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Chapter 7. CELL SHAPE AND MOTION

MORPHOLOGY OF CELLS IN CULTURE

The establishment of a permanent cell line from a primary culture containing many different types of cells involves the selection of those rare cells which are able to grow indefinitely in the conditions of cell culture. Cells which grow in culture assume a wide range of shapes that depend on, among other things, how well they anchor to glass or plastic surfaces, how rapidly they migrate, and what the culture medium is composed of. In other words, the shape a cell assumes when it is growing in vitro may not be the same as the shape its ancestors had when they were growing in an animal. Nevertheless, cells growing in culture are often described as epithelial or fibroblastic or lymphoid if their shape resembles that of the cells of one or the other of these tissues.

Epithelial cells in primary cultures grow to a single cell layer, and viewed from above an epithelial cell culture has a cobblestone appearance; in colonies epithelial cells adhere tightly to one another.

Fibroblastic cells in primary cultures grow to form a layer usually three or four cells thick. Fibroblastic cells tend to move around the culture dish by the extension of processes with ruffled edges, and as a result, in sparse culture small colonies are dispersed. These ruffled edges apparently have a sensory role, for when a fibroblastic cell migrates along the plate its leading edge is ruffled until contact is made with another cell. Contact is followed by immediate cessation of both ruffling and cell migration. As a consequence, fibroblastic cells line up next to each other so

that older colonies contain oriented streams of cells (Abercrombie and Heaysman, 1954).

Molecular basis of cell shape

A cell growing in culture is an aqueous suspension surrounded by a lipid membrane suspended in an aqueous solution; thus, the configuration of least energy that a cell could assume is a sphere. Such a description is, of course, a caricature of reality. Cells in culture are not spherical. Usually they are adherent and flat; they move and maintain local asymmetry between front and back and top and bottom (Buckley and Porter, 1967).

Their surfaces are locally differentiated into smooth areas, ruffled areas, and villous areas, and these shapes are continuously changing. In short, the overall shape of a cell oscillates during the cell-division cycle and only during mitosis does the cell temporarily assume a basically spherical configuration (Buckley and Porter, 1967). Clearly, the changes in the shape of a cell must ultimately result from changes in the pattern of polymerization of macromolecules within the cell. What molecules are involved, and how is the equilibrium between polymerization and depolymerization regulated? Recently, partial answers to the first question have been obtained. The proceedings edited by Goldman, et al., (1975) contain an excellent summary of recent work.

Microfilaments penetrate and probably support ruffles and microvilli and other asymmetric extensions of the cell surface. Furthermore, the microfilaments appear to connect with microtubules (hollow fibers about 25 nm in diameter), ribosomes, and the cell membrane.

In short, the microfilaments, the bundles of microfilaments, and the microtubules appear to be the skeleton of a cell. The amount and distribution of these structural elements vary from cell to cell, and it seems that their changing causes changes in cell shape as well as cell movement (Goldman, et al., 1973).

Immunofluorescence

Given an antibody specific for a structural protein, one can visualize the pattern of organization of that protein by electron microscopy or by

immunofluorescence light microscopy. The latter technique, enhanced by advances such as through-the-lens excitation of fluorescence, offers a low resolution compared to electron microscopy, but an enormous gain in convenience. Review of a statistically significant number of cells for a given protein's distribution is now straightforward.

Many polymerized cytoplasmic proteins, such as actin (Lazarides and Weber, 1974), filamen (Wang, et al., 1975), tubulin (Fuller, et al., 1975; Weber, et al., 1975), neurofilament protein (Jorgensen, et al., 1976), actinin (Lazarides and Burridge, 1975), tropomyosin (Lazarides, 1975), and myosin (Weber and Groschel-Stuart, 1974) localize in different but associated (Lazarides, 1976) arrays in spread fibroblasts. The most easily seen (and therefore most highly organized) cytoplasmic structures are bundles, or cables running the length of the spread cell, at the place of adherence to the substrate, just within the cell membrane. These structures, initially called stress fibers, are easily seen in live cells by phase microscopy. They have been shown by immunofluorescence to contain, inter alia, actin, tropomyosin and myosin. Intermediate filaments are another independent collection of structural proteins, with a major component of 58K monomers (Hynes and Destree, 1978). Tubulin is not in either of these structures; it is found in single hollow flexible rods, the microtubules.

The combined results of electron-microscopic, immunofluorescence, and biochemical studies have shown that the microtubules, intermediate filaments and microfilaments are composed of a class of proteins that are found in every cell and are indeed capable of reversible polymerization (Pollard and Weihing, 1974). Furthermore, we now realize that the ubiquitous proteins are very similar or identical to the proteins of the contractile apparatus of muscle and flagella. The so-called muscle and flagellar proteins occur at their highest concentrations in muscle and flagella but at lower concentrations in all cells. The terms "muscle proteins" and "flagellar proteins" are, therefore, most misleading; they reflect our initial ignorance of the universal distribution of these molecules, as did, many years ago, the terms "animal nucleic acid" for DNA and "plant nucleic acid" for RNA. As these major structural proteins are studied intensively, other new proteins that share the capacity for reversible polymerization are being reported (Wang, et al., 1975). Undoubtedly, cell shape requires interactions among all of these, as well as other, still unreported proteins.

Properties of major structural proteins

Actin.--Actin is the chief constituent of microfilaments, and in striated muscle it is the chief component of the thin filaments (Bray, 1972). The amino acid sequence of actin in striated muscle of mammals is highly conserved (Collins and Elzinga, 1975). But the amino acid sequences of human muscle actin and actin from platelets differ by at least one amino acid (Elzinga, et al., 1976). It seems likely, therefore, that separate structural genes code for muscle and nonmuscle actin. The molecules involved in regulating the polymerization of actin are poorly defined. At least one inner-face membrane protein, spectrin, copolymerizes with actin in solution (Pinder, et al., 1975). Filamen (Wang, et al., 1975) may be a fibroblast form of spectrin. In addition, in vitro actin polymerization is dependent upon potassium, magnesium, and chloride ions, and cytochalasin B inhibits the polymerization of actin into microfilaments in vivo (Spudich, 1972) while phalloidin, a drug obtained from the fungus that yields amanitin, specifically inhibits the depolymerization of microfilaments into monomeric actin (Low, et al., 1975). Both cytochalasin and phalloidin have the same effect upon liver; they cause the cells to round up into spheres, to become vacuolated, and to leak potassium ions. It appears therefore that the maintenance of the shape of liver cells depends upon the freedom of actin to polymerize and depolymerize.

Myosin.--Like actin, myosin is ubiquitous, and since different tissues of the same animal contain myosins of different antigenicities and amino acid sequences there must exist multiple structural genes (Adelstein, et al., 1972). Myosin is assumed to be a component of the microfilaments in nonmuscle cells on the basis of immunofluorescent localization, but it has not been convincingly attributed within nonmuscle cells to any single sort of filamentous structure (Weber and Groschel-Stewart, 1974; Pollard and Weihing, 1973).

Tubulin.--Tubulin is the subunit of the microtubules. Microtubules are dispersed singly through the cytoplasm of nonmuscle cells (Buckley and Porter, 1975). They must depolymerize and repolymerize in each cell cycle for in mitosis they are the major constituent of the mitotic spindle (Buckley and Porter, 1975).

Polymerization proceeds from a monomer to a dimer and then to a disc structure which stacks to yield the hollow microtubule 25 nm in diameter. The protein is ubiquitous, and polymerization depends upon the presence of a disc intermediate and a nucleation site or sites which include the centriole, the mitotic spindle, a chromosome, and possibly the cell membrane (Olmstead and Borisy, 1973). The polymerization occurs at calcium concentrations of 10^{-5} M calcium or higher concentrations microtubules depolymerize. The drugs griseofulvin, colchicine, and vinblastine all cause depolymerization. The tubulin molecule is itself a kinase able to phosphorylate other proteins, including perhaps histones.

Structural proteins and cell movement

In striated muscle the interaction of myosin and actin to yield contraction is regulated by a reversible inhibition of actin-myosin binding. This regulatory block is modulated by the concentration of free Ca^{++} in the muscle cytoplasm. Below 10^{-7} M Ca^{++} , tropomyosin and troponin attach to polymerized actin (the thin filament) as to block its interaction with polymerized myosin (the thick filament). Above 10^{-5} M Ca^{++} , the two regulatory proteins shift in their binding to polymerized actin, allowing the contact with myosin necessary for ATP-dependent sliding movement of one polymer with respect to the other (Pollard and Weihing, 1974).

Even before it was known that actin and myosin are present in nonmuscle cells it was thought that Ca^{++} might participate in the regulation of cell motility, since injection of dilute Ca^{++} solutions into giant amoebas caused localized cytoplasmic contraction (Pollard and Weihing, 1974). Investigation of the biochemical basis of nonmuscle motility has been guided by analogy with muscle: Movement has been shown to be dependent on Ca^{++} , and Ca^{++} has been shown to be regulated by Ca^{++} -sequestering membranes (Gail, et al., 1973).

However, it is essential to remember that nonmuscle cells must construct a contractile system that has the ability to reversibly collapse at every mitosis, a property striated-muscle actomyosin need not have. It is likely, therefore, that even if the ionic and protein cofactors of movement are the same for nonmuscle cells, novel cofactors for assembly and disassembly will be found (Stossel and Hartwig, 1975).

Tumorigenicity of cultured cells

Conditional tumorigenicity.--Both the 3T3 mouse cell line and the BHK hamster cell line are normal in that they serve as substrates for viral transformation. However, under certain conditions both cells can be rendered tumorigenic in the absence of tumor viruses. The 3T3 cell line will grow as a tumor if it is first permitted to attach to glass beads before injection into the animal. Neither the beads alone nor the 3T3 cells and the beads separately injected will yield tumors (Boone, 1975). BHK cells, if injected in large enough numbers (greater than 10^6 cells per animal) will grow as tumors in neonatal hamsters. These results are in sharp distinction to ones obtained with early-passage mouse or hamster embryo cells, neither of which will grow as tumors in any host regardless of immune compatibility.

Clonal instability of cell lines.--So-called normal cell lines suffer from the same somatic genetic instability. Subclones obtained from (for instance) 3T3 or BSC-1 will have varying degrees of growth control and varying degrees of susceptibility to viruses (Risser and Pollack, 1974). Again this distinguishes cell lines from primary cell cultures, in which clones, when they can be obtained (Green, 1975b), are remarkably similar to one another.

Tumorigenicity of cell lines.--Tumors grow in animals, not in petri dishes. Tumorigenicity cannot be studied in culture, although certain of the properties of tumor cells can be studied in vitro and then tests can be made to correlate particular properties that can be assayed in vitro with tumorigenicity in vivo. Unfortunately, however, the tests for tumorigenicity that are routinely used bear little resemblance to the events that we assume occur when an animal develops a tumor spontaneously. We assume that a tumor arises when some genetic or epigenetic change such as tumor-virus infection occurs in a cell which causes it to multiply under conditions which restrict the multiplication of normal cells. A mass of tumor cells -- a primary tumor -- accumulates and is invested without a host capillary net (Folkman, 1975). Sometimes a few tumor cells migrate from their site of origin (Sato, et al., 1976) and colonize another site to give rise to secondary tumors. This latter process is known as metastasis (Fidler, 1973). Tests of tumorigenicity of cell lines involve the injection, usually

subcutaneously, of large numbers (typically between 10^3 and 10^6) of cells in suspension. The animals are then monitored to see if a palpable tumor (at least 10^8 cells) develops at the site of injection. Clearly, this bioassay measures metastatic ability as much as tumorigenicity (Pollack, 1977). When a single cell of a tumorigenic line is injected, a tumor rarely if ever arises. In other words, the tumorigenicity assay is very inefficient, and one has always to consider the possibility that the assay selects for a rare type of cell unrepresentative of the majority of the population of cells that is injected. This problem also raises doubts about the significance of biochemical analyses of mass populations of tumor cells, most of which in vivo may not be tumorigenic.

The reasons why the present assays for tumorigenicity are so inefficient are no doubt manifold. On the one hand, the cells being injected may have a reduced viability because they are in suspension, and they may not be able to grow at sites beneath the skin although they might be able to grow in some other site in the body. On the other hand, the response of the host must be as critical as it is complex. The host may mount an immune response to the injected cells and it must provide nutrients and growth hormones. If a palpable tumor mass is to develop it must be supplied with blood, and vascularization of the tumor requires the proliferation of host cells in step with the proliferation of the tumor cells (Folkman, 1975). Very often 50 percent of the mass of a large tumor may be host endothelial capillary cells, and one could argue that a tumor is a mass of host blood vessels supported by injected tumor cells rather than the reverse.

Even if the tumor cells repeatedly give rise to the same type of tumor on serial passage through several animals, the fact remains that at each passage tumor growth depends on capillary proliferation. Specific inhibition of tumor-associated angiogenesis would be a novel form of tumor chemotherapy (Gimbrone and Gullaro, 1976). Recently the endothelial cells of major blood vessels have been cultured (Gimbrone, 1976). If capillary endothelia can be placed in culture as well, then the biochemical nature of tumor-associated angiogenesis will be open to study.

When a culture is made of a tumor, more than one cell type survives (Smith, et al., 1976). The capillary endothelial cells and the fibroblasts of

a tumor have been assumed to be "nontumor" cells. However, evidence is accumulating that these "nontumor" cells are themselves abnormal. In some cases, tumor-associated fibroblasts have been shown to be serum and density transformed. Further, fibroblasts from distant tissues of tumor victims have been shown to have abnormal cytoskeletons (Kopelovich, Conlon and Pollack, 1977), to be extraordinarily susceptible to transformation (Pfeffer and Kopelovich, 1977), and even to be spontaneously tumorigenic (Smith, et al., 1976). These exciting results suggest the possibility of a susceptible systemic defect in the growth control of persons bearing tumors. Is there a viral gene component to this "nontumor" defect?

"Nude" mice.--A successful tumorigenicity assay requires both a cell population that is able to proliferate and a susceptible host animal that can support the proliferation and does not mount an immune response against the injected cells. In the past the problem of immunity has been circumvented by (a) using isogenic inbred animals and cell cultures derived from them, (b) using neonatal animals that lack the ability to mount an immune response, (c) using animals given immunosuppressive treatment, such as irradiation or antilymphocyte serum (ALS), or (d) injecting the cells to be tested in a site inaccessible to the immune system, for example the cheek pouch of a hamster. More recently, however, mouse geneticists have identified an autosomal recessive mutation, the nude mutation, which renders mice not only hairless but also partially immuno-incompetent. "Nude" mice lack mature functional thymocytes or T lymphocytes (Flanagan, 1966). As a result these mice fail to mount a cell-mediated immune response and do not reject grafts of foreign tissue. Skin from humans, cats, chickens, lizards, and frogs has been grafted to the back of a nude mouse (Manning, et al., 1973). However, despite this tolerance of foreign tissues, suspensions of foreign normal cells injected subcutaneously, intravenously, or intraperitoneally fail to become vascularized and multiply in nude mice. (Freedman and Shin, 1974). In other words, the nude mouse discriminates between a foreign normal tissue and a suspension of foreign normal cells (Povlson, et al., 1973). The animal's capillary system proliferates only in response to the former. This finding suggests that normal tissue contains a factor or factors capable of eliciting a proliferative response on the part of the capillary cells, whereas normal cells capable of growing in vitro lack such factors.

Three aspects of tumorigenicity in the nude mouse are poorly understood. First, not every transformed cell grows as a tumor upon injection (Stiles, et al., 1976). Some SV40-transformed human cells apparently grow well in nude mice (Freedman and Shin, 1974) while others do not (Stiles, et al., 1975). Second, invasiveness in the nude mouse is defective. Although there have been rare reports of invasive tumors (Biovanella, et al., 1972), usually even the most malignant cell types, such as melanoma, do not break through encapsulation as they grow into huge solid tumors (Rygaard and Povlson, 1969). Finally, how the nude mouse survives for a normal life span while lacking a T cell immune response remains an enigma, but the fact is that these animals do not have an especially high incidence of mortality from spontaneous malignant tumors. Apparently, tumor immunosurveillance via the killer T cell does not occur in mice.

Selective assays for in vitro transformation

Transformation.--In order to determine whether tumorigenicity is a simple mutational event, one first needs an in vitro assay for selecting tumorigenic cells from a background of normal ones. On the reasonable assumption that tumorigenicity is related to the ability to grow under certain conditions that restrict the growth of normal cells, many such assays have been devised. In each, the presumptive tumorigenic cells is selected for its ability to overgrow; such overgrowing cells are called transformants.

In addition to the changes that serve to permit selection of transformed cells, many nonselective changes also occur.

One crucial question of tumor virology is which, if any, of the different selective transformation assays selects directly for a tumorigenic cell population. In order to determine the full spectrum of transformed phenotypes that SV40 is capable of inducing in a cell population, Pollack's group (Risser and Pollack, 1974) carried out extensive nonselective transformation experiments on 3T3 and rat embryo fibroblasts.

3T3 cells were infected with SV40 and then sparsely plated in dishes with a medium containing excess serum. Forty-five clones were picked and assayed separately in each of three different selective assays. In any one

assay, cells from different clones exhibited a continuum of different phenotypes ranging from that of an untransformed cell to that of a fully transformed cell. Similar results were obtained with rat embryo fibroblasts.

Therefore, SV40 infection does not create a single transformed phenotype, but rather a wide range of phenotypes are induced and particular phenotypes in this range are selected by particular assays.

Transformation and tumorigenicity.--Different tumor viruses are commonly assayed in different in vitro selective transformation assays, including any of the three described above (Pollack and Risser, 1974) as well as induction of cellular mutation (Marshack, et al., 1975), growth in low calcium (Freeman, et al., 1967), loss of hormone receptors (Todaro, et al., 1976), and induction of growth in a nonproliferating tissue such as neuroretina (Pessac and Calothy, 1974). Each different assay will yield a different subset of all possible transformants. It is possible, but not likely, that sets of transformed cell lines isolated through different assays will share a common pathway to tumorigenicity.

In fact, recent studies on the tumorigenicity in nude mice of transformed cell lines obtained nonselectively from primary rat-embryo cells after SV40 infection (Shin, et al., 1975) show that at least two different pathways to tumorigenicity exist. Shin's lab (1975) tested a wide variety of rat and mouse cell clones transformed by SV40 in nude mice and found that only the ability to grow in the absence of anchorage to a substratum correlated closely with tumorigenicity. The SV40 transformants that could grow in suspension gave rise to tumors irrespective of the saturation density the cells achieved in vitro or their ability to grow in low concentrations of serum. Conversely, cells that failed to grow in suspension failed to induce tumors in nude mice irrespective of their other properties. However, Gallimore, et al., (1977) have reported similar experiments with a set of rodent cells transformed by an adenovirus which gave very different results. They found no correlation between growth in suspension and tumorigenicity. Such discrepant results are at present hard to explain, and further work to separate initial selection from final tumorigenicity is needed before any generalization about the selection in vitro of a cell and its tumorigenicity can be made (Pollack, 1977).

Nonselective changes in transformation

Serum deprivation, extensive cell-cell contact, and anchorage deprivation are each sufficient to trap a normal fibroblast in the G₁ phase of the cell cycle. The mechanism by which any of these assays operates is not understood, but nonselective biochemical changes have begun to be described which provide clues.

Serum and hormones.--In its role in the body, the fibroblast proliferates to fill a wound. While normally all cells but endothelia are kept from serum, wounds fill with it. Thus, it is not surprising that fibroblasts need serum to grow in vitro. This serum requirement was thought to be quite high, but recent work has shown that serum can be reduced from 10 percent to 0.2 percent if the defined constituents of media are optimized (Ham, 1974). Serum can be replaced entirely by a mixture of polypeptide hormones, including insulin, transferin, and fibroblast growth factor (Hayashi and Sato, 1976).

These observations suggest that, in general, cells in culture require serum because it supplies particular essential hormones. Different types of cells are likely to require different sets of hormones, and from the tests that have been made so far this seems to be the case (Reinwald and Green, 1975b). For example, HeLa cells grow in the absence of serum if transferin, insulin, epidermal growth factor and fibroblast growth factor are added to the medium; the last two hormones are polypeptides and both were discovered by experiments with cell cultures (Hayashi and Sato 1976).

On the other hand, mouse fibroblasts of the 3T3 line in the absence of serum require insulin, hydrocortisone, and fibroblast growth factor but do not require epithelial growth factor or transferin (Holley, 1975). We can anticipate that as the hormonal constituents of serum are resolved further by bioassays with cultivated cells it will become possible to design supplemented media that will support the growth in culture of types of cells which are at present refractory to cultivation (Ham, 1974).

Viral transformation can result in a diminished requirement for serum (Holley, 1975), and therefore presumably in an altered hormone requirement for growth. Intracellular concentrations of cyclic AMP and cyclic GMP also change when cells are serum-transformed, which suggests the

possibility that cyclic nucleotide production may be perturbed by the viral gene as well (Oey, et al., 1974).

Density.--Normal fibroblasts block in G_1 when they are in close contact with each other. A cluster of 4-5 cells seems to be the minimum necessary to get this effect (Pollack, et al., 1968). Viral gene expression can lead to failure of fibroblasts to recognize each other, as a result of which they pile up in dense foci. Since this is a defect in cell-cell interaction, the external surface of a density-transformed fibroblast must differ from normal (Culp, 1974), and indeed this is so by direct biochemical analysis. The major change in proteins is the loss of a large, external, transformation-sensitive (LETS) glycoprotein (Hynes, 1974). This protein is exquisitely protease-sensitive, which suggests that the membrane change could be the consequence of local proteolysis (Pearlstein, et al., 1976). Such proteolysis could occur by the action of a serum protease, such as plasmin. Many density-transformants and anchorage transformants secrete a plasminogen activator (Pollack, et al., 1974). Since plasminogen activator is a normal secreted product of endothelial cells (Buonassissi and Venter, 1976), the viral gene in this case may be thought to be inducing an inappropriate differentiation in anchorage-transformed fibroblasts.

Anchorage and cell shape.--Dividing tissue cells are, by and large, always in contact with each other or with collagen, and are spread out at all times except for mitosis. In culture as well, the normal cell rounds up only in mitosis, and then the two round daughter cells spread again, reestablishing an anchored configuration.

Normal fibroblasts must be able to anchor on a solid substrate at the end of mitosis, or they do not proceed through G_1 . Expression of the viral gene relaxes this specific requirement, without at the same time preventing anchorage. Anchorage-transformants presented with a solid substrate will spread on it at all times except in mitosis, but need not do so in order to divide. Thus, in vitro, this cell-shape change is part of a normal growth control.

Cell shape is the sum of the states of homopolymerization and copolymerization of a small number of cytoplasmic proteins, including actin, myosin, filamen, tubulin, and spectrin (see above). The shape changes occurring in the division cycle of a fibroblast require a reversible polymerization-

depolymerization of these proteins. Normally, the fully spread interphase fibroblast shows the most highly organized states of polymerization, in which proteins such as actin are organized in bundles large enough to be visualized by immunofluorescent light microscopy (Lazarides and Weber, 1974). Microtubules are also abundant in such cells. Actin-containing bundles are both gone from anchorage-transformed fibroblasts, even well-spread ones (Pollack, et al., 1974; Edelman and Yahara, 1976). Changes in microtubule and intermediate filament distribution have also been described (Brinkley, 1976; Hynes and Destree, 1978).

Viral-chemical synergism in transformation.--A number of chemical agents or analogs have the ability to potentiate virus-mediated transformation in cell culture. For example, SV40-mediated transformation of cells in culture is enhanced (frequency) by agents like 4-mitoquinoline 1-oxide. The incorporation of 5-iododeoxyuridine into cellular DNA also enhances SV40 mediated transformation (Coggin, 1969). These drugs damage DNA and cause cellular repair processes of DNA to be activated and similarly UV irradiation and caffeine treatment stimulates the frequency of SV40 transformation (Ide, et al., 1975).

With a different class of chemicals it has been shown that treatment of cells in culture with glucocorticoid hormones increases the frequency of polyoma transformation. These cells must have the glucocorticoid receptor in order to be enhanced in their viral mediated transformation and so the hormone (used in physiological concentrations) appears to act in a normal fashion to promote higher transformation frequencies (Rabinowitz, et al., 1976). The glucocorticoid hormones can also induce mouse mammary tumor viruses from the appropriate mouse strains. Indeed a number of carcinogenic agents (tobacco tars) appear to induce endogenous RNA viruses of the mouse. Halogenated pyrimidines (Budr, Iudr) have long been known to induce these viruses from cells. In particular Iudr was recently employed to induce a transforming virus (MSV-Z) from mouse cells in culture (Nomura, et al., 1976) and this establishes a relationship between chemical treatment and transformation.

Chemicals like Phorbol esters (Phorbol myristate acetate, for example) are known to promote tumorigenesis with suboptimal doses of carcinogens and to mimic transformation in cell culture by inducing plasminogen activator, loss of the LETS glycoprotein and altered deoxyglucose transport

(Weinstein, 1976). Whether these Phorbol esters promote endogenous viruses or enhance viral tumorigenesis is under active study.

Reversion of transformation

Serum-sensitive, density-sensitive, and anchorage-sensitive revertant lines have been isolated from cell lines transformed by chemicals and by SV40, polyoma, murine sarcoma, or avian sarcoma virus. Virus usually is less rescuable from revertants than from their transformed parents (Vogel, et al., 1974), but most revertants are not cured of their virus, since they have viral DNA, RNA, and antigens (Deng, et al., 1974). Genetically, these revertants are therefore not back mutants of transformation, but rather second-site cellular mutations (Vogel and Pollack, 1974). Nevertheless, many nonselected aspects of the revertant phenotype resemble those of the normal cell. In particular, revertants seem to have regained the ability to assemble a complete cytoskeletal array of actin and tubulin containing filaments (Pollack, et al., 1975). Recent work has shown that true complete reversion through mutation of the integrated viral genome is possible in SV40 transformed cells. Apparently, such reversion occurs best in cells transformed by a single copy of SV40 DNA (Steinberg, et al., 1978).

Possibilities for new directions

New normal cell cultures.--Keratocytes, macrophages, arterial endothelial cells, capillary mural cells, and many hormone-dependent glandular cells have recently been put into culture (Rheinwald and Green, 1975a, b; Desmond, et al., 1976; Ross and Glomset, 1976; Gimbrone, et al., 1974; Buonassissi and Venter, 1976; Buzney, et al., 1975). Do these cells harbor any new viruses? SV40 was discovered only because of our ability to culture rhesus monkey kidney epithelial cells; cytopathic effect was seen only by passage of rhesus culture supernatant fluids onto African green monkey epithelia. Similar cross-checking should be done as each new cell type is cultured, both as a precaution, and as a new opportunity to detect the agents suspected of being responsible for such diseases as multiple sclerosis and diabetes (Buzney, et al., 1975).

New abnormal cell cultures.--Tumor cells are being cloned with ever-increasing proficiency. The old block-host fibroblast overgrowth is being

bypassed by careful cell dissociation, and by novel substrates and media. Again, each new cell type of tumor origin should be treated with respect as a possible source of new viruses, whether oncogenic or not. By the same argument, such cultures should be scanned for the presence of viral gene sequences and their products.

Multicell culture.--Mixed cultures will be a necessity if we are to carry into culture the later steps of tumorigenesis (Pollack, 1977). The step immediately following transformation itself is angiogenesis.

At least two cell types are required for this event, the transformed cell expressing at least part of the syndrome described above, and the host capillary endothelial cell. The tumor cell secretes a compound of unknown structure, TAF, which, as Folkman and others have shown, causes capillary endothelial cells nearby to proliferate so that capillary growth is directed toward the microtumor (Folkman, 1975). This is followed by continued vascularization of the microtumor, providing an inexhaustible supply of nutrients. Selective systems in vitro would be of two types: for TAF-secreting tumor cells, and for TAF-requiring endothelial cells. Neither system has been reported, but there is no difficulty in principle, since both transformed cells and capillary mural (Buzney, et al., 1975) and endothelial cells (Haudenschild, et al., 1976) grow well in culture.

Questions that should be asked with such systems include: How are TAF secretion and synthesis regulated? How can they be blocked? Do endothelial cells ever transform genetically in response to TAF, gaining the heritable ability to proliferate in the absence of the compound? Is tumor-secreted TAF tumor-specific, or is it identical to the factor(s) necessary for maintenance of capillary density in normal tissues? All these questions demand the courage to go beyond the limitations of single-cell-type cultures.

CONTRIBUTIONS OF VIROLOGY TO THE STUDIES OF CELL SHAPE AND MACROMOLECULAR TRANSPORT

We often think that cell shape reflects its function. For example, the ameboid shape of leukocytes reflects their motility, the spindle shape of fibroblasts, their role as structural components in connective tissue and so on. For heuristic reasons it is convenient to view the cytoplasm of the

cell as containing a scaffolding of microtubules supporting a network of filaments to which are anchored the plasma membrane and other membranes abutting to the cytoplasm. Immersed in this skeleton are the various cytoplasmic organelles. This grossly oversimplified model has obvious appeal. We could readily visualize how this cellular skeleton would direct and channel the movement of organelles, particulate matter and macromolecular complexes within the cells and indeed, the role of microtubules in intracellular movement was amply demonstrated (Bhisiy and Freed, 1971; Freed and Lebowitz, 1970). We could also readily visualize how wavelike propagation of collapse and reassembly of the scaffolding and network from one end of the cell to another would impel the cell to move in the direction opposite to the direction of wave propagation. Supporting this model is evidence that microtubules and microfilaments do disassemble and this correlates with changes in shape. Lastly, we could visualize how changes in the composition and organization of the plasma membrane could affect the integrity of the microtubules and microfilament interactions.

Viruses are excellent probes for the study of the structure and function of this skeletal structure for three reasons. First, virus particles do move within the cell. An example of such movement is the translocation of virus particles from cytoplasm to nuclei. Prime examples of viruses involved in such a movement are herpesviruses, adenoviruses and papovaviruses. In addition, most viruses that mature within the cells provide in some fashion for their egress. Second, most viruses that kill cells in the course of virus multiplication alter their shape as well, and this event frequently precedes total cessation of cell macromolecular metabolism. Lastly, many viruses specifically alter the structure of the plasma membrane by insertion of virus specific components. Correlated with this event are changes in adhesiveness and cell shape. Viruses comprising this group, such as herpesviruses and others, are excellent probes for the study of the interaction of microfilaments and microtubules with membrane components.

Interaction of Viruses with Microtubules

There have been several reports on the association of reoviruses and adenoviruses with microtubules. In the case of reovirus, elegant studies by Dales, et al., (1965) demonstrated that during infection the microtubules became coated by a dense material and that reoviruses adhere to this material. Immunoferritin studies indicated that this material is a

virus-specified antigen. However, the obligatory nature of the association between mature and immature virus particles with microtubules was not demonstrated inasmuch as disruption of the microtubules with colchicine did not affect virus multiplication. A question that has not been answered is whether the association with microtubules is required for egress of virus from cells rather than for synthesis of viral components and assembly of infectious progeny.

Dales in collaboration with Chardonnet (1973) also reported the association of adenovirus particles with microtubules. In this instance, however, the nature and purpose of the association are more clearly defined. It appears that disaggregation of microtubules induced by vinblastine, interferes with the transport of adenovirus from cytoplasm to the nucleus. Moreover in a series of elegant studies Luftig and Weihing (1975) and Weatherbee, et al., (1977) developed an in vitro system for binding of virus particles to microtubules and demonstrated that the binding is to the MAP component of the microtubules. This is an important observation, for it focuses attention on this high molecular weight component of the microtubules as a possible "transport" carrier.

Interaction of viruses with microfilaments

The most striking observations on the interaction of viruses with microfilaments is contained in a recent report (Stokes, 1976) on the release of a poxvirus-vaccinia from infected cells. It appears that the virus is enclosed in a membrane-like component of the cell cytoplasm. The egress of the virus takes place at the tips of microvilli; the movement of the enclosed virus particles to microvilli involves a cytoplasmic network containing microfilaments. It is noteworthy that the membrane-like structure was disrupted by cytochalasin B but not by vinblastine suggesting that its integrity depends on the structural integrity of microfilaments.

Association of viruses with membranes involved in transport

In contrast to the mechanism of egress of poxviruses from infected cells, herpesviruses are released through modified cisternae of the endoplasmic reticulum. Specifically, the virus becomes enveloped at modified patches of the inner lamellae of the nuclear membrane and accumulates in the perinuclear space and especially in the cisternae of the endoplasmic reti-

culum. Electron microscopic studies (Schwartz and Roizman, 1969; Jasty and Chang, 1972; Abraham and Tegtmeyer, 1970) indicate that the transport into the extracellular space is via a direct connection of the endoplasmic reticulum. Inasmuch as uninfected cells do not exhibit direct connections between the endoplasmic reticulum and plasma membranes, such connections in infected cells would imply an alteration of the existing membrane system. It is conceivable that this change in the cytoplasmic membrane system is mediated by the viral proteins known to be incorporated into the plasma membrane during viral replication (Roizman and Furlong, 1974).

Summary and recommendations

Most of the current studies probing the interaction of microtubules, microfilaments and membranes have made apparent the interaction of viruses with filaments and tubules and pointed to their probable role in movement of the viruses to and from the site of replication. The use of viruses as probes to study the functions of microfilaments, microtubules and membranes in determining cell shape, have only recently begun. The importance of these studies cannot be overstated inasmuch as changes in cell shape, mobility, and adhesiveness characterize cells transformed by both viral and nonviral carcinogens.

TRANSFORMATION AND RESULTANT MEMBRANE CHANGES

The cell surface-membrane complex can be considered the most important communication link between the cell and the outside world. It is here that initial transactions between cells and other cells, and cells and molecules take place. These transactions affect cell growth and differentiation, as well as cell movement (as seen in so-called contact inhibition of movement). Moreover, the cell exports molecules and molecular information to the outside world at the viral borders of the plasma membrane and its secretory apparatus.

In view of these complex functions, it is perhaps not surprising that determination of the structure of the cell surface and cell membrane should be a major challenge for basic research. In the last decade, there has been a quiet revolution in our view of the cell surface. This revolution

has opened new views of the structure-function problem and has clarified much that was previously obscure. Most important, it has provided a sound chemical picture and a basis for detailed explanations of processes as diverse as viral budding, changes in cell shape, and control of mitosis. Our changed view of the cell surface has come in large measure from the application of advanced physicochemical and microchemical techniques and we can expect much more development in these areas. Of course, these more sophisticated approaches rest upon the previous careful electron microscopic and compositional analyses of membranes and their components (proteins, lipids, glycolipids, etc.).

The main ideas that have come from the structural work are: The cell membrane is a fluid lipid bilayer which contains key proteins loosely attached or more intimately embedded in it. Some of these proteins (e.g., intramembranous particles) seem to have functional properties important for stabilizing the structure. Others such as the proteins of gap junctions form specialized structures for intercellular communications, and still others (e.g., insulin receptors, Ig's) carry out key binding events (for hormones or antigens) that are essential for specialized cellular transactions. Many cell surface proteins appear to penetrate the lipid bilayer to the cytoplasm. Many of the receptors are free to diffuse in the plane of the membrane independently of other receptors. This allows them to patrol the surface and enhances the possibility of interaction with other surface molecules. As a result of intracellular events or extracellular perturbations, the movement, distribution, or interaction of these cell surface proteins may be modulated.

There are two general kinds of modulation: local, involving interaction of a particular receptor with viral proteins, hormones, or other agents; and global, involving major alteration of all properties. An example of the latter is the capping phenomenon, in which receptors cross-linked by antibodies are swept by an active process to one pole of the cell. Equally striking is the global process of anchorage modulation, in which cross-linkage of certain glycoproteins at one point of the surface (for example, by lectins such as concanavalin A [Con A]) results in a propagated restriction of the motion of all of the freely moving receptors.

Local modulation is dependent upon the architecture of the cell surface, but is also dependent upon the particular specificities of the cell surface

receptors as gene products (i.e. as hormone receptors, viral receptors, or antibodies). This form of modulation is best discussed under the category of recognition of molecules at the cell surface.

Global modulation has extensive implications for the problems of viral transformation of cells, however, and requires emphasis here. Why is this the case? The main reason is that the key functional elements involved in viral transformation, namely changes in cell shape, adhesiveness, and motion, and the alteration or loss of growth control are also observed in anchorage modulation. Transformation by a tumor virus results in a very large number of chemical changes in the cell surface. These include glycolipid turnover, protein changes, alteration of agglutination patterns, morphology changes, transport changes, and alteration of surface modulation. The problem is to determine the causal connections among these various events and above all, their interactions with the products of viral infection that have resulted in the transformation.

It must be said that, so far, little progress has been made in disentangling these events. Nevertheless, a strategy and a series of hypotheses that may be unifying are beginning to emerge. This strategy comes from two main lines of investigation: a) the attempt to define src gene products of viruses such as Rous sarcoma virus; b) the implication of morphogenetically significant subcellular structures such as microfilaments (actin-like filaments) and microtubules in both modulation and control events.

As shown by a number of detailed studies of modulation events, it is clear that these submembranous structures can interact with surface structures, particularly glycoproteins and defined receptors. Addition, of antibodies to a receptor leads to formation of cross-linked patches of that receptor on the surface as a result of simple diffusion and nucleation. Subsequently, a metabolically dependent process involving microfilaments (and possibly myosin) gathers the patches in a cap. Induction of anchorage modulation by Con A prevents both patch and cap formation in a process that depends upon intact microtubules. Thus, there is an intimate connection between submembranous structures and global modulation events such as capping and anchorage modulation. When one considers that these submembranous cytoskeletal structures are responsible for cell shape and motion (and in certain cases for transport of hormones and other key macromolecules), the connection with modulation becomes even more significant.

This is all the more emphasized by the finding that anchorage modulation blocks cellular commitment to division, as does the dissociation of microtubules by agents such as colchicine.

To account for these effects, a model for a surface modulating assembly (SMA) involving transmembrane control has been proposed. This assembly consists of through and through membrane receptors, microfilaments, and microtubules, as well as any of their accessory proteins or regulatory molecules. One of the main challenges at present is to provide evidence for interactions of this kind that are postulated to account for surface modulation and its effects on function.

This challenge can be sharply focused in terms of virology by considering the relationships between RSV infection, the nature and target of the src gene product responsible for transformation, and the phenomena of surface modulation. It appears that RSV has sufficient genetic information to code for a protein of molecular weight as high as 40,000 that is responsible for the events of transformation. Work with temperature sensitive mutants indicates that this src gene product is a protein. Its target in the cell is one of the key problems facing research in the area; for a variety of reasons, it has been suspected that this target is the cell membrane.

More recently, it has been found that the components of the SMA are direct or indirect targets of the src gene product. This was shown by developing specific antibody stains to actin and tubulin so that the state of the SMA in RSV-infected chicken fibroblastic cells could be assessed. Uninfected cells showed radiating microtubule patterns and parallel fibers of actin-related microfilaments. In infected cells, however, these structures were practically gone and were replaced by diffuse staining. In cells infected with temperature sensitive viral mutants, the normal pattern was seen at 41°, the temperature at which the src gene product did not function. At 37° (the permissive temperature), the disordered pattern was seen. A switch in the patterns could be rapidly obtained by altering the temperature and did not depend on the synthesis of new cellular or viral proteins. Strikingly, cells at the permissive temperature, which had no orderly SMA structure, also had greatly reduced levels of anchorage modulation. The finding that the SMA is a target of the src gene product and that its structure and function are altered by this protein provides a

new lead in the effort to disentangle the causal network of events occurring in transformation.

In summary, structural and functional analyses at the nexus of virology, cell biology, immunology, and protein chemistry are beginning to reveal a picture of the cell surface as a complex supramolecular assembly with dynamic recognition and control functions. The directions of future research will include an extension of the structural approaches in molecular detail. This will entail the development of improved microchemical surface-mapping techniques, fractionation of the muscle-related proteins and tubulins of normal and virally infected cells, and reconstruction experiments to search for interactions among these components. In addition, there will be heightened efforts to isolate the src gene product and demonstrate directly the interaction with its putative targets.

There is a singular opportunity here to encourage development of methods of antibody-based microchemistry and affinity fractionation procedures. These methods, as well as more refined DNA and RNA chemistry to search for those parts of viral genomes specifying transforming genes, could use extensive support. This is particularly justified because it has been found that normal cells have src related genes. In addition, it will be valuable to have methods of examining specific areas of the cell surface in a dynamic way and correlating the results with structure and function. The method of fluorescence photobleaching microscopy is one example; others will no doubt be developed in the near future.

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