first-order kinetics⁵⁵. For normal cells this transition probability can be experimentally raised by increasing anchorage or serum concentration. Upon transformation, the transition probability remains stochastic, but becomes higher than for normal cells under equivalent amounts of anchorage and serum^{56,57}.

A UNIFYING HYPOTHESIS

The relation of TMP motility to growth control begins with the observation that hormone receptors are TMPs whose lateral movement seems prerequisite to their function^{58,59}. For the insulin receptor, the formation of cluster is sufficient for normal hormone action⁵⁹. Whole IgG directed against insulin receptor, or the (Fab)2 fragment antibody, will both suffice to stimulate a full lipogenic response in 3T3-L1 adipocytes. No insulin is necessary⁵⁹. Monovalent Fab prepared from the same antibody has no stimulatory effect in the absence of insulin. Though it binds to the receptors, it cannot cross-link to bring on the formation of a cluster of receptor molecules⁶⁰⁻⁶². Similar results have been reported with antibody to EGF and an inactivated EGF fragment⁶².

When one population of TMP is specifically clustered by an antibody or lectin, other classes of TMPs are not in general brought along into that cluster⁴⁹. In lymphoid cells, for example, anti kappa caps H2-K, but not H2D molecules. In fibroblasts, three separate TMPs have been each separately clustered by their respective antibodies, without coclustering of the other two⁴⁷.

In all cases of cellular functions that require TMP clustering studied to date, one hormone has been sufficient for stimulation. Thus, in the adipocyte, insulin or antibody to insulin receptor both stimulate specific protein phosphorylation with similar kinetics⁶³. Therefore a cluster with only one type of TMP is presumably a minimal signal for cellular response. Hormone-induced clustering is likely to require a specific state of actin on the inside of the cell. In the lymphocyte, the transplantation antigen H2 molecule is physically linked to actin and this linkage is enhanced by agents that patch H2 molecules²⁶.

A Paradox

The decision to proceed from M to S is regulated in normal cells by an address of more than one polypeptide hormone. Polypeptide hormones bind to specific receptors and receptor movement (clustering) is a prerequisite to hormone response. What then keeps a normal cell from responding to merely a single one of the hormones of its growth address? If any of the hormones of the address formed a receptor cluster, the cellular response would not be division, but differentiation. The problem is real, since some hormones (e.g. insulin) do act alone, through clustering to stimulate differentiated responses. Thus paradoxically, any one-hormone response must be restricted, if the complex multi-hormone signal for growth is to be received.

How can a normal cell ever get the whole address? Let us make the conservative guess that the cellular response to a signal of many hormones, is still one TMP patch or cluster. This one cluster would then be heterogeneous, containing receptors for each of the hormones of the growth address. By this hypothesis, the cell machinery that carries out clustering need not be different for stimuli requiring one, or more than one, hormone.

An immediate consequence of this hypothesis is that lateral movement of any one class of receptors participating in a multi-hormone response must be restricted from clustering until the probability is high that the full address has been received somewhere on the cell surface. By extension then, all such receptors must have restricted mobility. Otherwise, premature clustering of one or more receptor types into homogeneous patches will occur. These patches, by removing a receptor from the pool of molecules available for a heterogeneous patch, would lower formation of the probability of the latter, and hence of the signal. The probability of a passage from M to S would then be the probability of getting receptors of all necessary specificities into one heterogeneous cluster, before any one kind is self-clustered. Such competition would lead to a predictable average probability, but its outcome would be unpredictable for any one cell.

In the normal cell, spreading permits a complex hormone address to be read

Spreading, with the concomittant appearance of FN and actin cables, slows the formation of clusters. This could be sufficient to raise the probability of the critical

heterogeneous cluster being formed. Cross-linking of FN (to itself or by factor XIII for example) would have an additive effect, slowing patch formation by non-specific clogging. Once all receptors required by the address have been found by hormones and co-clustered, the cell would be past its indeterminate phase. Events from S to M are stereotyped. Protease synthesis and secretion are part of this stereotyped response; G2 cells synthesize and secrete proteases (including PA) to aid in their retraction from substrate prior to mitosis.

In summary, regulation of the normal cell cycle is hypothesized to be the result of the following mechanism linking serum and anchorage requirements.

- 1) polypeptide hormone receptors are TMP's
- 2) TMP linked to cytoskeletal actomyosin mesh
- 3) mesh needed to translocate TMP's into patch
- 4) patch is signal that hormone is received
- 5) normal spreading alters actomyosin mesh: cables form
- 6) spread configuration necessary to permit receptor patch

Therefore, rounded normal cells cannot divide even in presence of serum.

Transformations

There should be as many different transformants as there are ways to break the normal sequence outlined above. All of the transformants so far discovered fit easily into scheme, at the following different points of disruption.

Serum-transformation

This phenotype would be the consequence of any cellular change which increased the probability of heterologous cluster formation but which did not reduce the complexity of the hormonal signal for growth. Coordinate increases in hormone receptor number or affinity would be examples of such changes. However, the complexity of the address will still demand the restriction of receptor lateral motility, which will be detected as a retained anchorage requirement¹².

Density transformation

Saturation would be the density at which the probability of formation of a heterologous receptor cluster signalling growth drops sharply, due to the inability of crowded cells to become fully spread. Simple density-transformants that retain

an anchorage requirement for growth, would be cells whose ability to spread on each other permits them to receive hormone signals properly at higher than normal cell density. Saturation density would always remain proportional to serum concentration for such cells; that is, the probability of formation of the receptor cluster signalling growth would go up with increasing concentrations of required hormones.

Anchorage Transformation

Cells already transformed by the above criteria are not always able to grow without anchorage^{12,13}. Yet only cells that can grow without anchorage will express the full in vitro syndrome associated with cellular tumorigenicity¹⁶. Release of the anchorage requirement, whatever its mechanism, must therefore represent a qualitatively different event from either of the two transformations already discussed.

I have proposed that the function of anchorage is to restrict formation of patches of certain TMPs. The most direct way to lose the anchorage requirement would be to bypass this function. Were a cell to acquire the internal submembranous actin-gel organization characteristic of a heterogeneous receptor patch, it would be "blinded" to both the hormone and anchorage requirements normally prerequisite to the development of this configuration of its cytoskeleton. There are two direct but different ways to accomplish this: via proteases and via the cytoskeleton.

Proteases

The sequence of events in the growth of a normal cell is proposed to include a proteolytic event which occurs before mitosis, but after the signal generated by the heterogeneous cluster of hormone receptors. If a major consequence of the cluster is to signal the eventual synthesis of a protease, then a cell constitutively synthesizing the protease might cycle whether or not a cluster is formed. The stochastic event could occur even without serum, or any of the hormones of the normal cell's address. All parts of normal cell behavior given over to maintaining the restriction of receptor movement would then become gratuitous. In addition to release from the anchorage requirement for growth, the consequences of out-of-phase PA production would include the following. Cell bound FN would be reduced by the activity of plasmin and PA. Lectin agglutinability would rise as a consequence of increased TMP mobility. Cytoskeletal organization would be disrupted as a consequence of the protease⁶⁴, but could in any

event become disrupted for any other reason, such as altered TMP motility. Indeed they would have to be gratuitous, since the effect of the premature protease precisely would be to free the TMP receptors to form independent clusters. This would provide an explanation for the fact that many tumors remain differentiated, since rapid independent clustering is a prerequisite to hormone-stimulated differentiation.

Cytoskeleton

Not all anchorage-transformed, tumorigenic cells show an increase in production of plasminogen activator, or any other protease^{54,65}. Such an increase would be unnecessary in a cell that had discovered any other means of signalling to itself that all its hormone-receptors were clustered into a joint TMP patch. The most direct way to do this would be to change the mechanism of TMP clustering, so that receptors trap each other and the cluster forms spontaneously. Then the first hormone received would make the required heterogeneous patch and signal the cell.

Since TMP movement is a function of the submembraneous actin gel, a partitioning of excess actin into the gel at the expense of the cables might generate a hyper-clustering state. Under these circumstances the protease would still be necessary for the transition from G2 to M, but its synthesis would remain consequential, and part of the stereotyped cell cycle.

TMP biochemistry

By either the protease or the cytoskeleton pathway, anchorage-transformed and normal cells should differ from each other in TMP clustering responses when presented with a single growth hormone, or with the normal cell's whole address of hormones. Isolation of the hormone-receptor-actin-complex should show these differences. With lymphoid cells, myosin mesh can trap the TMP H2, via actin²⁶. If such a mesh can be used to trap the above complex, then a direct biochemical test of the hypothesis will be possible.

Contributions of viral genes to transformation

Perhaps the most direct function for a viral gene product would be the capacity to interfere with heterogeneous TMP patch formation. A close alternative role would be to induce the synthesis or activate a cell protein made in the normal course of events only as a result of patch formation. Recent work on the transforming gene products of SV40 and ASV is

consistent with these proposed functions^{66,67}. If a single hormone can become a sufficient growth signal, then synthesis and secretion of such a hormone, or of a compound that binds to such a hormone's receptor, would accomplish anchorage-transformation. Perhaps the EGF-receptor-binding compound of Ki(MSV) transformed cells⁶⁸, works this way.

Differentiation

Many differentiated states are induced by single hormones. In some cases, such a hormone (e.g., insulin) is also part of the address signalling cell division. Without ad hoc assumptions, the hypothesis explains the common observation that in normal cells such differentiations are accompanied by a reduced transition probability. A transformation that destroyed the growth-regulatory utility of a heterogeneous patch of hormone receptors would free those receptors for homogeneous patch formation. That is the hypothesis predicts the observation that differentiation combined with continued growth is a characteristic of abnormal cell behavior. Indeed, almost all tumors retain the differentiated markers of their cell of origin.

The successful signal of differentiation necessarily would remove a receptor from the pool of TMP's available for the heterogeneous, growth-signalling cluster. Agents that enhance patching of a single class of receptors should then direct normal cells from growth to differentiation. This can be tested with antibody to insulin receptor.

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