PRIMARY AVIAN TENDON CELLS IN CULTURE

An Improved System for Understanding Malignant Transformation

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ABSTRACT

Primary avian tendon (PAT) cells which maintain their differentiated state in culture are rapidly transformed by Rous sarcoma virus. By criteria of morphology, increased rate of 2-deoxyglucose uptake, and loss of density dependent growth control, PAT cells transform as well as their less differentiated counterpart, chick embryo fibroblasts. In addition, the percentage of collagen produced by PAT cells drops on transformation by an order of magnitude, from 23 to 2.5%, but is unaffected by viral replication of a transformation-defective mutant.

The responsiveness of normal and transformed PAT cells to various environmental factors changes dramatically upon transformation. Normal PAT cells respond to the presence of ascorbate and high cell density by raising the level of collagen synthesis from 5 to 23%. Transformed PAT cells are totally unresponsive. These and previously reported results lead us to postulate that the breakdown in the normal regulatory mechanisms used by the cell to maintain the differentiated state is related to or is responsible for the onset of malignant transformation.

KEY WORDS collagen synthesis Rous sarcoma virus malignant transformation differentiation ascorbate

Chick fibroblasts derived from the body wall of chick embryos (chick embryo fibroblasts, CEF) and their Rous sarcoma virus (RSV)-transformed counterparts have been used as models for normal and malignant states for many years. There can be no doubt that this system has brought advances in our understanding of cell transformation by viruses; nevertheless, a significant improvement could be obtained by using highly differentiated fibroblasts, which possess tissue-specific functions. A basic assumption in using mixed fibroblast cultures from many tissues is that they all behave the same. While no one would expect epithelial cells from liver, breast, and kidney to behave the same, fibroblasts from the above tissues are treated as if they are all equivalent. Using mixed fibroblast cultures has the added disadvantage of reducing one's ability to define with any specificity the differentiated state of the cells, since no clear in vivo reference point exists. Furthermore, cells are grown in complex medium without regard as to whether or not there are detrimental factors present or beneficial factors absent which are necessary for maintaining the differentiated state (7, 20, 21). Using a mixed fibroblast population in a medium which is most likely detrimental to the expression of differentiated function, hinders our ability to resolve differences between the nor-

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mal differentiated state and the transformed state of the cell (18). This may be extremely important if the mechanisms used by the cell to maintain its differentiated state are the ones blocked by the virus to create the transformed phenotype.

To answer the objections above, we have made use of primary avian tendon (PAT) cells (20, 21). These cells have several distinct advantages over CEF cells. First, they are derived from a tissue which is composed almost exclusively of a single type of fibroblast. Therefore, we are dealing with a homogeneous population. Second, PAT cells in the right environment will maintain their differentiated state in culture (21). In vivo, or in culture, PAT cells devote 25–30% of their total protein to collagen (8, 21) (the percentage increases by 1.5 × when one takes into account that collagen is first synthesized as a procollagen precursor [6]). This extremely biased synthesis in the direction of a differentiated function makes for an easy assay of changes that occur after transformation.

In the present communication, we have two aims: One, to show that highly differentiated PAT cells could be transformed and studied in the same way as less differentiated cell types. By using standard criteria of morphology, loss of density dependent growth control, increase in 2-deoxyglucose uptake, and decrease in collagen synthesis, PAT cells can be transformed as well as chick embryo fibroblasts. Two, to study the similarity and differences in the normal and transformed PAT cells' response to environmental factors which affect the stability of the differentiated state (21). An abstract of the work has appeared (22).

MATERIALS AND METHODS

Cell Culture

PAT cells were isolated from a modification (20, 21) of the Dehm and Prockop procedure (8). PAT cells (8×10^5 cells in 25-cm^2 flasks; Bio:Quest, BBL & Falcon Products, Cockeysville, Md.) were allowed to attach in 5 ml of F12 medium (12) for 40 min. The medium was then changed and the cells were grown in F12 with 0.2%-fetal calf serum (Grand Island Biological Co. [Gibco], Grand Island, N. Y.; deactivated ½ h at 56°C) with subsequent daily changes of medium. When ascorbic acid was used in the medium (50 μg/ml), it was added daily from 100× stock. The stock solution of ascorbic acid was freshly prepared every other day.

Virus Infection and Focus Assay

After PAT cells had attached, the medium was changed to 5 ml of F12 with 0.2% serum plus Rous sarcoma virus cloned from single focus. The cells were incubated for 1 h and then an additional 5 ml of medium was added. The ratio of virus to cell varied from 1:1 to 1:20 as specified in the figure legends. The rate at which infection spread appeared to be more a function of the strain of the virus than the size of the initial innoculum. Focus assays were performed as described previously (4).

2-Deoxyglucose Uptake and Reverse Transcriptase Activity

These methods have been described elsewhere (5, 24).

Collagen Assay

The cells were labeled with [^{3}H]proline for 3 h and were assayed using a purified collagenase as described (20, 15).

Cell Counts

Cells were removed from the flask with trypsin (0.05%) and gently pipetted to eliminate clumps. They were then counted in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

RESULTS

Morphology

One of the most dramatic effects of transformation of mixed chick embryo fibroblasts by Rous sarcoma virus is a change in the morphology of the cells: cells round up, pile up, and are more refractile under a phase microscope (4). With PAT cells the effect of transformation is even more pronounced. Normal PAT cells in medium which promotes the differentiated state, low serum (0.2%), and ascorbic acid (50 μg/ml), assume a very round and flat morphology at high density. Under the phase microscope this gives the appearance of a continuous sheet of cells (although under the electron microscope the distinct separation of cells is clear). PAT cells innoculated with Rous sarcoma virus on the day of isolation show a radical change in the morphology at the end of a week in culture. This is shown in Fig. 1. Transformed PAT cells pile up and are more spindle shaped than their normal counterpart. In addition there is an abundance of rounded cells. By the criteria of morphology PAT cells are transformed.

Growth Control

Another common criteria of transformation is a

lack of density-dependent growth control. Almost by definition a tumor in vivo has to have an impaired sensitivity for cell density, although the rate and the range of cell overgrowth can vary immensely depending on the type of tumor. In culture, transformed cells tend to exhibit what appears to be an exaggerated loss of density-dependent growth control. In this regard PAT cells are no exception: after infection by Rous sarcoma virus, PAT cells do not show the normal sharp decline in generation time on reaching a confluent monolayer. The growth curves for normal and virus-infected PAT cells (Fig. 2) show that normal cells change from a generation time of ~1 day in the early part of the week to zero growth at the end of the week, while the transformed PAT cells are much less inhibited and the generation time only increases from 1 to 2 days as the cells reach high density. It is also evident from Fig. 2 that PAT cells infected with a transformation defective virus grow at a rate which is similar to normal cells. This common control indicates that loss of density-dependent growth inhibition is a property of viral transformation and not of viral infection and replication. To assure that infection by the defective virus has indeed occurred we assayed for the presence of virus particles in the medium by looking for the presence of reverse transcriptase (24). On day 7, the level of enzyme activity on a per cell basis, as measured by incorporation of deoxythymidine triphosphate, was approximately the same for the wild type and the mutant virus (data not shown).

By exhibiting loss of density-dependent growth control, PAT cells appear to be well transformed.

2-Deoxyglucose Uptake

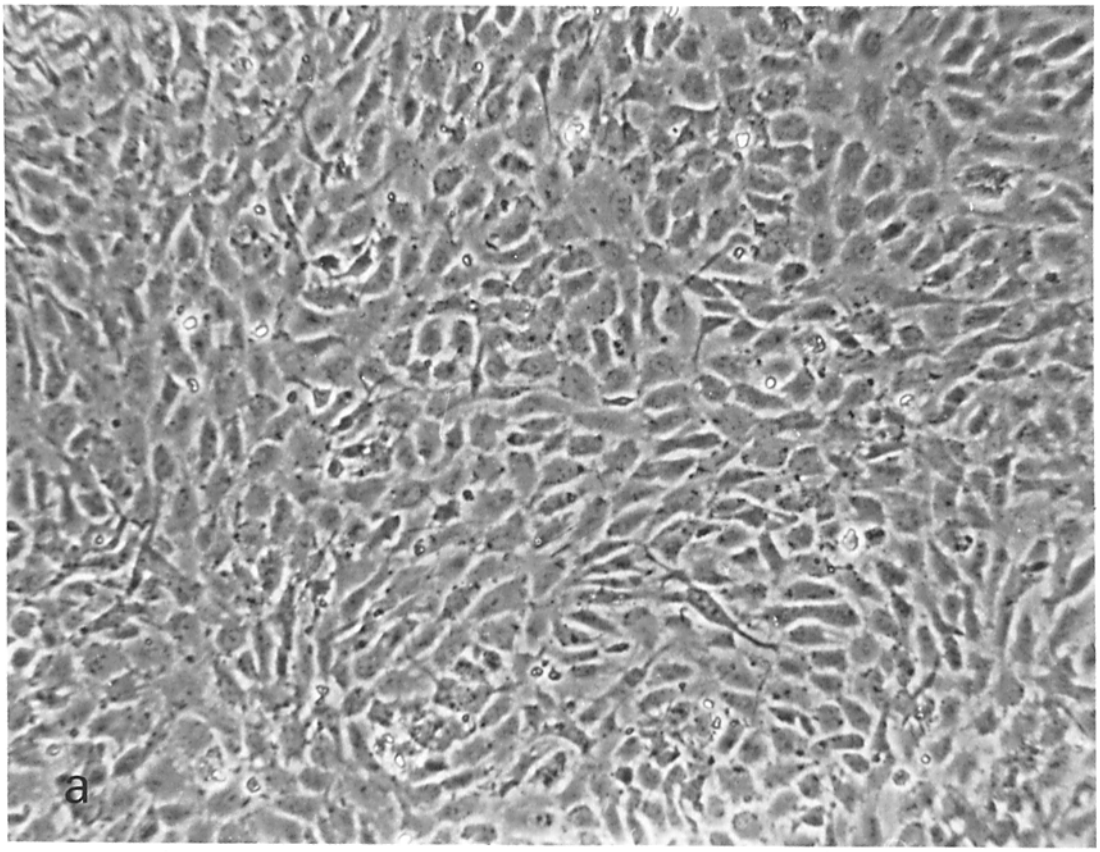
The rate of glucose uptake is a frequently used measure of transformation; transformed cells transport glucose much more readily than do their normal counterparts (3, 5, 10). By using the nonmetabolizable analog, 2-deoxyglucose, glucose uptake (plus the first step in phosphorylation) can easily be measured by accumulation of the label within the cell. In this respect also, PAT cells respond to being transformed in a typical fashion. The time course of 2-deoxyglucose uptake per μg of protein of normal and transformed cells is presented in Fig. 3 *A*. The rate is 15-fold greater in the transformed cells than in the normal cells.

As has been shown with CEF (10, 26) and other cell types, the rate of 2-deoxyglucose uptake varies as a function of the rate of growth of normal cells and additionally with transformation. To test the effect of growth on 2-deoxyglucose uptake, this parameter was measured on normal and transformed PAT cells over a 3-day period. In this experiment, only on time point on each day was taken (5 min), and a plot of this data is presented in Fig. 3*B*. With the use of primary cultures, the increased difference in uptake observed at the end of the week reflects both the slowdown in growth of the normal cells and the greater percentage of transformed cells present in the culture. Despite this complication, 2-deoxyglucose uptake remains a good indicator of transformation for two reasons. Even when growth rates are equal (day 4; Fig. 2), substantial differences between normal and transformed cells still persist. In addition, when normal PAT cells slow down their division rate, they also slow down their uptake of 2-deoxyglucose by half; transformed cells, on the other hand, slow down their growth rate at high cell density (although to a far less extent [Fig. 2]), but they increase their level of 2-deoxyglucose uptake by twofold. Taken together, the data reflects the fact that a large component of the mechanism for glucose uptake is directly transformation-sensitive, as has also been shown previously for CEF (10, 26).

Collagen Synthesis

While a drop in the level of collagen synthesis is not a widely accepted criterion of transformation, several laboratories over the past 15 yr have shown a correlation between transformation and a decline in the percentage of collagen synthesis (11, 13, 14, 17). However, the "normal" cells utilized in these studies synthesized from 6- to 30-fold less collagen than normal PAT cells. With the low level of differentiated synthesis, the question has been raised as to whether or not the changes observed on transformation reflect the actual process in vivo (17). With PAT cells, we can test

FIGURE 1 PAT cells grown for 1 wk in F12, 0.2% fetal calf serum, and 50 μg/ml ascorbate with daily change of medium. (*a*) Normal cells. (*b*) Cells infected on the day of isolation with Schmidt-Ruppin A subgroup of Rous sarcoma virus. The ratio of virus to cells was 1:20 as determined by focus forming units.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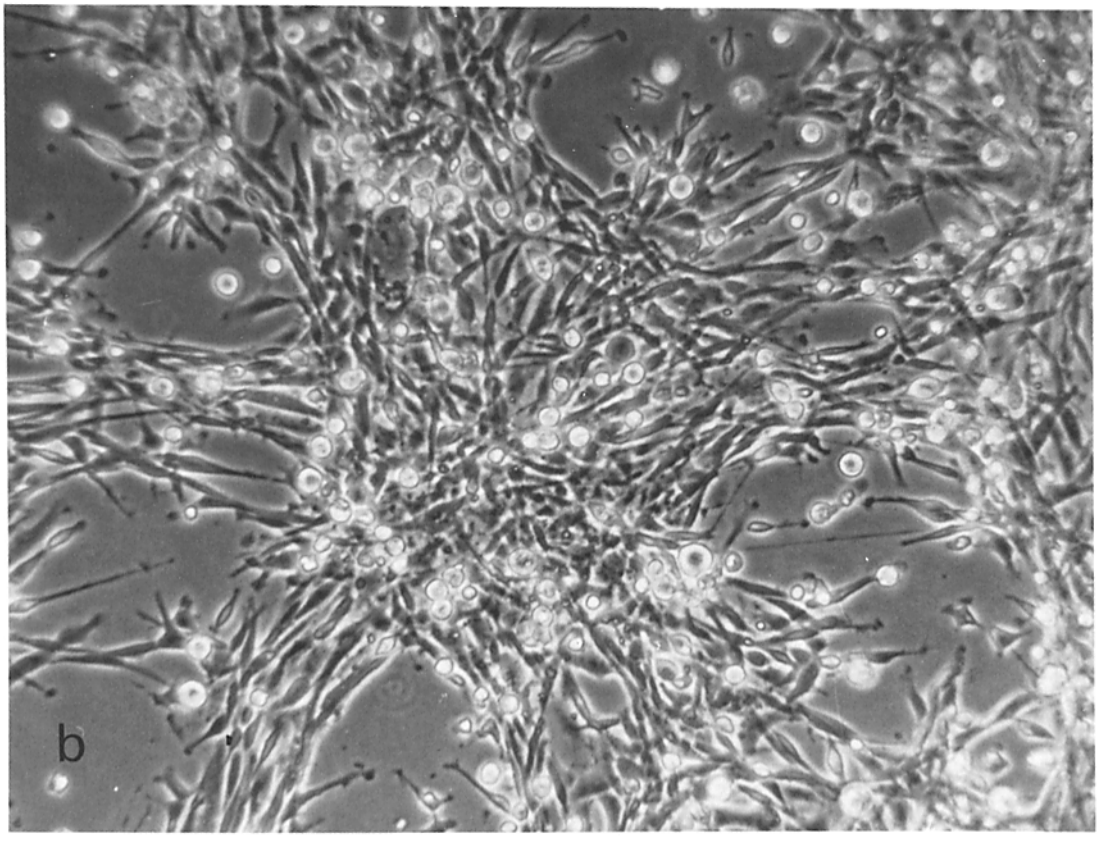
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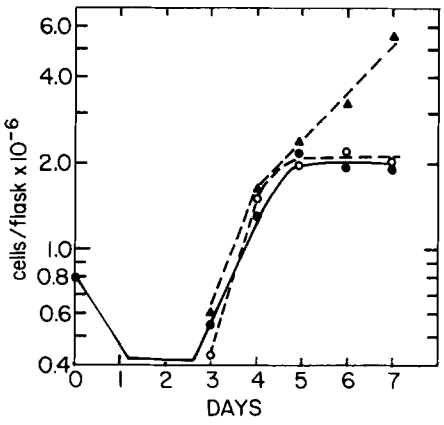

FIGURE 2 Growth curves for normal cells (●), cells infected with wild type Prague C, Rous sarcoma virus, (▲); cells infected with transformation defective mutant of Prague C, Rous sarcoma virus. (○). Cells were grown in F12, as described in the legend to Fig. 1. The ratio of wild type virus to the cells was 1:1.

the action of Rous sarcoma virus on a cell which approximates the in vivo situation much more closely (21).

To study the ability of PAT cells to synthesize collagen after transformation, cells were infected with Rous sarcoma virus and the level of collagen synthesis was measured over a 1-wk period. This was compared to normal cells and cells infected with a transformation-defective virus. Under this protocol little change was expected until 4–5 days when infection had spread to a majority of the culture. We therefore concentrated our analysis on the latter part of the week. The data is presented in Fig. 4. By the fifth day, as the cells began to show alteration in morphology, collagen synthesis began to drop. Over the next 2 days, transformation proceeded swiftly and collagen synthesis declined steadily. In this experiment, by the end of the week there was a drop in collagen synthesis from 23 to 7.5% upon transformation (in other experiments, where complete transformation of the culture was achieved within the week, collagen synthesis declined to 2.5% [Fig. 5]). This drop was a function of transformation and not just virus replication, since cells infected with a transformation-defective virus synthesized the normal percentage of collagen.

In addition, it should be pointed out that the sharp slowdown in growth rate observed in the normal cells has, at most, only a minor positive effect on the percentage of collagen synthesis (20). This is shown by the fact that normal cells on day 4 can grow with a rapid 24-h generation time (similar to transformed cells) and yet produce almost 20% collagen (Figs. 2 and 4). Therefore, the drop in collagen synthesis upon transformation is an effect which is independent of the release from density-dependent growth control. From this experiment, we can conclude that transformation of the cells has a decisive effect on collagen synthesis of PAT cells. This agrees with most of the observations reported in the literature with other fibroblasts (11, 13, 14, 17).

Responsiveness to External Factors

While a decline in collagen synthesis parallels the degree of transformation in PAT cells, their interrelationship is unclear from the above experiments. We would like to distinguish between two possibilities. One, that the control of collagen synthesis after transformation is still normal but at a reduced level. Two, that normal control of collagen synthesis has been disrupted and what remains is a residual synthesis which does not respond to "normal" control mechanisms. These two mechanisms are basically distinguished by whether or not a quantitative loss in collagen synthesis reflects a radical change in the responsiveness of the cell. Normal tendon cells in vivo or in culture are sensitive to their environment and only synthesize a higher percentage of collagen when ascorbate is present and the cells are at high density (21). The question can then be raised as to whether transformed PAT cells are also sensitive to these same factors.

To test this possibility, we looked at the ability

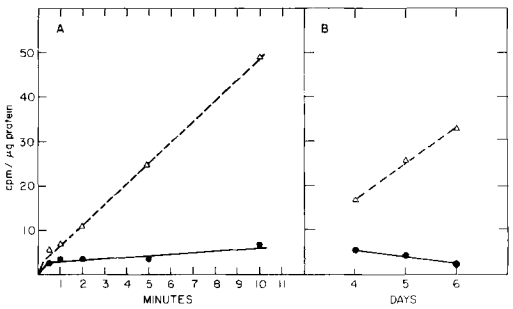

FIGURE 3 The uptake of 2-deoxyglucose in normal (●) and transformed (△) PAT cells. (*A*) Shows the time course of uptake on day 6 after isolation. (*B*) Shows the variations in the level of uptake in a 5-min pulse on days 4–6. The culture conditions are the same as those described in the legend to Fig. 1.

of normal and transformed PAT cells to modulate the level of collagen synthesis when ascorbate was added to ascorbate-deficient cultures. Normal PAT cells responded dramatically to ascorbate by increasing their collagen synthesis 3-fold from 8 to 23% (Fig. 5). Transformed PAT cells on the other hand, were insensitive to ascorbate, producing collagen at a level of ~2.5%, with or without the addition of the vitamin. Transformed PAT cells were not only insensitive to concentrations of ascorbate and to density dependent inhibition of growth (Fig. 2), but also to cell density stimulation of collagen synthesis. As has been shown before (20), and is displayed again in Fig. 5, normal PAT cells seeded at a low cell density respond to a small degree to a density increase even in the absence of vitamin C. If one compares normal cells with transformed PAT cells which were not given vitamin C, then one sees that as the cells reach high cell density at the end of a week in culture, the normal cells respond and raise their synthesis of collagen from 4.5 to 8%, while the transformed cultures remain unresponsive within experimental error. Thus, the difference between the sensitivity of the normal PAT cell to its environment and the lack of sensitivity of its transformed counterpart is clearly established.

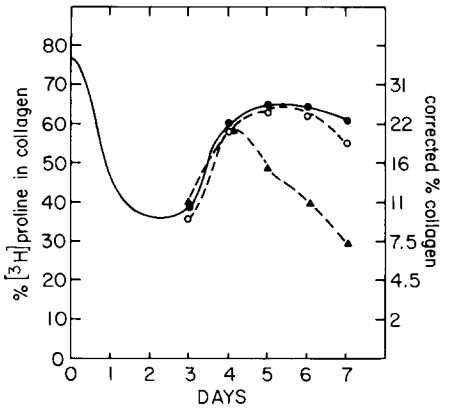

FIGURE 4 The percentage of collagen synthesis over a 1-wk period for normal cells (●); cells infected with wild type Rous sarcoma virus, (▲); and cells infected with a transformation defective mutant (○). The conditions were the same as described in the legend to Fig. 2. The left ordinate expresses the percentage of radioactive proline which was incorporated into collagen relative to total protein. The right ordinate corrects for the fact that proline occurs 5.2 × more often in collagen than in the average protein (9, 20). The corrected value is the one referred to in the text.

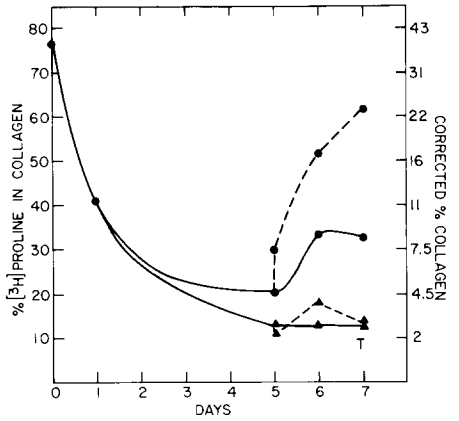

FIGURE 5 The effect of addition of ascorbic acid to the medium of normal (●) and virally transformed (▲) PAT cells. Normal PAT cells and cells infected with Schmidt-Ruppin strain of Rous sarcoma virus (20:1, respectively), were grown for 5 days in medium deficient in ascorbic acid (solid line). On the 5th day, half the cultures of each set were switched to medium containing vitamin C (broken line).

DISCUSSION

In this paper we have shown that a highly differentiated fibroblast culture can be transformed by RNA-tumor viruses as easily as its less differentiated predecessors. By several of the accepted criteria of transformation (altered morphology, increased rate of 2-deoxyglucose uptake, and loss of density-dependent growth control), PAT cells can be transformed by oncogenic viruses. Because the gap between the "normal" differentiated state and the transformed state is broader in PAT cells than in CEF or in established cell lines, several of the virus-induced changes have been magnified and are thus easier to study.

In addition, we have shown that the synthesis of collagen, the major differentiated product of PAT cells, is impaired after transformation by more than one order of magnitude. This result appears to agree with a number of previous studies which have shown several fold drops in collagen synthesis upon transformation by oncogenic viruses (1, 11, 13, 14). While in these studies the relative change in the level of collagen synthesis after transformation was similar to that in PAT cells, the initial level of synthesis of the "normal" cells was either less than, or only slightly higher than, a transformed PAT cell. Comparing in several cell systems the ratio of collagen synthesis before and after transformation becomes confus-

ing when such large differences exist in the "normal" cells. Two perspectives can be taken with respect to the large quantitative difference between the "normal" level of collagen synthesis in PAT cells and other cell systems (17). First, that the ability to respond is critical while the actual quantitative level achieved is of only minor importance. Second, that the type of response of a cell has to transformation can be radically different depending on its initial state. In the latter perspective one would argue that a change in collagen synthesis from 23 to 2.5% (for PAT cells) may be by quite a different mechanism than a drop observed from 2 to 0.5% (for 3T3 [16, 17]). While we have no direct evidence that the changes that occur when PAT cells are transformed are different from those of various cell lines, we do know that the response of the respective normal cells is different towards various external factors. For example, in trying to mimic scurvy in cell culture, PAT cells respond to the absence of vitamin C by a similar mechanism to cells in vivo (2), (i.e., lowering the percentage of collagen produced [21] and reducing the level of hydroxylation of proline [Schwarz and Bissell, unpublished observations, and reference 23]). Other cells in culture appear only to alter the level of hydroxylation (16). Therefore, changes in the initial level of synthesis can affect the type of response as well. PAT cells approximate the "normal" differentiated state more accurately and they may also approximate the transformation process more faithfully.

One reason why PAT cells respond more dramatically to the transformation process is that they will grow at very low serum concentration (0.2%). High serum concentrations (>1%) cause the same changes as viral transformation, only to a lesser degree: collagen synthesis declines, the cells no longer respond to ascorbate or as strongly to density-dependent growth control, and morphology is changed in the direction of virally transformed cells (19, 20, 21, and unpublished results). Therefore, starting with "normal" cells in high serum could significantly reduce the effect of viral transformation.

The similarity of effects of serum and viral transformation on PAT cells raises the question as to whether their mode of action is similar. What these two entirely different agents appear to have in common is that they desensitize the normal cell to its environment. PAT cells which can no longer translate high cell density into a slowdown in generation time, will no longer be able to detect cell density and ascorbate to make high levels of collagen. This multifaceted action of both serum and the virus could be because they have individual components (or branching steps) which interact with the cell in a variety of ways; or, on the other hand, these two effectors could disrupt a common pathway of the cell which is essential for maintaining its differentiated state. The fact that serum causes the same changes to occur within the cell as viral transformation, but only to a lesser degree, makes the latter case more probable. The value of the PAT cell system is that one can approach the problem of viral transformation from two directions. One can use the common approach of trying to decipher the primary action of the transforming gene (src gene[25]) against a background of a myriad of secondary characteristics acquired after transformation. An additional approach is to study the control mechanisms used by the normal cell to maintain the differentiated state; in the case of PAT cells, these include an understanding of the mode of action of cell density and ascorbate on collagen synthesis. If the virus does interfere with an essential step that the cell uses to maintain its differentiated function, then these two approaches will come together to a single and more clearly defined focus on the mechanism of transformation.

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