The Cytoskeleton in Cultured Cells: Coordinate in vitro Regulation of Cell Growth and Shape

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1 This work was supported by grants from the NSF and the NIH. The technical help of Sue Conlon is gratefully acknowledged.
Introduction

We have observed two striking sets of similarities between a model system for studying neoplasia and the actual behavior of cells from persons who have an inherited propensity for colonic neoplasia. The model system is SV40 transformation of murine embryonic fibroblasts. The human cells under study are early-passage skin fibroblasts from subjects with adenopolyposis of the colon and rectum (ACR).

The first similarity is genotypic. SV40 transformation and ACR are inherited as simple Mendelian dominant mutations. In the case of transformation, the 'mutation' is the addition to the host genome of at least one functional copy of the early region of the SV40 genome (109). In the case of ACR, family histories show that the disease is inherited as an autosomal dominant Mendelian trait, with a 90% penetrance.

The second similarity is phenotypic. SV40 transformation generates many different changes in the growth-related properties of murine embryonic fibroblasts. Tumorigenicity is very tightly correlated with a subset of these changes, including anchorage-independence, reorganization of cytoskeletal actomyosin, and inappropriate activation of plasmin. Other changes, such as loss of the ability to overgrow to high cell density, serum independence, and low cAMP, seem to be much less well-correlated with tumorigenicity.

SV40 infection can generate intermediate-transformed clones, i.e. clones which are transformed by some, but not all of these assays. Quite often, such intermediate transformants are not tumorigenic. Recent work suggests the amount and specificity of viral protein made in the transformed cell from integrated SV40 early sequences may be responsible for the existence of different phenotypes, with the set of changes related to tumorigenicity occurring only in cells with a sufficient amount of all early viral gene products. In ACR as well, more than one different phenotypic difference between affected and normal subjects can be detected. When skin fibroblasts are examined in culture, Remarkably, the skin fibroblasts from ACR patients have precisely the same pattern of expression of transformed characteristics as do intermediate transformants including an altered distribution of cytoskeletal actin.

Viral Transformation

Cancer can be the consequence of viral perturbation of normal, controlled cell growth. This perturbation may be thought of as a probe, albeit a blunt one, of the various growth controls by which normal tissues maintain a constant cell mass. Our current understanding of these growth controls is bounded by our inability to grow many normal cell types in culture. This inability blocks us from
using our probe *in vitro* in all but the earliest steps of the progression from normal to malignant phenotype (69). It also blocks us from studying the cell types that most often become cancerous. However, tumor viruses have enabled us to analyze the first steps in oncogenesis in some detail. The sum of these early steps is the process of oncogenic viral transformation.

Cells in tissues regulate their mass by at least two different mechanisms. In the first mechanism, cell mass is kept constant in the presence of continued cell division by coupling division to differentiation. Providing that the terminally differentiated cell cannot divide, and that one daughter of each cell division eventually differentiates, steady-state constancy of cell number is maintained. This form of regulation is typical of the epithelial linings of the body: skin, gut, and glands.

The second mechanism for maintaining cell mass is the shutdown of cell division. Usually cells are shut down immediately after mitosis, in such mesenchymal tissues as liver, bone, and connective tissue. In statistical terms, cells in such a state of regulation are said to have an extremely low probability of passing through the cycle (87). While both forms of growth control are currently available for *in vitro* study with clonal cell cultures (68, 77, 78), only the second regulation has so far been found to be subject to *in vitro* perturbation by tumor viruses. Such perturbations are known as transformations, and they are multifacetted.

Indeed, one of the major complications in attempts to understand transformation by the small papovaviruses SV40 and polyoma has been the demonstration that growth control in fibroblasts is subject to a number of environmental regulations, each of which is perturbed separately as a result of papovavirus infection.

Serum deprivation, extensive cell-cell contact, and anchorage deprivation are each sufficient to retard or bring to a halt the proliferation of a population of normal cells. Each assay has been the subject of biochemical analysis, and some penetration to mechanism has begun. Apparently, selection for the loss of sensitivity to one such regulation after transformation does not necessarily lead to the loss of sensitivity to any of the others (17, 29, 80).

*Serum Sensitivity*

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 to 0.2% if the defined constituents of media are optimized (34, 56). Serum can be replaced entirely by a mixture of polypeptide hormones, including insulin, transferrin, and fibroblast growth factor (10, 14, 35, 56).
Viral transformation results in a diminished requirement for serum (36, 80, 93, 96), and therefore presumably for an altered hormone requirement for growth. Indeed, addition of the human hormone EGF to cultivated normal human fibroblasts causes them to behave like serum transformants (10, 16). This suggests that serum transformation may be related to a reduced requirement for this hormone.

Intra-cellular concentrations of cyclic AMP and cyclic GMP also change when cells are serum-transformed, suggesting the possibility that cyclic nucleotide production may be perturbed by the viral gene as well (3, 47, 63, 84).

**Density Sensitivity**

Normal fibroblasts block in G₁ 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 (71a). 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, and indeed this is so by direct biochemical analysis. The major change in proteins is the loss of a large, external, transformation-sensitive (LET₅) glycoprotein (11, 37, 42). This protein is exquisitely protease-sensitive, suggesting that the membrane change could be the consequence of local proteolysis (1). Such proteolysis could occur by the action of a serum protease such as plasmin. Many density-transformants secrete a plasminogen activator (40, 74, 76). Since plasminogen activator is secreted by cultured vascular endothelial cells but not fibroblasts (9), viral genes in this case might be thought to be inducing an inappropriate differentiation as they transform fibroblasts.

**Anchorage Sensitivity**

Dividing tissue cells are, by and large, always in contact with each other, with basement membrane 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 may not proceed through G₁. Expression of transforming viral genes 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.

The reason why cells do not spread and anchor on certain substrates such as agar and teflon is not clear. Well-spread cells deposit a complex set of glycoaminoglycans and proteins, especially collagen, on their substrate (15). Perhaps
these compounds will not adhere to agar or teflon. Unpurified agar is less able to support the growth of transformed BHK cells than in agarose, an agar depleted of acid polysaccharides (58). Collagen seems to overcome the inhibitory effect of acid polysaccharides. Addition of 0.2% native collagen to agar specifically elevates the plating efficiency of untransformed cells. This result suggests that collagen, deposited either by the fibroblasts themselves or from serum, may be essential for satisfactory anchorage by normal fibroblasts.

**Transition Probability and the Cell Cycle**

The cell cycle is conventionally divided into four phases (G₁, S, G₂ and M). While G₁, S and G₂ are constant and relatively independent of growth conditions, G₁ is highly variable. Even within a single population, cell synchrony is always lost rapidly after mitosis because some cells seem to have extended G₁. Until recently, it had been assumed that such cells entered a non-cycling compartment, usually called G₀. A new concept of the cell cycle has recently been proposed, which predicts cell cycle kinetics more accurately than the G₀ model. This proposal divides the cycle of all cells into two parts, one of fixed duration comprising S, G₂, M and parts of G₁ (the ‘B’ phase), and the other a phase of indeterminant length (the ‘A’ state) (90). According to this scheme, a cell may stay in the A state for any length of time, providing that the probability of cells in A going to B is constant. This means that cells leave the A state exponentially with respect to time they have spent there. All of the restrictive assays would then lower this transition probability, and any transformation would be an increase in it. If the transition probability model is correct, there is no need to hypothesize that normal cultures contain a subpopulation of G₀ blocked cells in any of the restrictive assays (87) and the elucidation of the mechanism by which a cell regulates the transition probability becomes an essential problem.

**Viral Antigens in SV-40 Transformed Cells**

The multiplicity of different transformed phenotypes raises the question whether SV-40 viral genes and their products could be responsible for all or even part of such a panoply of different changes in cell behavior. The only viral gene product able to be easily detected in transformed cells is nuclear T antigen. Nuclear immunofluorescence, using sera from hamsters bearing tumors of SV-40 transformed cells, enables us to relate viral gene products (Tag) to the different phenotypes. Two complications have recently arisen. We now know that SV-40 specific tumor antisera can recognize at least two different viral gene products, the A and F proteins. Also, an assay of antigenic specificity can only detect the accumulation of viral proteins, and not their functional fraction.

However, within these limitations, the amount of Tag detected in different transformed clones is related to the clonal phenotype in a very simple way (80):
Table I. Classes of SV40 mouse 3T3 clones

<table>
<thead>
<tr>
<th>Class</th>
<th>% clones</th>
<th>T antigen</th>
<th>Response to selective assays (average of at least 5 clones)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>serum²</td>
<td>density³</td>
</tr>
<tr>
<td>Normal</td>
<td>12.5</td>
<td>-</td>
<td>78</td>
<td>8.5</td>
</tr>
<tr>
<td>Transformed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal</td>
<td>37.5</td>
<td>-</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>Partial</td>
<td>25</td>
<td>ε</td>
<td>34</td>
<td>25</td>
</tr>
<tr>
<td>Full</td>
<td>25</td>
<td>+</td>
<td>34</td>
<td>47</td>
</tr>
</tbody>
</table>

1 From Risser and Pollack (80).
2 Doubling time, hours, in 1% calf serum plus DME.
3 Cell/cm² × 10⁻⁴ at saturation in 10% calf serum plus DME.
4 Efficiency of plating in metrocil.

clones with no detectable Tag are transformed in only one selective assay; clones with very little Tag are transformed in more than one selective assay but not in all, and clones with the highest amount of Tag are transformed by all selective assays (81). Furthermore, within each assay, clones with the most Tag are more completely transformed (table I) (80). These data lead to the hypothesis that SV40 gene products indeed are quantitatively as well as qualitatively responsible for the maintenance of the transformed phenotype (table I).

Other Tumor Viruses

The many possible phenotypes following infection have not been searched for in as systematic a way with other tumor viruses. Conversion from a spread to rounded cell shape, and appearance of dense foci, are the most common selective assays used with the avian and murine retroviruses and the adenoviruses. Nevertheless, some recent studies suggest that transformation by these viruses is very similar to SV40 transformation, from the point of view of the cell.

Adenovirus-transformed hamster cells are typically selected for their ability to grow in media with reduced calcium. 1–2 mM of the ion is present in normal medium. If that concentration is reduced to 0.1 mM, normal cells will not proliferate, and eventually they will die. Adenovirus infection followed by plating in medium with 0.1 mM calcium yields cells able to grow at either calcium concentration (51). These cells may also be, but sometimes are not, transformed by the three criteria selective for SV40 transformants. Adeno transformants also contain virus-specific T antigens. The minimal piece of the adeno-virus 5 genome capable of generating stable Tag-positive, calcium-trans-
formants is 6% from the left end (31). This may also be the location of the
gene(s) coding for adeno T antigens, as well as for transformation. This segment
of virus DNA is also found stably integrated in transformants obtained by adeno
2 or adeno 5 virion infection (23). Adeno 12 transformed cells, as well, carry
integrated viral DNA (32).

Retrovirus transformation proceeds through the intermediate steps of virus
DNA synthesis, DNA circularization, and integration (98, 99, 102). Apparently,
one gene must be carried by the retrovirus if it is to stably transform mammalian
fibroblasts, and this gene is not necessary for viral replication. In avian retro-
viruses, this gene is called src, in murine retroviruses onc. src has been mapped to
the 3' end of the viral RNA genome. The gene products of src and onc, if any,
have not yet been characterized. Because these genes are unnecessary for viral
replication, there is no reason to expect nuclear localization of their products
(100). Temperature-sensitive avian sarcoma and Rous sarcoma viruses have been
isolated which generate transformed rat and chicken cells whose phenotypes are
at least partially temperature-sensitive (4, 18, 29). However, the selective assays
for SV40 transformation have not yet been systematically applied to src-transformed
or onc-transformed cells, to determine whether intermediate states of
transformation can arise. With a few exceptions, morphological conversion
remains the most commonly selected src-transformed phenotype.

Anchorage and Tumorigenicity

Persistent expression of SV40 viral genes is likely to be necessary for
overgrowth in the density and anchorage selective assays, but not in the serum-
selective assay (table II). In addition to these two selective assays, persistent
SV40 gene expressions must generate a large number of non-selective biochemi-
cal differences in cells, since as many as 30% of the major proteins of a cell are
altered upon SV40 transformations (97). Remarkably, in this study the same
pattern cellular changes were seen in both Ki MSV (onc) and SV40 (Tag)-directed
transformations. If this result is confirmed, it will have to be explained in the
light of the very close similarity of mRNAs in normal and SV40-transformed
human fibroblasts (105).

The most important of these changes, from our point of view, are the ones
most closely related to tumorigenicity. It is difficult to test causality between in
vitro non-selective assays and tumorigenicity, but it is possible to determine if
any selective assays also select for tumorigenicity, and vice versa.

Many ways exist to deaden the immune system of an animal about to
receive cells in a test of their tumorigenicity, including administration of anti-
lymphocyte serum and X-irradiation. We have chosen to use the partially
immunoincompetent athymic mouse mutant nude (nu/nu). Nude mice will
Table II. In vitro correlates of tumorigenicity

<table>
<thead>
<tr>
<th>A. Phenotypic syndrome of complete transformation in vitro</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Growth without anchorage</td>
<td></td>
</tr>
<tr>
<td>(2) Loss of internal actin-containing cables (immunofluorescent)</td>
<td>(71b)</td>
</tr>
<tr>
<td>(3) Production of plasminogen activator</td>
<td>(72)</td>
</tr>
<tr>
<td>(4) Tumorigenicity</td>
<td>(73)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Binary linkages within syndrome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anchorage-actin</td>
<td>(72)</td>
</tr>
<tr>
<td>Anchorage-plasminogen activator</td>
<td>(73)</td>
</tr>
<tr>
<td>Anchorage-tumorigenicity</td>
<td>(79)</td>
</tr>
<tr>
<td>Actin-plasminogen activator</td>
<td>(73)</td>
</tr>
<tr>
<td>Actin-tumorigenicity</td>
<td>(88)</td>
</tr>
<tr>
<td>Plasminogen activator-tumorigenicity</td>
<td>(64)</td>
</tr>
</tbody>
</table>

produce immunoglobulin directed against non-thymus-dependent antigens, but they cannot mount a classic T-cell response to xenografts (85). Many mammalian and avian cell lines which are tumorigenic in isogenic animals also are tumorigenic in nude mice (88).

For SV40 transformed murine cells, and also for a wide assortment of tumors and spontaneously transformed cells of many mammalian species, the anchorage assay selected for cells able to grow in nude mice (21, 88, 95). Furthermore, selection in the nude mouse by passage as a tumor (94), also selected for sublines with increased anchorage independence as assayed by growth on agar (52) in methyl cellulose (80) or on teflon sheets (7).

Where exceptions to this correlation were found, almost always they were in the form of lines which grew without anchorage but did not grow in nude mice. Stiles et al. (95), for example, reported that W18VA2, a human fibroblastic SV40-transformed line, had a plating efficiency of 0.31% in methocel, but grew only nodules, and not tumors, in nude mice. Another unexplained exception to the correlation is the report of an adenovirus 2-transformed rat cell line, selected in low calcium medium, which made tumors in nude mice handily, but did not grow in methylcellulose (11).

Nevertheless, two recent reports strengthen the hypothesis that anchorage independence and cellular tumorigenicity are usually very closely linked. The anchorage-dependent, permanent mouse lines Balb 3T3 and C3H10T1/2 are not
tumorigenic. Boone et al. (7) have shown that release of the anchorage-block, by implantation of plastic sheets bearing cell monolayers, was sufficient to permit both of these lines to form tumors in isogenic mice. Further, they showed that the tumors which grew out on the anchoring sheets contained anchorage-independent variants.

The second observation linking anchorage and tumorigenicity was a genetic one. Transfection by calcium-precipitation of metaphase chromosomes is a reproducible technique for transferring genetic markers from one cell line to another, even across species barriers (54, 83, 89). Spandidos and Siminovich (94) recently showed that anchorage independence could be transfected into normal hamster fibroblasts via chromosomes from an anchorage-transformed, tumorigenic hamster line. Each transfected anchorage-independent fibroblast cell line was also tumorigenic. In light of the fact that less than 0.1% of a donor's genome is transfected, this result implies that anchorage-transformation and tumorigenicity may be genetically as well as correlatively linked.

Proteolysis and Anchorage Transformation

Evidence that proteolytic enzymes may be involved in crucial aspects of malignancy and cellular transformation has been recently reviewed (38, 62, 82). Numerous studies have suggested that the production of an activator of the serum protein plasminogen is a specific attribute of the transformed state (26, 48, 53, 64, 73, 101). The activated serum protease plasmin is reputed to determine the morphological alterations which accompany transformation (64). Furthermore, there is evidence suggesting that plasmin determines the ability of virus-transformed cells to multiply in semisolid medium (48, 64, 74). The possibility that the latter property is the in vitro criterion most closely linked to oncogenicity has been discussed above. However, in some studies with other transforming agents, a simple relationship between growth in semisolid medium and plasminogen activator production was not discerned (41, 61, 107).

Cytoskeleton and Anchorage Transformation

In recent years, it has been discovered that the biological movements of all living cells may be due to less structured arrangements of actin and myosin, the proteins of the highly organized contractile mechanism of skeletal muscle (75). Initially, the demonstration of non-muscle actin was achieved by extraction procedures or by less direct methods, such as heavy meromyosin binding, ultra-structural observation of depolymerization, paracrystal formation, peptide mapping, coelectrophoresis with isolated actin, or content of N-methyl-histidine
By these various parameters, the actin derived from species ranging from *Acanthamoeba* to man and from tissues as diverse as brain and platelets appear remarkably similar.

Myosin, about which less is known than actin (at least that within the cytoplasm of non-muscle cells), is defined by its capacity to bind actin reversibly and by its ATPase activity. In its other properties, myosin appears quite heterogeneous and may be much less conserved in the evolutionary sense.

Still less is known regarding the control proteins, tropomyosin, troponin, or the actin-linking protein, α-actinin, but they may be widespread as well. Tubulin, the major component of microtubules, is a well-established component of the mitotic spindle, neurotubules, and flagella. It would appear, however, that this fibrous protein is much more ubiquitous and that, like actin, it may be universally present in mammals (59). The same would seem to be true of desmin, the protein of striated muscle similar to the protein of the 100 Å intermediate filaments (6, 49).

Until the development of specific antisera against these cellular components, it was not possible to easily visualize the arrangement of the cytoskeleton in architecturally intact cells. With the advent of sophisticated purification procedures and immunization schedules employing partially denatured proteins (which in their native forms are weakly antigenic), laboratories have been able to prepare a series of antisera directed against these structural proteins (50, 86). Immunofluorescent, immunoenzymatic, and ferritin labeling have allowed direct visualization of the fibers and networks at the cellular and ultrastructural levels. The further demonstration that antibodies against 'smooth muscle' (in patients with chronic active hepatitis) are partially actin-specific have provided another potential source of antiserum for localization and distribution studies (22).

Within the limits of the technique, many laboratories studying fibroblasts in culture have observed a change in the immunofluorescence pattern of actin with transformation.

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 (50, 103). Actin-containing bundles are diminished in anchorage-transformed fibroblasts (4, 18, 19, 57, 71b, 104). This difference in pattern may be in part the result of changes in cellular adhesion as well (106). To the extent that adhesion is maintained by membrane-associated proteins like LETS (1), it may also be under hormonal control by EGF (12).

In any event, the viral gene product is in some way capable of disrupting the ability of a cell to assemble its cytoarchitectural proteins in their most ordered state. Proteases, especially plasmin, offer a possible mechanism here. Anchorage-transformed fibroblasts secrete plasminogen-activator, and plasmin can specifically remove actin bundles from normal fibroblasts (72, 74).
A Syndrome of Changes in Oncogenic Transformation of Cultured Murine Cells

To summarize, when SV40 transformation leads to tumorigenicity, it may, but need not, generate a syndrome of phenotypic changes all pairs of which are found to be covariant (table II). Exceptions to this syndrome have been described (106) and clearly cells which break the syndrome can be selected (106, 107). However, the syndrome may be a direct viral pathway to tumorigenicity when no selection is exerted since transformed cells generated by ts src ASV or ts A SV40 are ts for the in vitro parts of the syndrome (4, 5, 71b, 107).

Cultured Cells from Individuals at Increased Risk of Cancer

At present, it is rarely possible to detect persons at risk for malignancy before the appearance of a frank invasive or metastatic growth. Although many biochemical assays have been proposed as indicators of premalignant states or cryptic early tumors, most have eventually been shown to be related to age, sex, drug treatment, or other variables linked to, but not clearly indicative of a malignancy. Two serious problems limiting attempts to detect neoplastic states or a disposition to eventual neoplasia are the inherent low frequency of incidence of any single type of tumor and the difficulty of obtaining identical sample material from prospective or actual patients once they are located.

Our approach to the first of these two problems has been to study in detail an inherited syndrome, ACR (24, 55, 60) whose ultimate manifestation by the third decade of life is colonic neoplasia. Because ACR is inherited as an autosomal dominant mutation, children of ACR patients represent a mixed population, about half of whom have the usual low probability of developing colon neoplasms and the other half of whom have a probability of 1.0 (55). Thus, ACR families offer a chance to monitor children who have no oncological symptoms but who will with certainty develop them.

Our approach to the second problem has been to concentrate on the in vitro properties of cells obtained by cutaneous biopsy from patients and their families (45, 46, 67) rather than on cells grown from biopsy material of tissues at risk (33, 65, 91) or of biopsied tissues per se. By using only cultured skin fibroblasts (SF), we are able to examine a single cell type, under reproducible conditions, for presumptive cellular differences between ACR+ and ACR− individuals.

ACR: Clinical Studies

ACR is a disease in which numerous adenomatous polyps develop from the mucosa of the large intestine. The polyps vary in number and are distributed throughout the large bowel with high densities in the rectum. At present it is
believed that the disease is carried by an autosomal dominant gene (24). However, it seems probable that additional genes may pleiotropically modify its expression (55). The close association of ACR with malignancy has been established in a large number of studies. In such instances, frank malignancy is believed to develop from polyps (2). The Gardner syndrome is considered a special variant of ACR, although no sharp distinction between the two can presently be made (2, 43). Thus, ACR may be regarded as a disease in which there is a general tendency to develop both benign and malignant growths from normal flat epidermoid tissue of the colon with or without fibroblastic desmoid tumors and other extra-colonic manifestations. The list of tumors referred to as extracolonie is extensive and has been described elsewhere (2).

Previous studies have concerned the nature of proliferative processes involved in the development of colonic epithelial cells in normal subjects and those with ACR. Differentiation of normal colonic epithelial cells in man occurs during cell migration to the surface of the mucosa, concomitant with repression of DNA synthesis and proliferative activity. On the other hand, development of colonic epithelial cells from ACR subjects was characterized by abnormal phases of cell growth.

The earliest phenotypic expression that identified a genotypic propensity for cell transformation was a failure of colonic epithelial cells to repress DNA synthesis during migration to the surface of the mucosa. This phenotypic alteration occurred in cells prior to polyp formation and did not in itself indicate neoplasia. Rather, additional changes were observed during the evolution of neoplastic transformation. These were characterized by a second phenotypic change, i.e. cell accumulation and initiation of polyp formation in the colonic mucosa (110).

The results suggested that these alterations may identify major steps in a common pathway leading to malignant transformation of colonic epithelial cells. These modes of analysis of colonic epithelial cells are currently used in a screening program of individuals with latent or early ACR and subjects at high risk for colon cancer.

**ACR: Cell Culture Studies**

Because ACR is an inherited autosomal dominant trait, *in vitro* cultures derived from any cell in the body might in principle be expected to be different in some way from normal. Although detection of the difference would in no way be assured, culture studies of skin fibroblasts from ACR subjects showed that the cells were indeed different from normal skin fibroblasts (66, 67). In particular, the SF from ACR subjects and from some of their children responded as if they were transformed, when they were placed in some of the selective assays normally used to recover different classes of SV40 transformants from murine fibroblasts.
SF from ACR patients and from about one half of their children have lost serum- and density-sensitive growth control in culture (46, 67). Compared with SF from normal persons, ACR SF grow better in low serum concentration and grow to a higher density (67). However, the ACR SF are identical to normal SF in their failure to grow in the absence of anchorage and in their failure to form tumors in nude mice (66). Infection of normal or ACR SF by Kirsten murine sarcoma virus yields fully transformed tumorigenic cell lines, and this transformation is more efficient on ACR than on normal SF (66). SV40 transformation of SF from ACR subjects has not been reported.

Thus, the ACR mutation partially decreases the growth control of SF. Specifically, with regard to selective assays, they resemble the stable partial transformants of murine fibroblasts generated by SV40 which express low amounts of T antigen per cell (80). That is, the ACR cells and the intermediate-transformed mouse cells both are serum- and density-transformed but anchorage-sensitive and both types of cells are unable to make tumors.

The skin fibroblasts from ACR subjects and their families have also been studied for their expression of two non-selective parameters of in vitro transformation, plasminogen activator production, and cytoskeletal organization. In both, the ACR fibroblasts were intermediate in their expression, between normal human SF and tumorigenic Kirsten MSV-transformed sublines derived from normal human SF.

Plasminogen activator activity was determined on Triton X-100 extracts of SF grown to near confluency, as described by Riskin and Pollack (79). In a small sample, activator in SF cell extracts from 15 ACR individuals and from two F1 ACR progeny was significantly higher (p < 0.001) than that found in cell extracts from normal SF (46). However, the activity in ACR cells was less than the activity found in extracts of tumorigenic cells (79).

ACR: Cytoskeleton of Skin Fibroblasts

Immunofluorescent anti-actin staining of most normal cells revealed a multitude of long, well-organized, actin-containing cables in SF obtained from normal subjects. These cables frequently ran for many micrometers in length and were up to 1 µm in width (46). In contrast, most SF from ACR phenotypes were deficient in actin-containing cables (46). We do not yet know the reason for this change in pattern. In some transformed cells, a loss of cables is accompanied by a loss of microfilament bundles (4). In others, the change in actin pattern is accompanied by merely a reorientation of cables (27).

About 70% of SF cells from normal individuals in cultures contained actin cables. In cultures of SF from children of ACR individuals, only about 30% of cells contained cables. The age (range, 5–53 years) and gender of individuals from whom the SF were derived did not appear to affect actin distribution (table III).
Table III. Analysis of distribution of actin cables in SF from ACR phenotypes

<table>
<thead>
<tr>
<th></th>
<th>Number of individuals</th>
<th>Experiments</th>
<th>Mean ± SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Symptomatic ACR</td>
<td>11</td>
<td>48</td>
<td>31.6 ± 2.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2. Asymptomatic progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACR phenotypes</td>
<td>7</td>
<td>17</td>
<td>31.2 ± 3.70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-ACR phenotypes</td>
<td>4</td>
<td>5</td>
<td>76.8 ± 4.17</td>
<td>NS</td>
</tr>
<tr>
<td>3. Normals</td>
<td>9</td>
<td>25</td>
<td>77.6 ± 3.15</td>
<td></td>
</tr>
</tbody>
</table>

NS = Not significant.

1 From Kopelowich et al. (46).

2 By Student's t-test; for difference from mean of group 3.

In previous work, we found that cultured SF from clinically asymptomatic children of ACR patients segregated into two groups; asymptomatic positive and asymptomatic negative. Asymptomatic positive SF lacked contact inhibition of division, grew in low serum concentration, secreted large amounts of plasminogen activator, and were abnormally susceptible to viral transformation. Asymptomatic negative SF were identical to normal SF with regard to all of the above characteristics.

Actin patterns in SF of ACR children also fell into two classes. In one class, the actin distribution was identical to that found in SF from ACR patients; in the other class, the distribution was identical to that found in SF from normal persons (table III). The classes of actin distribution and of growth control overlapped exactly. That is, SF from asymptomatic positive children of ACR patients were deficient in actin cables, and SF from asymptomatic negative children had normal actin patterns (46).

Recently, we have found a decreased serum requirement for growth and a disruption of actin cables in the SF of three children of a clinically afflicted patient whose type of colon cancer (genetically undefined at present) indicated a predisposition to ACR. A prospective study has thus been initiated on the predictive value of the actin test for the early detection of the ACR phenotype.

In our assay for cytoplasmic structure, we chose an arbitrary threshold of visualizable actin cables and score the fraction of spread SF capable of this degree of organization. This fraction is a stable measurement with no overlap between ACR and normal (table III). However, Kirsten murine sarcoma virus-transformed clones of ACR or normal SF have a lower percentage of cells containing actin cables than the ACR SF studied here (Pollack and Kopelowich, preliminary data). This suggests that the ACR SF are in an intermediate state of actin organization.
We have begun to examine SF from ACR subjects and their families with antibodies to tubulin and myosin. Our preliminary result is that myosin antibody detects the same change as actin antibody, while tubulin antibody does not detect any major difference in pattern of cytoplasmic microtubules between ACR* and ACR+ individuals.

Cultured Cells from Human Tumors

Most human tumors are of epithelial or endothelial origin (table IV). Therefore, knowledge of the properties of epithelial cells cultured from such tumors would be of great interest. Before epithelial cells from common human tumors could be characterized in vitro, they had to be simply cultured. Unfortunately, establishment of human tumors cells in tissue culture has been a relatively rare event. In most cases, normal (or abnormal, cf. ACR, above) fibroblastic stromal cells overgrow the tumor cells. Furthermore, a number of reported tumor cell lines are the result of contamination by a few cultures such as Hela which grow readily in culture. Indeed, as of 1975, Foghi and Trempe (20) could find reports of fewer than 20 sarcoma and 60 carcinoma cell lines which had been characterized in any detail.

Cell heterogeneity is another problem in the in vitro study of human tumor cells. When a culture is made of a tumor, more than one cell type survives (33, 65, 91). The capillary endothelial cells and the fibroblasts of a tumor have been assumed to be 'non-tumor' cells. However, as we have just discussed, evidence is accumulating that certain 'non-tumor' cells may themselves be abnormal (46, 91). Culture heterogeneity must be overcome to determine whether or not these
observations mean that such systemic defects in 'normal' cells are common in persons bearing tumors.

In the past few years, the cell culture laboratory of the School of Public Health of the University of California at Berkeley has developed simple methods for isolating and culturing epithelial cells from human carcinomas (33, 65, 91). Their results suggest that at least some types of carcinomas can now readily be cultured (92).

When tested for the selective and non-selective in vitro parameters of fibroblast transformation, each tumor cell line had a unique combination of aberrant properties (92). Apparently, epithelial cultures from human tumors do not display the syndrome of transformation expressed in ACR skin fibroblasts and SV40-transformed murine cell lines.

_Cultured Cells from Vascular Endothelium_

Endothelial cells form the inner lining of blood vessels. In the past few years, techniques have been developed to study endothelial cells in culture. Short-term monolayer cultures of bovine aortic and human umbilical cord endothelial cells, and one established rabbit aortic endothelial cell line, have been described (9, 25, 28). Endothelial cells respond mitogenically to the polypeptide hormone fibroblast growth factor (FGF), but not to EGF. Infection with SV40 DNA yields fully transformed clones of human endothelial cells (25).

We have studied the clone of rabbit endothelial cells isolated by Buonassisi and Venter (9). So far, we have not been able to transform it with SV40 virus (500 PFU/cell) or with SV40 form I DNA (1 μg/10⁶ cells). We have, however, been able to show that the cytoskeleton of endothelial cells differs from that of fibroblasts in its sensitivity to the serum protease plasmin.

The actin pattern of rabbit endothelial cells is highly organized (fig. 1). The actin pattern in rat fibroblasts can be disrupted by lowering the concentration of divalent cations (70) or by proteases (72). In preliminary work, we have found that these structures are not disturbed by growth of the cells in rabbit serum (fig. 1), despite the presence in this serum of high levels of plasmin (9). Direct administration of plasmin (urokinase plus plasminogen) also failed to alter actin distribution, again in contrast to the sensitivity of fibroblast actin structures to this protease (72).

The cytoskeleton is, however, as sensitive to divalent cation deprivation as are rat fibroblasts (Pollack, preliminary results). Taken together, these results suggest that the surface of the cultured endothelial cell, which presumably differs from the surface of a fibroblast with regard to hormone receptors (table V), also differs in the protease sensitivity of its trans-membrane linkages to the cytoskeleton.
The Cytoskeleton in Cultured Cells

Fig. 1. Distribution of actin in rabbit endothelial cells. Cells were grown for 24 h at low (a, b) or high (c, d) density in either 10% fetal calf serum (a, c) or in 10% rabbit serum (b, d). No difference in actin distribution is apparent, despite the presence in b and d of high levels of plasmin. Bar = 10 μm.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mitogenic response to</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FGF</td>
<td>EGF</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Epithelial keratinocyte</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Endothelial cell</td>
<td>+</td>
<td>-</td>
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</table>
Pollack/Kopelovich

Summary

Adenopolyposis of the colon and rectum (ACR) links the well-characterized phenomena of murine oncogenic virus transformation with the progression of a human cancer. The same syndrome links defects in fibroblast growth control and cytoskeletal organization to a tumor of epithelial origin.

Since skin fibroblasts are involved in this colonic tumor, the syndrome is very likely to be systemic. That is, one element of normal growth regulation of epithelial cells in situ may be provided by the fibroblasts residing beneath their basement membrane. These observations have led to a novel approach to early detection of persons at risk for a tumor, via the behavior of their skin fibroblasts in culture.

At present, it is rarely possible to detect persons at risk for malignancy before the appearance of a frank invasive or metastatic growth. Although many biochemical assays have been proposed as indicators of premalignant states or cryptic early tumors, most have eventually been shown to be related to age, sex, drug treatment, or other variables linked to, but not clearly indicative of, a malignancy. Two serious problems limiting attempts to detect preneoplastic states or a disposition to eventual neoplasia are the inherent low frequency of incidence of any single type of tumor and the difficulty of obtaining identical sample material from prospective or actual patients once they are located.

Our approach to these problems significantly departs from the common dependence upon isolation of cells from the site of a tumor. Clearly, it is an easier task to scan skin fibroblasts for disrupted cytoskeletal patterns than it is to obtain epithelial cells from most tissues at high risk for malignancy. This line of work, buttressed by information derived from the model system of SV40 transformation, may provide a novel mode of early detection of other human malignancies, as it has for ACR.

References

The Cytoskeleton in Cultured Cells

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bundles and cell shape are related to adhesiveness to substratum and are dissociable from growth control in cultured fibroblasts. Cell 10: 375–380 (1977).


