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STUDIES ON GUINEA PIG SKIN CELL CULTURES

IV. Collagen Synthesis

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Abstract. Six cell lines derived from the epidermis of adult guinea pig ear skin have been compared with three cell cultures of WI 38 fibroblasts, as regards their ability to synthesize and excrete collagen, in time sequence studies. The statistical method of principal component analysis revealed that only one out of 3 WI 38 fibroblast cultures exhibited significant fibroblastic characteristics. It also showed that two types of cell lines can be grown from epidermal suspensions: one which is fibroblastic in nature and one which is not. This comes as a confirmation of previous papers in this series in which it was suggested that cell lines derived from adult epidermis are not obligatorily overgrown by dermal fibroblasts but can be composed of morphologically altered keratinocytes.

It was shown in the first paper of this series (14) that long-term cultures can be established from separate primary cultures of epidermal keratinocytes and dermal fibroblasts. Irrespective of their origin, all these long-term cultures are composed of fibroblast-like cells. However, epidermis-derived cells are leucinaminopeptidase (LNase)-negative, whereas dermis-derived fibroblasts react positively (2, 3, 7, 14). Since on frozen section of guinea pig skin, the epidermis is LNase-negative and dermal cells are LNase-positive, it has been proposed that long-term cultures derived from epidermal cell suspension are composed of more or less dedifferentiated keratinocytes which have lost their morphological characteristics, but have retained at least one cytoenzymatic trait (4, 7, 12).

In order to lend support to this assumption, it was thought that quantitative studies of collagen synthesis by these fibroblast-like cells would be of interest.

Indeed, regular fibroblasts have been shown to produce large amounts of collagenous materials in tissue culture (5, 6) and collagen synthesis has been proposed to classify various cell types (6).

In a preliminary report from these laboratories (13) it was said that epidermis-derived cultures were in fact capable of producing some collagen but actually yielded about five times less of this material than reference fibroblasts (WI 38). In addition, the ratio of collagen present in the culture medium to that remaining in the cell fraction was far greater (4.3) in WI 38 cells than in epidermis-derived cultures (0.8). These data were found inconclusive, however, since rather wide variations were observed for a given time from culture to culture. These variations could have been explained on the basis of differing phase activity and this prompted us to re-evaluate collagen synthesis by epidermis-derived cultures according to time.

In the present paper we report on time sequence studies of collagen synthesis by epidermis-derived cells as compared with reference fibroblasts (WI 38).

It was agreed that a given cell should be characterized by its ability not only to synthesize, but also to excrete collagen into the external medium. Accordingly the fibroblastic function was defined as the combination of collagen synthesis and excretion. In order to evaluate this function, four parameters have been considered: incorporation of intracellular proline, hydroxylation of intracellular proline, secretion of proteinic proline, and secretion of proteinic hydroxyproline. Finally, to analyse these four parameters simultaneously, the statistical method of principal component analysis has been used.

MATERIALS AND METHODS

(A) *Cell cultures*

Fibroblasts of the reference cell line WI 38 (human embryonic) were grown as monolayers on Falcon plastic tissue culture flasks 250, starting with a trypsinized seed lot of 1.5×10^6 cells per flask in 15 ml of tissue culture medium (TCM).

Table I. *Means of dpm and $\Delta C/\Delta P$ percentage values in seven WI 38 cell cultures pulsed stationary in phase*

	Cell fraction			TCM fraction			Cell + TCM fractions		
	Hypro	Pro	$\Delta C/\Delta P$	Hypro	Pro	$\Delta C/\Delta P$	Hypro	Pro	$\Delta C/\Delta P$
Means	3 480	35 900	2.94	5 050	8 870	21.8	8 540	44 200	6.09
S.D.	2 240	15 500	1.31	3 840	4 770	19.2	5 780	18 200	3.16
Mean S.D.	848	5 864	0.5	1 452	1 803	7.3	2 186	6 889	1.2

Adult guinea pig (Hartley SPF, outbred) ear epidermis derived cultures were propagated through serial subcultures using either trypsin or collagenase (Worthington, crystallized) detachment. In this latter case the technique of Lasfargues 1971 was used (8). Various TCM have been employed. WI 38 cells were grown in Eagle's BME supplemented with 10% preheated calf serum. Epidermis-derived cells were cultured either in Eagle's MEM or Ham's F12 medium without proline (Eurobio, Paris, France). When MEM was used, it was supplemented with 10% (2 experiments) or 20% calf serum (1 experiment) or 10% fetal calf serum (1 experiment) or 25% horse serum (1 experiment). F12 medium was supplemented with 10% fetal calf serum. All serums were from commercial source (Eurobio, Paris, France). Fifty micrograms of ascorbic acid per ml were added to all TCM. TCM was changed every other day. Twelve hours before harvesting, the cells were pulsed with 4 μCi of DL (5 ^{14}C) proline (specific activity 42 mCi/mM; CEA, Saclay, France) in 15 ml of TCM.

After incubation, the TCM was removed and precipitated with 95% ethanol (1:4), 4 hours at +4°C. The precipitate was centrifuged 30 minutes at 10 000 rpm and the pellet was washed three times in 95% ethanol. The cells were detached mechanically by a rubber policeman after washing with saline supplemented with cold proline (Mann). They were then spun down at 4 000 rpm for 5 minutes and the pellet was again washed three times in cold proline containing saline.

Both TCM precipitates and cell pellets were kept separately, frozen, for further use.

(B) *Estimation of collagen and non-collagen protein synthesis*

Cultured cells washed three times with saline solution (cell fraction) as well as TCM proteins after ethanol precipitation (TCM fraction) were hydrolysed with 6 N HCl for 8 hours at 100°C under 1 kg/cm^2 in an autoclave. After hydrolysis, hydroxyproline and proline were separated by column chromatography on Dowex W X 8 according to the technique described previously (1).

Radioactivities of chromatographic fractions were measured in a Packard Tri-Carb scintillation counter in presence of 1 ml of H_2O and 13 ml of Bray's fluid. The amount of proline measured in the cell fraction corresponded to total incorporation of this amino acid whereas the amount of hydroxyproline accounted for cellular synthesis of collagenous proteins. In the TCM fraction, the amount of protein proline represented the secretion of total proteins whereas the amount of protein hydroxyproline accounted for collagen excretion.

(C) *Factor analysis (principal component analysis)*

In trying to achieve a concise description of the observed data we have applied the methods of Factor-Analysis, in particular "principal component analysis" (9). This method gives a simple interpretation of the given body of data and thus affords a fundamental description of the set of variables analysed.

The raw data are the values of n variables for each of N individuals. We can represent this data as a $n \times N$ matrix. The matrix may be interpreted as containing by rows the rectangular Cartesian coordinates of n points in an N space. On the other hand, the same numbers in the matrix may be read in sets by columns to give N points in an n space. In the latter case there will be a swarm of N points in the point representation of the n variables.

The n points whose coordinates are given in the matrix, are all contained in a linear space of dimension m, where m is the rank of the matrix. The loci of the swarm of points are m dimensional ellipsoids. The axes of these ellipsoids correspond to the principal components.

The method of component analysis, then, involves the rotation of coordinate axes to a new frame of reference in the total variable space, i.e., an orthogonal transformation wherein each of the n original variables is describable in terms of the new principal components.

An important feature of the new components is that they account, in turn, for a maximum amount of variance of the variables. More specifically, the first principal component is that linear combination of the original variables which contributes a maximum to their total variance; the second principal component, uncorrelated with the first, contributes a maximum to the residual variance; and so on until the total variance is analysed. The sum of the variances of all principal components is equal to the sum of the variances of the original variables.

For a practical problem only a few components may be retained, especially if they account for a large percentage of the total variance.

Since the method is so dependent on the total variance of the original variables, it is most suitable when all the variables are measured in the same units. Otherwise, by change of units or other linear transformations of the variables, the ellipsoids could be squeezed or stretched so that their axes (the principal components) would have no special meaning. Hence, it is customary to express the variables in standard form, i.e., to select the unit of measurement for each variable so that its sample variance is one. The analysis is then made on the correlation matrix (or covariance matrix) with the total variance equal to n.

In our experiment the choice was made as follows: 4

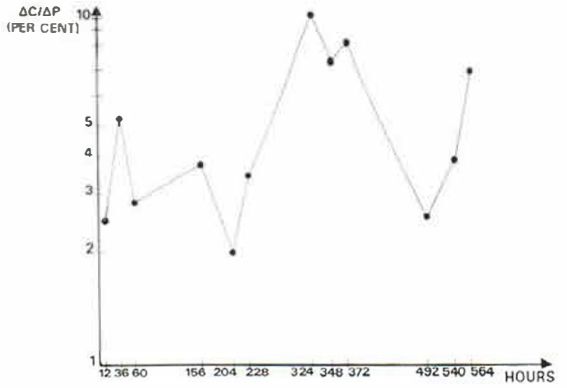

Fig. 1. The ratio of collagen synthesis (ΔC) to synthesis of total protein (ΔP) was calculated according to Green et al. 1966 and plotted against time on a logarithmic scale. WI 38 cells (experiment 1), 30th subculture in Eagle's BME supplemented with 10% calf serum.

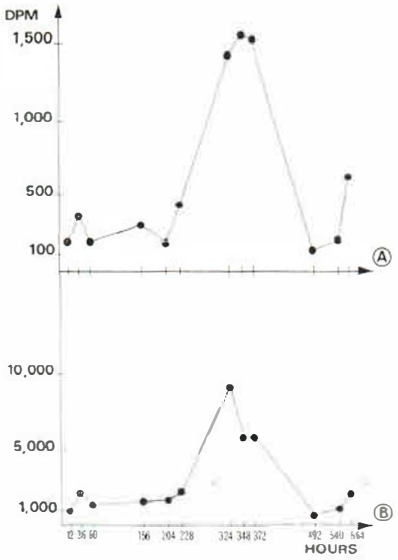

Fig. 2. Hydroxyproline (in dpm) according to time in cell fraction (*A*) and in TCM fraction (*B*). Same cell cultures as for Fig. 1.

variables which are, proline incorporated into the cells (Cell Pro), hydroxyproline formed into the cells (Cell Hypro), protein proline (TCM Pro) and protein hydroxyproline (TCM Hypro) delivered into the culture medium; the individuals are the values obtained at each kinetic time measurement.

The results of the principal component method are plotted in the following manner: the first two principal component axes were considered being responsible for the major part of the variance, and in this two-axis space were plotted the points representing the variables and the individuals.

Thus, using the above considerations, it is possible to obtain a qualitative description of the variables, depending on their coordinates in the space defined by the two principal component axes. According to the proximity of a point variable and an axis we have concluded in the greater or lesser importance of each variable on the variance.

RESULTS

1. *Expression of fibroblastic function by reference fibroblasts (WI 38)*

(*a*) Previous experiments in stationary cultures pulsed at 372 hours.

The ratio of collagen synthesis (ΔC) to that of total proteins (ΔP) was calculated according to Green et al. (5, 6). Cell fractions and TCM fractions were studied separately for each culture experiment.

Means of recorded dpm and $\Delta C/\Delta P$ percentage values for this series of seven (previous) experiments are shown in Table I.

(*b*) Time sequence studies of collagen synthesis have been performed in three different series of experiments.

Fig. 1 represents the curve of collagen synthesis as expressed in percentage $\Delta C/\Delta P$ in the first of these 3 experiments. One can see that there is a wave of collagen synthesis which culminates between 324 hours and 372 hours. Fig. 2 shows the curve of collagen synthesis as expressed in hydroxyproline dpm accoring to time in the cell fraction (*a*) and the amount of hydroxyproline-containing protein in the TCM fraction (*b*) in this same first experiment.

One can see that the two curves have some similarities as regards the timing of maximum collagen synthesis (*a*) and excretion (*b*). Both peaks are located between 228 and 492 hours of culture with a maximum around 348 hours.

The application of statistical principal component analysis to this first experiment is shown in Fig 3. One sees that the points representing cultures studied at 12, 36, 60 and 156 hours are located far from the circles and squares representing the variables (Cell Pro; Cell Hypro; TCM Pro; TCM Hypro). Around 204–228 hours the points are getting closer to the variable Cell Pro. At 324 hours, they are closer to TCM Pro than at 348 and 372 hours. At these latter times, the points are close to Cell Pro and Cell Hypro. After 372 hours of culture, the points are back to non-characterized zones. One can also see that the two variables characteristic of hydroxyproline are about equally distant from the F_1 axis which represents 71% of the variance. There-

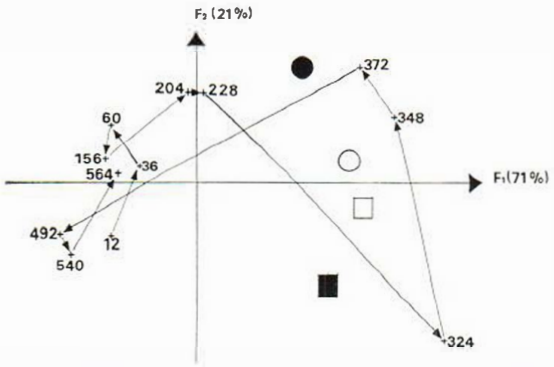

Fig. 3. Principal component factor analysis of the kinetics of collagenous and non-collagenous material synthesis and excretion. ○ = Collagenous material synthesis in cell fraction (Cell Hypro); □ = its excretion into the TCM (TCM Hypro). ● = Non-collagenous protein synthesis in cell fraction (Cell Pro); ■ = its excretion into the TCM (TCM Pro). F1 and F2 axes correspond together to 92% of total variance (71 + 21). Numbers indicate at which time, in hours, the cultures were tested. Same cell cultures as for Figs. 1 and 2. There is a significant tendency of these cells to synthesize and excrete collagenous proteins.

fore, the main function of the cell culture is considered to be directed at manufacturing and exporting hydroxylated (collagen) proteins. Since such a function is taken as characteristic of the connective tissue cell called fibroblast, the above results are taken as consistent with the fibroblastic nature of the cultured cells.

Fig. 4 represents the statistical principal component analysis of the second time sequency study.

Here, one can see that from 132 to 372 hours, the points representing the cultures are far from the

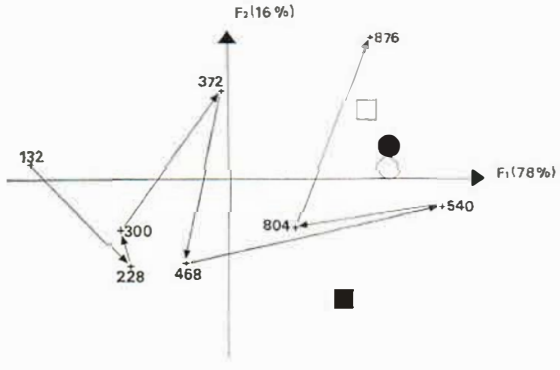

Fig. 4. Same graph representation as in Fig. 3, but cell cultures corresponded to experiment 2 with WI 38, 30th subculture in Eagle's BME supplemented with 10% calf serum. There is no definite trend for these cells to synthesize and excrete collagenous materials. They remain undetermined.

Table II. *Expression of fibroblastic function (collagen synthesis + excretion) in WI 38 cells cultured in BME + 10% calf serum*

	Exp. 1	Exp. 2	Exp. 3
Cell Hypro	+	+	+
TCM Hypro	+	−	−
Cell Pro	−	±	+
TCM Pro	−	+	−
Expression	+	?	?

circles and squares which correspond to the variables Cell Pro; Cell Hypro; TCM Pro and TCM Hypro. At 540 hours, the culture is closer to the empty circle representing hydroxyproline synthesized in the cells, but it is far from the empty square representing excretion of collagenous material. In addition, these two hydroxyproline parameters are located on the same side of the F_1 axis which accounts for 78% of the variance.

Therefore, the fibroblastic nature of the cultured cells is not evidenced in this second experiment.

These two figures, 3 and 4, having been given as examples of the method as it was applied in the present study, and, since this kind of factor analysis is actually essentially qualitative, we thought that in an effort to simplify we could be allowed to represent the various factors by their proximity to the axis of principal variances. Thus, when a factor was obviously near this axis, the sign + was used for this factor.

The sign − was applied to the oppsite situation and the sign ± was used when the location of the point did not permit any evident conclusion.

In applying such a notation the results for our series of three time sequence experiments with WI 38 cells are presented in Table II. This table shows that only in the first experiment was it possible to assess the fibroblastic nature of the cultured cells. In experiments 2 and 3, nothing could be said either for or against. In these last two experiments the cells remain undetermined in terms of fibroblastic function (question mark).

2. *Expression of fibroblastic function by epidermis-derived cells*

Results of six series of experiments are presented in Table III.

On can see in this table that the first four cell cultures remained undetermined. On the other hand,

Table III. *Expression of fibroblastic function (collagen synthesis + excretion) in epidermis derived cell cultures*

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
TCM	MEM	MEM	MEM	MEM	MEM	F 12
Serum	10 % calf	10 % calf	20 % calf	25 % horse	10 % fetal calf	10 % fetal calf
Cell Hypro	+	−	+	−	+	−
TCM Hypro	−	+	+	±	+	−
Cell Pro	+	+	+	−	±	+
TCM Pro	−	−	+	+	±	+
Expression	?	?	?	?	+	−

experiment 5 yielded conclusive evidence for the fibroblastic nature of the cell culture. This is shown in Fig. 5 in which one can see, in particular, that the two hydroxyproline parameters are about equidistant from the main F_1 axis which represents 77 % of the variance.

Experiment 6 also yielded conclusive evidence, but in the opposite direction. As shown in Fig. 6, the two proline parameters are close to and symmetrically equidistant from the main axis which accounts per 72 % of the variance and there is no point close to hydroxyproline cell synthesis. In this experiment, there is no tendency of the cultured cells toward collagenous protein production. On the contrary, there is a definite trend to the synthesis of non-collagenous materials.

DISCUSSION

The present study yields some information as regards (i) the tempo of collagen synthesis by fibroblasts in culture, and (ii) the use of collagen synthesis as a criterion for the characterization of cells in culture.

Statistical factor analysis of the two cell cultures which in this study significantly expressed both collagen synthesis and excretion (experiment one for WI 38 fibroblasts and experiment five for epidermis-derived cell cultures), indicates that the synthesis of proteins, collagenous and non-collagenous, occurs as a succession of phases. In the first phase, there is first synthesis and second excretion of non-collagenous materials. In the second phase, which extends from about 340 to 500 hours, there is synthesis followed by excretion of both collagenous and non-collagenous proteins. Then, a third phase begins, in which protein synthesis remains undetermined. It has been suggested by Manner (10) that during the stationary phase, which corresponds grossly to the second phase referred to in the present study, collagen synthesis is only apparent, due to a decrease in non-collagenous protein synthesis. Data presented here do not support this suggestion.

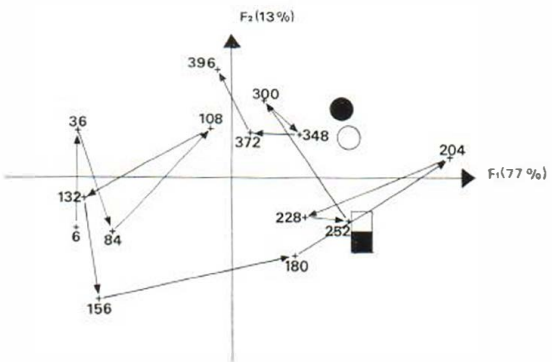

Fig. 5. Same graph representation as for Figs. 3 and 4. Adult guinea pig ear epidermis derived cultures (experiment 5), 8th subculture in Eagle's MEM supplemented with 10 % fetal calf serum. These cells do synthesize and secrete collagenous material (compare with Fig. 3).

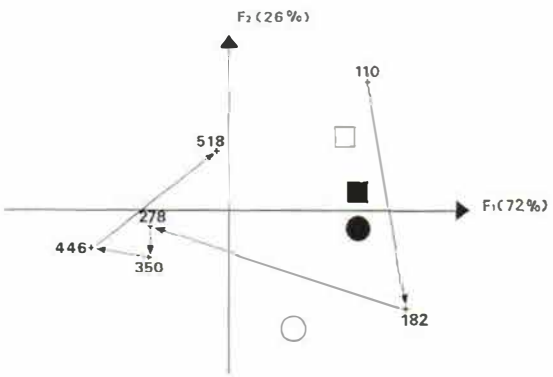

Fig. 6. Same graph representation as for Figs. 3, 4 and 5. Adult guinea pig ear epidermis derived cells (experiment 6) at 9th subculture in Ham's F 12 TCM, without proline and supplemented with 10 % fetal calf serum. There is significant expression of non-collagenous protein synthesis and excretion, i.e. opposite to fibroblastic function.

since in phase II here, active synthesis of both collagenous and non-collagenous proteins has been found.

As regards the wide variations which were observed in collagen synthesis in previous experiments (13) as shown in Table I, they can easily be explained in terms of phase activity. In doing (as we did) our pulses only at 372 hours after seeding the cells, we assumed that we were in phase II in which collagen synthesis does take place. Indeed, according to the present study we were actually in phase II since it begins at about 340 hours. But, if one takes in consideration all variables inherent to the process of cell culture, one will easily admit a margin error of 10% or more. Thus, when phase II is said to begin about 340 hours it means plus or minus 34 hours at least.

Accordingly, there is little doubt that in pulsing at 372 hours, some pulses were made in phase II and some others were not.

This indicates that the estimation of collagen synthesis by cells in culture will be better based on time sequence studies than on single-pulse experiments.

If one now considers collagen synthesis as a criterion for the characterization of cell cultures, one first observes that the fibroblastic function, which, in this study, is defined as the combination of collagen synthesis and excretion, is significantly expressed by reference fibroblasts WI 38 in only one out of three kinetic experiments. This is in contrast to collagen synthesis alone since, as shown in Table I. $\Delta C/\Delta P$ mean value for Cell+TCM fractions at 372 hours of culture (a time which as we have just seen might not be ideal), is 6.09, a figure which is in agreement with that given by Green et al. (5, 6) for this type of cell. Thus, in our effort to characterize cells in culture on the basis of kinetics and combined parameters, we are much more exacting than in looking only for collagen synthesis *in toto*.

In fact, collagen synthesis did take place in cells in all three experiments, as shown in Table II. It is apparently the excretion of hydroxyproline-containing materials which seems to have been impeded. Or, it may be that the excreted material remained stuck to the cell fraction and therefore was counted as part of cellular collagen.

As regards now the epidermis-derived cell lines, only two out of six exhibited significant characteristics. In experiment 5, the cells were conclusively fibroblastic. In experiment 6, they were conclusively non-fibroblastic. Thus, two kinds of cell lines can be derived from epidermal suspensions, one made up of fibroblasts and the other made up of a different cell type.

As regards the nature of this different cell type, it is clear that it must be dedifferentiated keratinocyte. Indeed, it has been shown previously that of the four cell constituents of epidermal suspensions, namely keratinocytes, melanocytes, Langerhans cells and fibroblasts, only the first and the fourth exhibit consistent growth in vitro (11, 12).

This is in good agreement with other papers on the subject. Cytochemical studies have shown that non-fibroblastic cell lines can be established from epidermal suspensions (2, 3, 7), and numerical studies have shown that the nature of the cell lines depends upon the number of dermal fibroblasts which are being admixed to the epidermal suspension during the process of splitting the epidermis from the dermis (15).

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