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Cell Biology

Dependence of the differentiated state on the cellular environment: Modulation of collagen synthesis in tendon cells

(ascorbate/cell density/cell culture)

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ABSTRACT In an adequate environment, primary avian tendon cells are capable of retaining both the full expression of differentiated function and a correct morphological orientation for 1 week in culture. At high density and in the presence of ascorbate, they are fully stabilized in that they devote 25–30% of their total protein synthesis to collagen, a level comparable to that in tendon cells *in ovo*. However, either at low density or in medium without ascorbate, they synthesize collagen at only a third of this level. If plated on a collagen matrix, these cells will orient themselves in a manner similar to that of tendon cells *in vivo*. Furthermore, they are capable of fully modulating the percentage of collagen synthesis upon addition or removal of ascorbate and serum. The variation in the percentage of collagen produced is a result of alterations in collagen synthesis rather than of changes in total protein synthesis or hydroxylation of proline in collagen. Primary avian tendon cells, therefore, provide a suitable model for understanding the stability of the differentiated state, the mechanism of action of ascorbate, and the regulation of collagen biosynthesis.

Understanding what regulates gene expression at cellular and molecular levels requires a rigorously defined and controlled environment. Despite efforts of the cell biologists for the past 70 years, most cells that are removed from the organism and placed in culture lose their ability to remain differentiated (1–5). One conclusion that can be drawn from this universal phenomenon is that very few, if any, tissue-specific functions are "constitutive." That is, despite the fact that synthesis of differentiated products is stable *in vivo*, its continued expression relies on factors that are no longer present in culture. To study function in cells in culture, two approaches are therefore possible. One is to allow the cells to adapt to and reach an equilibrium with their new environment, with invariable loss of qualitative and quantitative expression of function. This is the approach traditionally taken in the past. Alternatively, the culture environment can be modified to maintain the differentiated state of the cell. This latter approach, initiated by Schwarz *et al.* (5, 6) for avian tendon cells, has been pursued in this research in order to achieve a culture system that more closely resembles the *in vivo* state of cells in terms of collagen biosynthesis.

The previous research on collagen synthesis, using "fibroblasts" that had adapted to standard cell culture conditions, has been confusing for several reasons. First, most cells in the body have the capability to synthesize and secrete some collagen; it is the type and the quantity that vary (7–9). Therefore, the level of differentiation retained by the cell in culture can only be delineated with a knowledge of the *in vivo* origin of the fibroblast. This information is unavailable for almost all cell lines used currently. Second, in the early research on collagen synthesis by fibroblasts in culture, the assay used did not distinguish between increased percentage of collagen synthesis and increased activity of prolyl hydroxylase, the enzyme involved in hydroxylation of proline in collagen (10, 11). This assay relied on determining the ratio of hydroxyproline (which is almost exclusive to collagen) to total proline incorporated as a measure of collagen synthesis. The role of factors, such as lactate and ascorbate, that could affect collagen synthesis by changing either the degree of hydroxylation of proline or the percentage of collagen synthesized, or both, could not be adequately described by using this assay (11). Third, although a more recent assay for collagen synthesis is independent of the degree of hydroxylation (12), the cell lines utilized are not stable with time in their ability to produce collagen or to respond to such factors as lactate and ascorbate. Cell lines 3T3 and 3T6, the most frequently used culture systems for research on collagen synthesis, have largely lost their ability to synthesize collagen (10, 11, 13–16). Fourth, Schwarz *et al.*, (5), using primary avian tendon (PAT) cells, have shown that serum levels in excess of 0.5% inhibit the percentage of collagen synthesized. Because almost all earlier research used serum in excess of this level, interpretation of the results becomes even more difficult. It is therefore reasonable to question whether the cell systems used in previous studies of collagen biosynthesis in culture adequately reflect the *in vivo* cell, a point also raised by Peterkofsky and Prather (16).

To avoid these problems, we use PAT cells which are known to produce 25–30% collagen before being placed in cell culture (5, 7); we grow them at low serum levels (except when we want to test the action of serum); and we use an improved assay for collagen (5, 12). In this paper, we explore the complex interaction between the cell and its environment, which results in the stabilization of differentiated function and correct morphological orientation. We show that, under appropriate conditions, loss of function need not occur when cells are placed in culture. We further show that, when functional loss does occur, it is a phenotypic response of the cell to its environment in that it is fully reversible when conditions are corrected.

MATERIALS AND METHODS

Isolation of Cells. Tendon cells were isolated from 16-day chick embryos as described (5–7), with small modifications as follows. To slow down the rate of tissue dissociation, trypsin was decreased to 0.15%, serum was increased to 5%, and the number of tendons per ml of solution was increased. The optimal time for dissociation was found to be about 60 min.

Cell Culture and Media. Cells were seeded at 1.2×10^6 cells per flask (Falcon, 25 cm^2) in 5 ml of F12 medium (17) (Gibco) with streptomycin sulfate (0.1 g/liter) and penicillin (10^5 units/liter) but without serum. After approximately 45 min, when the cells had attached, 10 ml of fresh medium with 0.5% fetal calf serum (Gibco; deactivated at 56° for ½ hr) was added.

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Abbreviation: PAT, primary avian tendon.

In lactate-containing medium, sodium lactate (Gibco) was included in the preparation. Because ascorbate is unstable in culture medium (11), a 100-fold concentrated stock solution of sodium ascorbate (Gibco) was freshly prepared every other day for addition to the medium. Culture medium was changed daily. Variations from the above procedures are described in the figure legends.

Collagen Assay. Cells were pulsed with 1 ml of medium containing 50 μCi of [^{3}H]proline (2′,3′-^{3}H, New England Nuclear) for 3 hr. The pulse was stopped by addition of 5 M NaOH to a final concentration of 0.25 M. Collagen was assayed by a collagenase method (12) modified as described (5). The percentage of collagen made on day "0," the day of isolation, was found to be very reproducible. As a result, day "0" assays were not performed for every experiment; instead, mean (± SD) point derived from previous experiments (six) was used (76% ± 3.3).

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.

Collagen-Coated Flasks. Purified acid-soluble collagen (Calbiochem; 5 mg/ml) was dissolved in 0.5 M acetic acid. This solution (0.2 ml) was spread over the surface of the flasks. The collagen was precipitated by addition of 3 ml of 20% (wt/vol) NaCl and fixed by the addition of 0.1 ml of 2% (vol/vol) glutaraldehyde (Poly Science Co.). After 6 hr, the flasks were rinsed with saline, and 10 mg of bovine serum albumin (Sigma) was added to inactivate any remaining glutaraldehyde. After incubation at 38° for 12 hr, the flasks were rinsed several times with saline and used as described above except that cells were allowed to attach in the medium containing serum.

RESULTS

Stabilization of Differentiation by Ascorbate and Density. The major role of ascorbate in collagen synthesis appears to be reversed when cultured cells are compared to *in vivo cells*. In the body, ascorbate deficiency leads to a marked decrease in the ability of cells to make sufficient collagen (18). To a lesser extent, ascorbate appears to affect the hydroxylation of proline in collagen. In late-passage cell lines in culture, ascorbate influences only the degree of hydroxylation and not the percentage of synthesis (11, 14). In early-passage cells, ascorbate does affect the percentage of collagen synthesis, but only to a small degree (19). The inability of the culture cell to reflect the *in vivo* situation could stem from the fact that the conditions previously used for cell growth tend to inhibit not only the percentage of collagen synthesized but also the ability of the cell to respond correctly to environmental stimuli. A primary cell grown in medium that does not inhibit the expression of collagen, on the other hand, might respond to ascorbate as cells do *in vivo*, by increasing the synthesis of collagen. Therefore, the ability of ascorbate to affect the level of collagen synthesis in PAT cells was tested. Furthermore, because it was already demonstrated that the percentage of collagen synthesis in PAT cells is sensitive to lactate addition (5), ascorbate and lactate effects also were compared.

PAT cells were grown in F12 medium (17) with the following four additions: (*i*) 0.5% serum; (*ii*) 0.5% serum plus sodium ascorbate (50 μg/ml); (*iii*) 0.5% serum plus 37 mM sodium lactate (the NaCl concentration was decreased by 37 mM in order to maintain a constant Na^+ concentration; this resulted in a better cell growth); (*iv*) 0.5% serum plus sodium ascorbate (50 μg/ml) plus 37 mM sodium lactate (the NaCl concentration was decreased by 37 mM). The cells were assayed for collagen synthesis at several times during the week. The data (Fig. 1 *upper*) could be grouped into two distinct periods, corre-

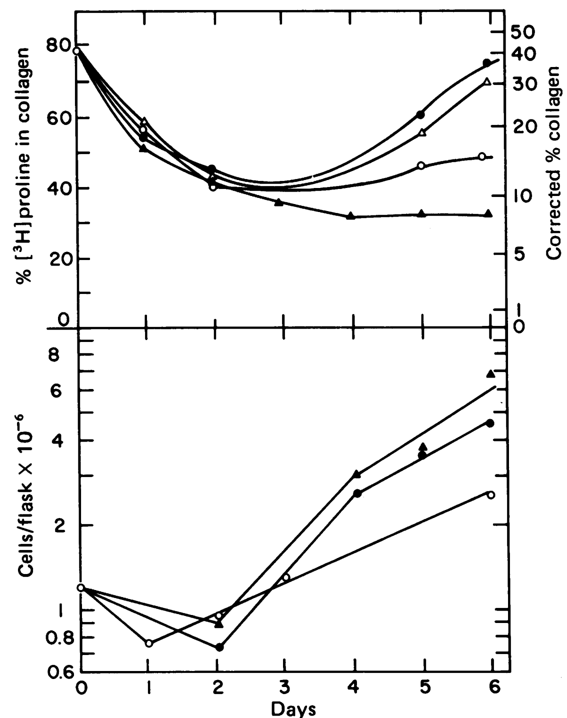

FIG. 1. (*Upper*) Collagen synthesis by PAT cells over a 1-week period in medium F12 plus 0.5% serum (▲) and this plus sodium ascorbate at 50 μg/ml (●), 37 mM sodium lactate (○), or sodium ascorbate at 50 μg/ml and 37 mM sodium lactate (△). The left ordinate shows the percentage of incorporated [^{3}H]proline sensitive to collagenase; the right ordinate is this value corrected for the ratio (5.2) of proline content in collagen and in other cellular proteins [correction based on the method used by Diegelmann and Peterkofsky (21)]. (*Lower*) Growth curves for PAT cells in the above media.

sponding to the beginning and the end of the week. As the cells adapted to culture conditions, there was a rapid 2- to 3-fold decrease in the percentage of collagen synthesized. This was independent of the addition of either ascorbate or lactate. At the end of the week, the cells were sensitive to the medium components, responding most strongly to ascorbate. By day 6, the collagen synthesis in the ascorbate-containing medium was completely restored to the original level (30%). The data also indicated that ascorbate and lactate effects were not synergistic—i.e., the addition of both compounds was no more effective than addition of ascorbate alone. However, because even in the absence of added lactate the cells excrete this compound into the medium (19), its role as a necessary cofactor cannot be ruled out.

The second period, corresponding to the end of the week, is a time when the cells are crowded together and thus grow more slowly. To clarify whether or not the slow rate of growth was responsible for increased collagen synthesis, cell number was determined simultaneously with collagen synthesis (Fig. 1 *lower*). PAT cells propagated rapidly with and without ascorbate. The generation time increased from 1 to 2 days as the cells approached a high density. It may be deduced from the data that the similarity in the growth rates did not correlate with the widely differing percentages of collagen synthesized. In addition, the slow generation time of cells in medium containing lactate was not beneficial to collagen production. This agrees with the previously reported results that the slower growth caused by removal of serum did not affect the percentage of collagen synthesized (5). Therefore, a shift down in the rate of cell growth by itself does not promote a high percentage of collagen synthesis.

It is obvious from Fig. 1 *upper* that PAT cells responded

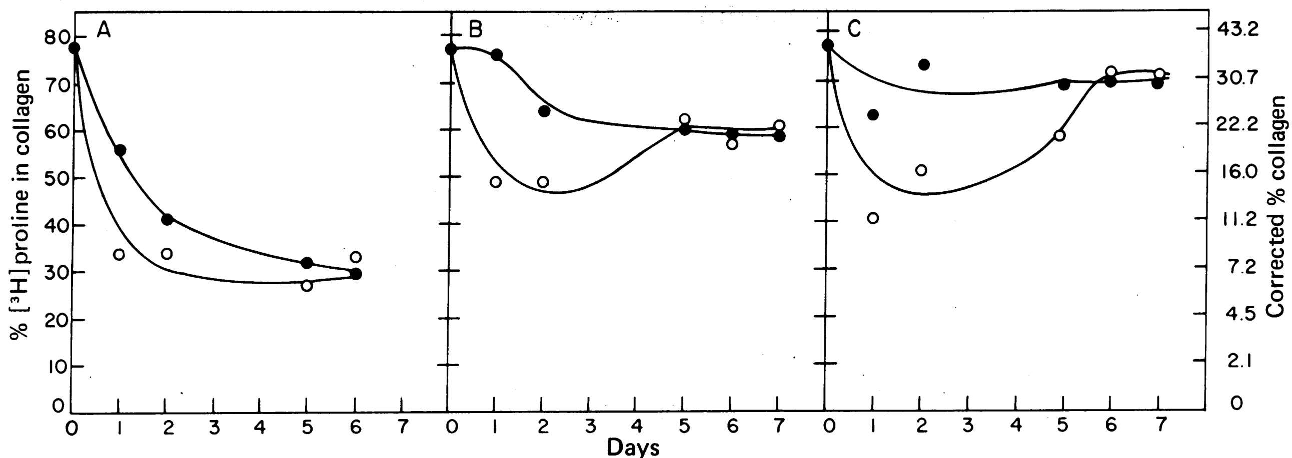

FIG. 2. Effect of initial cell density on the collagen synthesis of PAT cells. Flasks were inoculated with 5×10^6 (●) and 1.2×10^6 (○) cells and grown in F12 medium containing: 0.5% serum (*A*); 0.1% serum + 37 mM sodium lactate (*B*); or 0.5% serum + sodium ascorbate at 50 μg/ml (*C*). (The use of 0.1% instead of 0.5% serum in *B* is not critical; with lactate and certain batches of serum, the collagen level at the end of the week was slightly improved.)

differently to ascorbate at the beginning and at the end of the week. The simplest hypothesis to explain this is that the higher density attained by the end of the week played a role in the modulation by ascorbate. To test this, PAT cells were grown for 1 week starting at a high cell density (5×10^6 cells per flask) and at the usual density (1.2×10^6 cells per flask). The effect of initial cell density on the percentage of collagen synthesis is shown in Fig. 2. In all cases, a high initial cell density helped to stabilize the percentage of collagen synthesis in the early part of the week. With ascorbate present, no decrease in percentage of collagen synthesized was observed. Thus, high cell density by itself—or factors present at high density—is necessary for stability of collagen synthesis in these cells.

In summary, PAT cells in culture responded to ascorbate by increasing the percentage of collagen synthesis in a manner similar to cells *in vivo*. Slower growth rate did not necessarily lead to a higher level of collagen synthesis. A high cell density was a necessary but not a sufficient condition to stabilize the differentiated state in these cells. A combination of high density and ascorbate stabilized collagen synthesis at 25–30% for 1 week in culture.

Modulation of Collagen Synthesis. When PAT cells are placed in culture under a condition other than that described above, there is a decrease in the percentage of collagen produced (5). This loss of function may or may not be reversible (3, 4, 16, 20, 21). In this paper, we refer to irreversible changes as "terminal dedifferentiation" and reversible events as "modulations." Placing PAT cells in a detrimental medium that leads to a low percentage of collagen synthesis and then switching them to a favorable medium should determine whether or not the high percentage of collagen synthesis is a reversible event. PAT cells were grown in F12 medium with either 0.5% or 3% serum but without ascorbate for 4 days and then half of the flasks were switched to the favorable condition: F12 medium plus 0.5% serum plus ascorbate. As a further control, the reciprocal switch was also carried out: 5 days with ascorbate, and 2 days without ascorbate in F12 medium plus 0.5% serum. The data presented in Fig. 3 show that collagen production is a modulated function in these cells. Altering the environment of the cell clearly affected the percentage of collagen produced within 24 hr. Moreover, even the detrimental effect of higher serum levels was fully reversible. It should be pointed out that the addition of ascorbate to medium containing 3% serum was not sufficient to induce a higher percentage of collagen synthesis (data not shown). In other words, the inhibitory effect of serum was more dominant than the positive effect of ascorbate.

As the cells reached a very high density, there was a slight but significant decrease in the percentage of collagen produced (Fig. 3). This decline at the very end of the week appeared to be dependent on the final cell density in the flask, which in turn was related to initial serum levels (and the particular batch of serum). Preliminary experiments indicate that this may be due to an inability of standard cell culture medium to support cell function nutritionally at this high cell density.

Ascorbate Affects the Percentage of Collagen Synthesized by Specifically Increasing the Absolute Level of Collagen Synthesis. We have so far expressed the synthesis of collagen as a percentage of total protein. This is a critical value in determining whether PAT cells are in a normally differentiated state. In addition, the treatment of the data as a percentage corrects for fluctuations in the uptake of proline and overall metabolism. However, changes in the percentage value can be brought about by altering either the absolute level of collagen synthesized or the total amount of protein synthesized. Either case has implications for the mode of regulation of collagen biosynthesis in PAT cells. In order to determine whether or not ascorbate increases the absolute level of collagen synthesis, it is important to consider any additional effects that it may in-

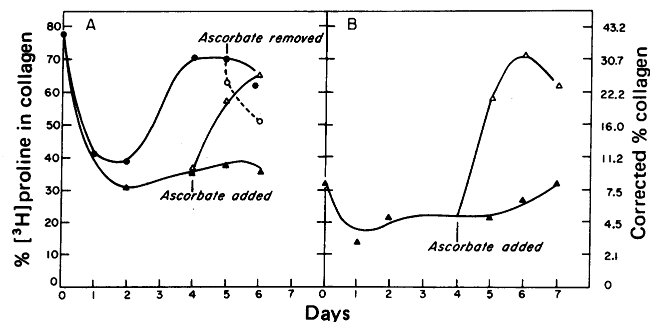

FIG. 3. Modulation of collagen synthesis by altering the medium. (*A*) Cells growing in medium F12 plus 0.5% serum plus sodium ascorbate at 50 μg/ml (●) were switched on day 5 to medium without ascorbate (○). Cells growing in medium F12 plus 0.5% serum (▲) were switched on day 4 to medium that also contained sodium ascorbate at 50 μg/ml (△). (*B*) Cells growing in medium F12 plus 3% serum (▲) were switched on day 4 to medium F12 containing 0.5% serum plus sodium ascorbate at 50 μg/ml (△). [The initial low percentage of collagen synthesis is due to the presence of 3% serum (5).]

Table 1. Effect of ascorbate on absolute collagen and noncollagen protein synthesis

	Cells grown with and without ascorbate, corrected cpm incorporated/flask						Cells modulated with ascorbate on day 4, corrected cpm incorporated/flask					
	Day 2		Day 4		Ratio (day 4/day 2)		Day 4		Day 5		Ratio (day 5/day 4)	
	−	+	−	+	−	+	Control	Modulated	Control	Modulated	Control	Modulated
Collagen	4,500	8,380	8,110	43,060	1.8	5.1	8,110	12,790	18,570	47,870	2.3	3.7
Noncollagen protein	54,830	68,580	73,360	92,740	1.3	1.3	73,360	106,720	165,440	180,650	2.3	1.7
Corrected % collagen	8	11	10	32			10	11	10	21		

The data used in preparing Fig. 3A have been recalculated to present absolute incorporation into collagen and noncollagen proteins. The presentation as "corrected cpm" adjusts for the use of radioactive proline as the probe (see legend, Fig. 1).

duce in cultured cells. The presence of ascorbate in the medium did not seem to affect the overall cellular metabolism. This is reflected in the fact that growth rate was unaffected by the presence of ascorbate. In addition, ascorbate did not alter the rate of proline uptake: in the early part of the week, the total incorporation of [^{3}H]proline into protein (as well as the percentage of collagen produced) was comparable in control and ascorbate-containing cultures. Thus, the rate of incorporation of [^{3}H]proline into collagen and noncollagen proteins after ascorbate addition can be compared with minimal possibility of artifacts.

The absolute synthesis of collagen and noncollagen proteins was calculated from the data obtained in the experiment presented in Fig. 3A. In Table 1, two cases are presented: a comparison between cells on day 2 and day 4 that were grown in medium with and without ascorbate; and a comparison of cells that were or were not modulated with ascorbate for 1 day (day 4 to day 5). The two situations chosen represent conditions under which the percentage of collagen synthesis changes most dramatically. The data show that, in both cases, the addition of ascorbate has a significant effect only on the rate of collagen synthesis. Because the growth rates for cells with and without ascorbate were unchanged, reporting the results on a per cell basis would not alter the conclusion: ascorbate affects the percentage of collagen synthesis by increasing the absolute synthesis of collagen.

Maintenance of Morphological Characteristics. In the final analysis, what makes a cultured tendon cell "normal" may prove to be much more complex than the ability of these cells to produce the correct amount of collagen. A normal tendon *in ovo* has other characteristics such as a specific metabolic pattern and characteristic morphologic features (8). With regard to the latter, we examined the ability of PAT cells to line up between long rows of collagen fibers, similar to cells *in vivo*. In a plastic flask in culture, no clear orientation of the cells exists. This may be due to the surface to which the cells are attached. We coated the surface of the flask with collagen to mimic more closely the natural situation *in ovo*. PAT cells grown on a regular plastic flask and on a flask coated with salt-precipitated collagen are shown in Fig. 4. PAT cells on the collagen surface were highly oriented whereas those on a plastic surface were randomly directed [when the percentage of collagen synthesis was optimal, growth on collagen did not significantly alter the rate of collagen synthesis (data not shown)]. This result clearly indicates that morphologic features as well as function can be manipulated in culture to correspond more accurately to those of tendon cells *in ovo*.

DISCUSSION

A differentiated cell must be defined in experimental terms so that it may be studied in culture. In the past, such definitions were confined to the ability of the cell to express a given function and not to the quantitative expression of that function (3, 22). This definition, however, has its limitation, both *in vivo* and in culture. To begin with, it assumes that all differentiated functions are unique to one cell type. This clearly is not the case. Many cells in the body have the capacity to synthesize some collagen. However, only a few cell types—for example, tendon or bone—have the mission, and thus the capacity, for collagen to be their major cellular product. In these cells, aside from the type, the quantity of collagen synthesized best defines the cell—i.e., quantity becomes quality.

In culture, a definition of differentiation that relies on the ability to express, and not on the level of expression, is even less adequate. In addition to earlier work by Green *et al.* (9), Langness and Udenfriend (23) recently used cloned nonfibroblast cell lines to show that cells that were not known for their collagen production *in vivo* indeed make appreciable amounts of collagen in culture when compared to fibroblast cell lines. Therefore, the mere qualitative synthesis of collagen is a poor criterion of differentiation for fibroblasts in tissue culture.

We define a differentiated cell in culture not only in terms

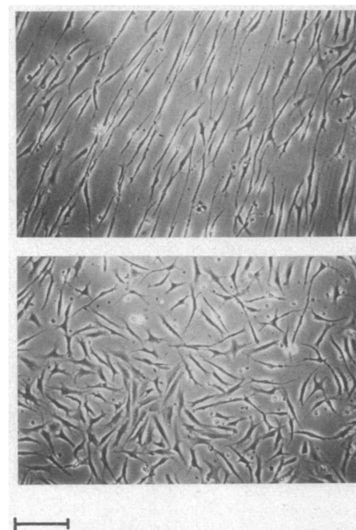

FIG. 4. Phase contrast micrographs of PAT cells on day 3, grown in F12 medium plus 0.5% serum plus sodium ascorbate at 50 μg/ml. (*Upper*) Cells attached to a plastic flask coated with collagen. (*Lower*) Cells attached to a regular plastic flask. The orientation of the cells does not extend continuously across the flask but instead occurs in many patches. This limitation we relate to our preliminary method of precipitating and orienting the collagen coating on the flask. (Bar represents 100 μm.)

of its ability to express tissue-specific function but also in terms of its potential to express function at a level and percentage comparable to those *in vivo*. Quantity of synthesis is not just an arbitrary definition of differentiation. This definition reflects the fact that, for a cell to function normally *in vivo*, it must regulate presisely both the type and the quantity of its products. The percentage value is also critical because it accounts for the fact that in a tissue the synthesis of each component is regulated in amounts relative to other components. Numerical differences in levels of synthesis have generally been ignored as being insignificant, but they may indeed signal important changes in the general state of the cell. For instance, cells synthesizing high levels of collagen respond to their environment differently than do cells making lesser amounts. Thus, PAT cells and cells *in vivo* respond to ascorbate by increasing the synthesis of collagen; established cell lines, which make collagen at a much lower level, respond by merely increasing the activity of prolyl hydroxylase.

Maintenance of the differentiated state in a proliferating cell population outside the body was the original aim of cell culturists (4). Whereas cells in general will either not grow or will lose their ability to express function, PAT cells are capable of both growing and maintaining their function if conditions are properly modified. However, the success in retaining full expression of function in PAT cells may reflect the fact that a tendon is a relatively simple tissue, made of only one kind of cells (fibroblasts) whose main function is to form collagen fibers. It is expected that complex tissues such as liver, which have diverse functions and an intricate structure, will require a much more complex microenvironment for the maintenance of the differentiated state.

Despite the importance of achieving stable conditions for expression of function, the decrease in function that occurs in primary cell cultures could be looked at as an asset rather than a liability: it contains information about the regulation of that function. A eukaryotic cell with a diminished or lost function can be looked at as the next best thing to a "mutant," providing a unique insight into important physiological questions. This is true, of course, only if the loss can be modulated. An irreversible loss cannot be used to study the regulation of function. In other words, both positive and negative modulations are needed. PAT cells are an ideal system in this respect. First, they produce collagen at a level that is more than an order of magnitude greater than that of commonly used cell lines: 30% versus 2% (5, 11). Second, they can modulate the level of collagen synthesis from 5% to 30%, depending on the cell density and the concentration of ascorbate, lactate, and serum. Third, they modulate not only synthetic function but also morphological orientation. And fourth, they respond to modulation in a manner similar to that of cells *in vivo*.

The mechanism by which PAT cells translate an environmental change into a change in the percentage of collagen synthesis is unclear at this point. A mechanism that relates a slowdown in growth rate to a "turn on" of differentiated function would be unsatisfactory because a reciprocal relationship is not always observed. Any model for the regulation of collagen synthesis must be complex enough to account for the necessary involvement of both high density and ascorbate. To decipher the actual mechanism will require a more thorough understanding of the metabolic state of cells at high density and the mechanism of action of ascorbate.

There are at least three further questions that may be raised with regard to this research. The first is the length of time that the cells can remain fully responsive to changes in their environment. Could collagen synthesis be manipulated in the same fashion after second or third subcultures? Does "terminal dedifferentiation" ever need to occur? The second is whether other less dominant differentiated functions of tendon cells are controlled coordinately or separately when compared to collagen synthesis. The third, and much more complex, question concerns the reversibility of development. If the environment of the cell is correctly modified, can a tendon cell undergo transdetermination (25) to become a totally different cell type? Although these questions are not all directly related to the maintenance of a differentiated cell in culture, the answers are critical for an understanding of the mechanisms of gene regulation and the intriguing process of development.

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