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Effects of tumour viruses on cell growth

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Cells in higher organisms, from hydra to human beings, are subject to marvellously precise control of growth, and the loss of this control may lead to cancer. Cultured cells *in vitro* are also subject to control of growth in a rather crude form, which may nevertheless bear some relation to the systems operating *in vivo* in whole organisms. Since tumour viruses modify these growth controls in cultured cells, model systems are available which could teach us something about real cancer.

Up to the present nearly all studies have been made on fibroblasts, the name loosely applied to the spindle-shaped cells which predominate in culture. Primary cultures, which are heterogeneous but mostly fibroblastic, and homogeneous populations of fibroblasts, obtained by cloning of cell lines such as BHK 21 hamster cells or 3T3 mouse cells, are used, and although there are some important differences, all show similar general features.

G_+ and G_0

In suitable conditions fibroblasts grow unrestrictedly, passing in 12 to 24 hours through the cell cycle with mitosis (M) and DNA synthesis (S) separated by two gaps, the G_1 and G_2 phases respectively. We call this whole cycle of growth 'G_+' (Stoker, 1972).

Various changes in the environment inhibit cell growth and the cycle slows or stops. This type of inhibition is reversible, and when re-started the cycle begins again at apparently the same place in the G_1 phase, so that, after a delay, DNA synthesis begins and is followed by mitosis. This happens even when the original inhibition was applied to asynchronous cells, at all stages of the cycle. The inhibition therefore takes place over a short part of the full cycle, in the early G_1 phase, and we suppose that some form of switch operates at this point, determining the commitment, or not, to the next cycle of growth.

Inhibited cells could simply remain in the pre-switch state, as a freezing, or rather extension, of part of the normal cycle. On the other hand, the inhibited cell may be shifted into an alternative 'holding' regulation state, not represented in the normal cycle at all. There are a variety of superficially unrelated metabolic changes in the stationary cells, which Hershko, Mamont, Shields, and Tomkins (1971) call a pleiotypic response, and though these changes could represent a transient undetected stage in the normal growth cycle, they have been taken as evidence of the latter concept, ie, an alternate regulation state generally called G_0 and not represented in the normal G_+ cycle.

Cyclic AMP levels are high in most G_0 cells which have been tested, and there have been several studies recently which suggest that this important substance is involved in the control of growth and perhaps has a rather similar role to that of guanosine tetraphosphate in stringent bacteria (Otten, Johnson, and Pastan, 1971).

Let us now turn to the factors which influence the critical control point in the cell cycle, and decide whether a cell is to remain in G_0 or continue the growth cycle in G_+ If we stick to physiological influences and leave aside unnatural factors such as drugs and substantial changes in temperature, pH, and so on, cell growth is affected in two ways: by the general environment, that is, the medium circulating freely in the culture and affecting the population as a whole, and by the local environment of a particular cell, including neighbouring cells.

General Factors Influencing Cell Growth

In this first class are nutritional deficiencies. When fibroblasts in otherwise adequate medium are deprived of certain essential amino acids, they stop growing but remain viable so that when the amino acid is replaced growth re-starts. Removal of other metabolic precursors has the same effect. But in addition to metabolic precursors there are also macromolecules, probably glycoproteins, which are essential for cell growth. These are normally supplied to cultured cells by the addition of fresh serum but they are presumably synthesized by cells *in vivo*, perhaps fibroblasts, perhaps more specialized cells, in which case they are a class of hormones. These essential growth factors, which may or may

not be important growth regulators *in vivo*, are imperfectly characterized and understood, but it is possible that their effect results from binding to the cell membrane and not penetration of the cell.

In this connexion I should mention the effect of the proteases, such as trypsin, which, even when applied for a short time in very low concentration, will initiate the growth of cells held in G_0. This occurs even when the enzyme is bound to large beads and cannot enter the cell, so clearly the action is effected by an alteration in some surface component (Burger, 1970; Sefton and Rubin, 1970). It is not known if naturally occurring growth factors are proteases.

In theory the growth of fibroblasts might also be controlled by negative feedback through accumulation of inhibitors in the medium, but although inhibitory substances have occasionally been reported, it has proved to be extremely difficult to show that inhibitors, as opposed to nutritional deficiencies, exert an important influence on growth control in cell cultures.

Local Factors Influencing Cell Growth

In addition to nutritional deficiencies and lack of growth factors, fibroblasts are sensitive to short range influences, involving the direct interaction of cells with one another or with substrates to which they adhere.

A cell floating in liquid or a soft gel is spherical, and in this form remains in G_0 and does not grow. An essential requirement for the switch to G_+ is anchorage to a surface on which the cell can spread (Stoker, O'Niell, Berryman, and Waxman, 1968). In recent studies in our laboratories on cells attached to glass fragments of different sizes and shapes, Maroudas (personal cummunication, 1972) has made the interesting observation that growth is more dependent on linear extension over a minimal distance than general area extension. Thus a long fibre is a much more efficient attachment site than a flat sheet with the same surface area. This shows at least that the requirement for spreading is not likely to be a simple need for feeding through the cell surface in contact with the substrate. If, as many expect, the growth stimulus comes from a change in the cell membrane, it may mean that a particular sort of distortion is involved, perhaps similar to the distortion caused by proteases.

Another factor which affects entry into G_+ or G_0 is cell-to-cell contact. Most fibroblasts, under standard experimental conditions, will not grow on a flat surface beyond a certain density, the saturation density, a layer which may be one or a few cells thick. When the cells are allowed to separate from one another by subculture at a lower density or by migration from the edge of a confluent layer the free cells begin to grow. Contact also inhibits movement, but though movement may be necessary for the switch from G_0 and G_+ it is not sufficient, and some additional change in the topographical relationship involving loss of contact seems to be required. This has led Dulbecco (1970) to propose the term 'topoinhibition' to describe the phenomenon and distinguish it from contact inhibition of movement. Naturally it is of particular interest because of its possible relevance to growth control and homeostatic mechanisms *in vivo*.

One or Many Controls?

Given these various factors which affect the G_0 and G_+ change we may now ask if the same control mechanism operates for all of them. For example, does absence of serum affect the same controls as those concerned in cell to cell contact? Some earlier studies suggested that they were indeed clearly linked because it was possible to overcome one type of block, for example, high density or anchorage, by an overdose of another stimulus from serum (Clarke, Stoker, Ludlow, and Thornton, 1970). There are, however, cell variants which lack response to one sort of signal, while retaining the response to another. Thus certain virus-transformed 3T3 cells remain very sensitive to topoinhibition but do not require serum for growth (Smith, Scher, and Todaro, 1971). All we can say at present is that there may be several pathways but they are probably interlinked.

Recently Kerr, Wyllie, and Currie (1972) have made the suggestion that homeostasis in some tissues *in vivo* might be due to controlled cell death, which they call 'apoptosis'. It would be as well to stress therefore that increased cell death could only be a minor factor in the maintenance of constant cell numbers in the stationary fibroblast cultures we have discussed. DNA synthesis and mitosis are reduced to low levels, and there is no regular substantial loss of cell viability as determined by colony-forming efficiency.

To summarize so far, therefore, we believe that there is a control system in the normal growth cycle which is sensitive to environmental signals, perhaps mediated by the cell membrane, and which changes the metabolic state of the cell to and from an alternative out of cycle regulation state. This non-cycling or G_0 state in cultured fibroblasts may resemble the state of most cells in the tissues of fully grown animals.

General Effects of Tumour Viruses and Cell Growth

Carcinogenic viruses stimulate cell growth even

under conditions which normally maintain the cells in G_0. Once stimulated, such cells remain in G_+ so long as the virus is active, and they are insensitive to the inhibitory environmental influences which we have just outlined. Thus cells infected with tumour viruses may grow even in low concentrations of serum which would be insufficient for growth of normal fibroblasts. They do not depend on anchorage and grow in suspension. Finally, infected cells go on dividing in crowded conditions, in close contact with one another, to form layers many cells thick, in which each cell ignores the others.

It seems as if tumour viruses make the cell deaf to inhibitory environmental signals. This state may in fact be hazardous for the cell since the increased metabolic activity associated with growth continues even if essential nutrients are lacking and so the cells soon die (Stoker, 1972). Whether infected cells ignore all inhibitory signals is not at present clear and I have already referred to virus-infected cells which lose the requirement for serum but still respond to topoinhibition. Nevertheless, the general effect of tumour viruses is to make cells unresponsive to their environment.

Mechanism of Virus Action

With one main class of tumour viruses, the DNA viruses, the growth stimulation may be transient. These viruses (polyoma, SV40, adeno- and herpes-type viruses) always kill the cells in which they multiply so that a change in host-cell growth can only be detected as a temporary stimulation of DNA synthesis, and occasionally mitosis, before disintegration of the cell. However, infection of certain types of cell, called non-permissive, results in a defective virus growth cycle without production of progeny virus or cell death, due to restriction of the expression of about half the viral genome. Those viral genes that do function in non-permissive cells include the gene (or genes) responsible for abnormal cell growth, which therefore continues for as long as the viral gene is active. Even with non-permissive cells, however, this abnormal growth continues in the majority for only a few cell divisions, after which the daughter cells return to their original state, presumably when the virus genome is lost or the gene concerned is no longer expressed. But in a small proportion of cells, one or a few copies of the functional viral genome becomes permanently associated with the host-cell genome, and this so-called integrated virus is then faithfully replicated and transmitted to daughter cells, all of which show abnormal growth. These rather rare survivors of the original infection by the DNA tumour viruses eventually outgrow the rest and constitute the virus-transformed cells.

The other main class of viruses are the C type particles, or RNA tumour viruses, sometimes called oncornaviruses or leucoviruses. Leucoviruses include the viruses of leukaemia and sarcoma in rodents and birds. These viruses do not kill the cells in which they grow, and new virus is continuously released by budding from the surface of viable and dividing cells and their progeny, all of which may be transformed and growing abnormally. The absence of cell killing by leucoviruses means that an incomplete virus infection is not an essential requirement for transformation, though incomplete cycles due to defective leucoviruses are actually very common.

Mutants which lack the ability to affect cell growth have been isolated from polyoma virus and Rous sarcoma virus, representing the two classes of tumour viruses (Eckhart, Dulbecco, and Burger, 1971; Martin, Venuta, Weber, and Rubin, 1971). These have provided valuable information about the role of the virus: for example, the effect clearly requires the action of virus genes, and since the mutagenesis is arranged to make single lesions in the virus, one viral gene may alone be responsible. Furthermore the mutants are conditional, and only expressed at high temperature which suggests that a protein rather than RNA specified by a gene is required. Finally, since alternating between high and low temperature allows the effect on cell growth to be activated or reversed at will, the transformed state requires the continued and not just the transient initial action of the viral genes. This has been particularly well demonstrated with Rous sarcoma virus mutants, and is of considerable importance because it means that the virus does not act on a hit-and-run basis.

Changes in Surface of Infected Cells

The abnormal growth is probably due to virus-specified proteins, and we may look for clues about the mechanism involved by a study of other changes in the structure and function of cell components affected by the virus and its mutants.

The most striking set of changes relate to the cell surface. Thus infected cells become agglutinable by naturally occurring glycoproteins called lectins which attach to specific sugar-containing sites on the surface (Burger and Goldberg, 1967; Inbar and Sachs, 1969). The agglutinability may be due to exposure or to an alteration in the spatial distribution of these surface-binding sites (Nicolson, 1971). It is of special importance that a similar agglutinable

state is found after protease stimulation of cells and also temporarily during mitosis.

Transport of several small molecules, such as sugars, amino acids, and purines, is also stimulated and there are general changes in the surface chemistry affecting glycopeptides and glycolipids. The infected cells alter in shape, the rate of movement increases, and they lose contact inhibition of movement (see Pardee, 1971; Stoker, 1972).

These surface changes are not just a characteristic of the G_+ state, but they could nevertheless be secondary events in the initiation of abnormal growth and not steps in a causal sequence. Some of our recent experiments on the sequence of changes following first exposure to a tumour virus indicate that surface modifications occur early and coincide with the commencement of the first G_+ cycle. This does not prove that they are causal but it shows that the modifications are not just a consequence of abnormal growth (Stoker, Thornton, Riddle, Birg, and Meyer, 1972).

Non-identity of Virus Effects on Cell Growth

Thus far virus-induced growth has been considered in rather general terms and not in relation to individual tumour viruses. We should now consider whether all tumour viruses affect cell growth regulation in an identical way. This brings back the question of one or many switches in the normal cycle.

It is already clear that there are some important differences between the two main groups of viruses, the DNA tumour viruses and the leucoviruses. We have already seen that leucoviruses can replicate without simultaneously killing cells, so transformation does not depend on an incomplete virus cycle. But there is another important feature of these viruses, namely, the variability in transforming capacity. Whereas agents such as Rous and murine sarcoma viruses regularly transform fibroblasts and alter their growth, a wide range of very similar leucoviruses grows in these cells without changing their behaviour at all. Some of these non-transforming viruses are the agents which cause leukaemia and lymphomas, but not sarcomas in animals, and so transformation and altered growth may depend on the target cell. Other viruses, however, may be completely non-transforming and non-oncogenic, so far as we know at present. The leucoviruses contain fragmented RNA genomes, and there is some indication that the transforming viruses of chickens, at least, contain larger RNA fragments, so called A-fragments, not detected in the non-transforming viruses (Duesberg and Vogt, 1970). Perhaps the extra part contains the growth-promoting genes.

The multiplication of DNA tumour viruses and stimulation of the cell cycle are nearly always associated, but since there are a number of leucoviruses which can replicate freely without altering the normal regulation of fibroblast growth, it suggests that abnormal growth is not an essential requirement for replication of the leucoviruses. This difference between the DNA tumour virus and the leucoviruses is clearly shown by the conditional virus mutants which affect transformation and to which I have referred. The available mutants of a DNA tumour virus (polyoma virus) which, in non-permissive conditions prevent the abnormal behaviour of the cell, simultaneously stop the replication of the virus. Amongst the mutants of a leucovirus (Rous sarcoma virus) there are admittedly some of this type, but there are others which in non-permissive conditions prevent the altered cell behaviour without affecting virus growth (see Vogt, 1972). This means that cell growth stimulation, whilst an essential requirement for DNA tumour viruses, is incidental for the leucoviruses. The DNA tumour viruses have presumably evolved with selection of this growth-stimulating function, but the lack of obvious selective advantage of cell growth stimulation for the leucoviruses suggests that they may have evolved in a different way altogether.

Despite these important differences between the viruses, the principal changes in the cells transformed by them are all rather similar, and we presume that they all affect the regulation mechanism which determines whether a cell can enter the G_0 state. But do these viruses affect the G_0/G_+ regulation in an identical way? Have the proteins specified by the transforming genes of the different viruses the same primary targets?

It was shown by Wyke (1971) that rare hamster fibroblasts with a normal phenotype could be selected from populations of polyoma-transformed cells. These apparently normal cells, however, still carry the viral genome and judging by the presence of viral-specific antigen there is expression of at least some viral genes. Such revertants may be cell mutants affecting, for example, a protein which is normally a target for the virus-transforming gene. If so it is possible to ask which other viruses will transform these cells, and presumably act through a different target. Wyke showed that re-exposure to polyoma virus fails to retransform the cells but that a leucovirus, hamster sarcoma virus, could still efficiently transform these revertant cells.

Recent more direct evidence that leucoviruses affect different targets to DNA tumour viruses has now come from work by Renger (1972) using an SV40-transformed 3T3 cell, in which the trans-

formed state is only expressed at a temperature of 33°C and not 38°C.

The failure to show abnormal growth at the high temperature could be due to a defective protein specified by a mutant virus gene or alternatively a mutant cell gene specifying a protein in the normal regulation pathway. But the virus which can be rescued from these cells is non-mutant, so the current view favours a change in a cellular gene concerned with growth regulation. If so, it follows that another tumour virus acting on cell growth through the same target or pathway should also be unable to stimulate cell growth at 38°C. As expected, SV40 virus itself fails to transform at this temperature but Renger has now reported that mouse sarcoma virus transforms the mutant SV40 transformed cells at the high temperature as well as normal cells. This strongly indicates that the cellular mechanisms which are affected by these two viruses are either completely different, or they each affect different successive steps in the same pathway, with SV40 early, and mouse sarcoma virus later, in the sequence.

It would be encouraging to think that the whole growth regulation mechanism of a fibroblast might be elucidated by work of this type with a series of cell and virus mutants. New virus mutants will surely be forthcoming but the deliberate selection of useful cell mutants affecting growth regulation is not feasible at present, and it will be necessary to rely a good deal on the variants provided by chance.

Meanwhile there are the biochemical approaches such as the isolation of proteins peculiar to either normal or transformed cells and the analysis of their role in growth control. In our laboratories Burk (1973), for example, has found that virus-transformed fibroblasts synthesize and release a macromolecular factor, which could be a protein of molecular weight 38 000, and which is absent or not detected in significant amounts in cultures of normal cells. This factor makes normal cells move actively and abnormally like transformed cells, and it also stimulates their growth. The factor is being further characterized to find out if its synthesis is promoted by virus infection, and whether it is subject to virus gene control. Another biochemical approach, being pursued by Dr Crawford and his colleagues, is the attempt to synthesize *in vitro* the proteins specified by the genomes of DNA tumour viruses. This is a difficult task but if proteins active in cell growth can be synthesized and introduced into cells, they might lead us to the targets through which these viral products act in the regulation system.

The final solution of cell growth control and its modification by carcinogens will certainly require the close integration of genetics and biochemistry, which has been so successful with simple micro-organisms. Whether that will lead us to a cure of the abnormal cells remains to be seen.

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