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STUDIES ON GUINEA PIG SKIN CELL CULTURES.

VII. *Statistical Analysis of Growth and Maturation*

C. Delescluse, S. Cordier, J. Frey,
M. Regnier and M. Prunieras

Laboratory on Human Skin Tumors, Fondation Ophthalmologique Adolphe de Rothschild, Paris, and the Biochemistry Laboratory, Faculty of Medicine, Saint-Etienne, France

Abstract. Thymidine uptake; incorporation of amino acids into perchloric acid extract and HCl extract (corresponding grossly to poorly and highly organized proteins respectively); cell fraction DNA and cell fraction protein contents have all been measured daily from day 1 to day 8 in primary cultures of epidermal keratinocytes (EK) and dermal fibroblasts (DF). Correlations between these five biochemical parameters (or variables) have been sought when using the statistical method of principal component analysis. The analysis of whole data of EK and DF populations taken together revealed that DNA content is a major distinguishing factor between these two cell types. The analysis of variables of each cell type taken independently showed that DF are essentially characterizable by their tendency to synthesize both poorly and highly organized proteins, whereas EK are more prone to DNA and highly organized protein synthesis. Thus, EK and DF in culture can be readily distinguished statistically by analysing their growth and maturation characteristics. It is even likely that time study of thymidine and amino acid incorporation would suffice to characterize these two cell types in vitro.

Key words: Skin culture; Guinea pig; Growth maturation; Statistical analysis

In the sixth paper of this series it was shown that the growth curves of epidermal keratinocytes (EK) were about the same when formed with values of the ratio of simultaneous incorporation of ^{3}H-thymidine and ^{14}C-amino acids (^{3}H : ^{14}C ratio) or with values of the incorporation ratio of ^{3}H-thymidine to μg of cellular DNA (^{3}H : μg DNA ratio). The growth curves of dermal fibroblasts (DF) were different (1).

This situation may reflect differences in the relation between growth and differentiation in these two cell systems. Indeed, EK belong to the class of "retaining cells" in which differentiation proteins accumulate inside the cell boundaries, whereas DF are "secreting cells" which produce collagen molecules some of which remain and others leave the cells and are to be found in the culture fluid. Consequently, the amount of protein contained in the cell fraction should theoretically be directly proportional to the number of cells in EK, whereas in DF it need not necessarily be so.

In order to document this point it was decided to study according to time not only the synthesis of DNA (representing cell growth) and the synthesis of proteins (corresponding to maturation), but also to evaluate daily both DNA and protein contents of the cells in culture. And, since during the process of protein extraction the cells were submitted first to PCA and second to HCl partial hydrolysis (which grossly speaking correspond to the extraction of poorly and highly organized proteins respectively) a total of five biochemical parameters (or variables), ^{3}H-thymidine, μg DNA, ^{14}C-amino acid-PCA, ^{14}C-amino acid HCl, and mg protein, have been considered.

Correlations between these five variables have been sought by using the statistical method of principal component analysis.

It will be shown that DNA synthesis and DNA content, as well as protein synthesis and protein content, are not the same in EK and DF and that these two cell populations can be easily characterized by statistical methods.

MATERIAL AND METHODS

Cell culture

EK and DF have been cultured according to condition 2 as published in the sixth paper of this series (2).

Table I. *Meal values of the 5 parameters (variables) from 4 different cultures of epidermal keratinocytes (EK)*

Figures in italics represent standard error

Days	^{3}H-thymidine (dpm)	μg DNA	^{14}C-AA-PCA (dpm)	^{14}C-AA-HCl (dpm)	(mg) Protein
1	1 554 404 *±222 000*	14.11 *±2.57*	8 990 *±2 215*	126 080 *±4 495*	0.261 *±0.010*
2	287 876 *±98 500*	12.84 *±3.17*	4 154 *±494*	48 565 *±7 100*	0.282 *±0.004*
3	135 606 *±50 500*	10.63 *±2.50*	4 071 *±860*	44 815 *±4 880*	0.250 *±0.014*
4	144 904 *±11 600*	10.23 *±2.25*	5 054 *±1 350*	43 915 *±3 280*	0.256 *±0.010*
5	151 575 *±53 450*	9.88 *±2.40*	4 541 *±1 470*	36 920 *±5 935*	0.269 *±0.017*
6	239 523 *±88 000*	9.59 *±2.75*	5 095 *±2 920*	24 460 *±3 635*	0.271 *±0.007*
7	166 453 *±87 500*	9.88 *±2.45*	3 381 *±1 060*	25 310 *±7 600*	0.276 *±0.012*
8	144 615 *±46 000*	7.90 *±1.85*	3 851 *±1 220*	26 920 *±2 935*	0.276 *±0.005*

Incorporation of ^{3}H-thymidine

The cultures were pulsed daily with 1 μCi/ml of ^{3}H-thymidine for 1 hour and washed. After dissolution in 1 N NaOH and neutralization with HCl, the cells were precipitated with PCA. The precipitate was hydrolysed successively in 0.5 N PCA for 10 min at 95°C and in 6 N HCl overnight and the radioactivity of the hydrolysate was then measured in a liquid scintillator. The details of the above technique have been given elsewhere (3).

Measurement of cellular DNA

Two aliquots of the supernatant obtained after hot PCA hydrolysis as described above were used in order to measure the amount of cellular DNA. The technique used was that of Burton (1).

Incorporation of ^{14}C-amino acids

Cultures were pulsed daily with 1 μCi/ml ^{14}C-amino acid mixture (spec. act. 25 mCi/milliatom of C. Amersham) for 1 hour. After hydrolysis in hot PCA as above, one aliquot of supernatant was counted and recorded as ^{14}C-AA-PCA.

The pellets were hydrolysed in 6 N HCl overnight. One aliquot of the hydrolysate was counted for radioactivity and recorded as ^{14}C-AA-HCl.

Measurement of total protein

The culture medium was discarded, the cells were dissolved in 1 N NaOH and the amount of protein measured by the technique of Lowry (6).

Statistical analysis

It will be seen in the results that data show important kinetic variations from one culture to the other. In order to interpret each experimental point in its relation to the others and to evaluate the influence of each variable, it is necessary to use a statistical method which allows each single event to be situated in the context of the experiment taken as a whole.

The method of Factor-Analysis, in particular "principal component analysis" (5) has been applied. This method gives a simple interpretation of the given body of data and thus offers a fundamental description of the set of variables analysed. It has been described in detail previously (4). Four series of EK cultures and four series of DF cultures, with 8 points (one per day) per culture have been used for computation. Thus each variable (or biochemical parameter) has 4×8=32 dimensions for each type of culture. In addition, since there are 5 biochemical parameters which are being considered at any one day, there are 5 dimensions for each point and for one experiment. In this paper, the following notations will be used: variables with 32 dimensions=V (32); variables with 2×32 dimensions (EK+DF)=V (64); and point with 5 dimensions for one single experiment=P (5).

RESULTS

A. *Presentation of Data*

Computed mean values of each parameter (^{3}H-thymidine, μg DNA, ^{14}C-AA-PCA, ^{14}C-AA-HCl and mg protein) are given in Table I for EK and in Table II for DF.

The variations of these mean values in EK have been plotted against time from day 1 to day 8 in

Table II. *Mean values of the 5 parameters (variables) from 4 different cultures of dermal fibroblasts (DF)*

Days	^{3}H-thymidine (dpm)	µg DNA	^{14}C-AA-PCA (dpm)	^{14}C-AA-HCl (dpm)	Protein (mg)
1	91 941 ±30 250	3.96 ±0.395	1 041 ±301	1 595 ±491	0.046 ±0.008
2	815 856 ±293 000	3.68 ±0.885	1 695 ±363	2 655 ±690	0.069 ±0.011
3	2 706 991 ±224 000	7.04 ±1.54	6 420 ±2 845	34 600 ±16 550	0.111 ±0.015
4	2 729 986 ±1 215 000	6.09 ±0.95	6 990 ±3 315	40 025 ±13 850	0.144 ±0.030
5	5 138 843 ±2 000 000	9.29 ±1.22	10 928 ±2 290	83 195 ±26 950	0.220 ±0.031
6	3 125 228 ±1 660 000	8.48 ±1.42	17 616 ±8 550	143 885 ±64 000	0.233 ±0.041
7	1 278 020 ±459 500	11.21 ±1.81	10 749 ±3 905	107 235 ±30 100	0.229 ±0.045
8	1 358 609 ±296 000	10.61 ±0.67	11 754 ±3 855	132 520 ±39 050	0.238 ±0.055

Fig. 1. Curves of ^{3}H-thymidine, ^{14}C-AA-HCl (and total ^{14}C) all follow the same shape. There is a sharp decline between days 1 and 2. From day 2 to day 8 the amount of DNA decreases slowly, whereas that of protein remains fairly stable. PCA-extracted proteins have a very low value and the curve remains almost flat.

For DF cultures the variations of the same parameters as above are shown in Fig. 2. The amount of DNA increases more or less evenly from day 1 to day 7. The incorporation of thymidine increases up to day 5 and then declines sharply. As regards the synthesis of proteins, there is a continuous increase of incorporation of amino acids into both PCA and HCl extracts during the first 6 days. The amount of proteins also increases steadily and plateaus from day 6 to day 8.

B. *Statistical Analysis*

1. *Analysis of whole data of EK and DF cultures taken together*

(*a*) *Study of correlation.* The limits of significance of linear correlation being p 1% $r \geq 0.3248$ and p 1‰ $r \geq 0.4078$, significant correlations have been found between the following four pairs of variables: 1) ^{14}C-AA-HCl and ^{14}C-AA-PCA ($r=0.864$); 2) the sum of ^{14}C-AA-HCl+^{14}C-AA-PCA and ^{3}H-TdR incorporation ($r=0.5$); 3) the sum of ^{14}C-AA-HCl+^{14}C-AA-PCA and total protein content ($r=0.5$); 4) total DNA and total protein content ($r=0.433$).

(*b*) *Comparison between EK and DF.* As shown in Fig. 3, discrimination between EK and DF populations is visible along the F2 axis (27.43% of the variance) which is linked with the variation of the variable V(64) representing DNA content.

2. *Analysis of DF*

As regards variables V(32) the majority of the variance corresponding to the F1 axis (63.83%) is linked with both ^{14}C-AA-PCA and ^{14}C-AA-HCl as well as with the amount of total protein (Fig. 4).

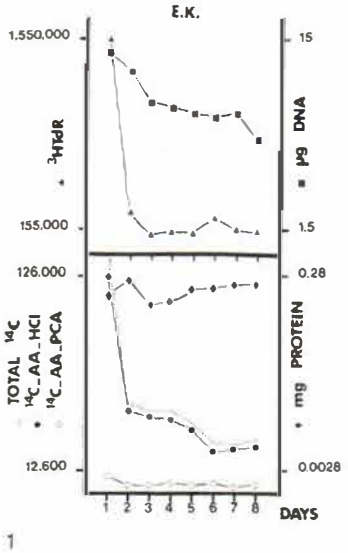
There is correlation between total protein and DNA content ($r=0.452$ for p 1%=0.4487).

Correlation between protein content and protein synthesis (^{14}C-AA-PCA+^{14}C-AA-HCl) is barely significant for p 1%.

It can be seen in Fig. 5 that P(5) plotted against time increases more or less evenly.

3. *Analysis of EK*

As regards variables V(32) the correlation between ^{14}C-AA-HCl and ^{14}C-AA-PCA is weak though significant. There is significant correlation between ^{3}H-TdR incorporation and ^{14}C-AA-HCl ($r=0.9$) and there is no correlation between ^{3}H-TdR and ^{14}C-AA-PCA ($r=0.39$). The correlation between total



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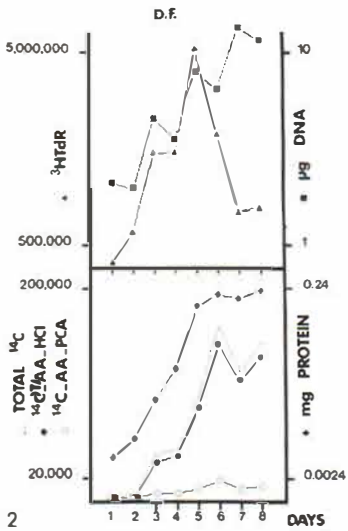

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Figs. 1 and 2. Variations in mean values of: ^{3}H-TdR incorporation, amount of DNA (μg/0.1 ml) incorporation of ^{14}C-amino acids into perchloric acid extract (^{14}C-AA-PCA) and HCl extract (^{14}C-AA-HCl), total amino acid incorporation (total ^{14}C representing ^{14}C-AA-PCA+^{14}C-AA-HCl) and, amount of protein (mg/0.1 ml) in EK cultures (Fig. 1) and DF cultures (Fig. 2) according to time. All incorporation values are given in dpm. The standard errors of these mean values are not shown on these figures but are given in Tables I and II. In Fig. 2 the curves of mg protein and that of total ^{14}C are similar. This is in contrast to Fig. 1, in which they are dissimilar. Also, the curves of μg DNA and mg protein in DF are grossly parallel, whereas in EK the amount of DNA decreases while the amount of protein remains fairly stable. All determinations have been made on the cell fraction after elimination of the culture fluid.

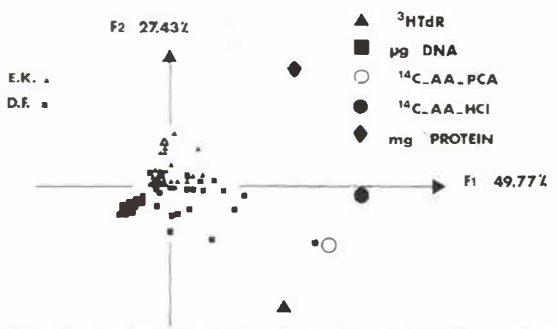

Fig. 3. Analysis of whole data of EK and DF populations combined. Along the F2 axis, EK (small triangles) and DF (small solid squares) show distinct groupings. The large solid square representing the amount of μg DNA is the one of the 5 parameters which is situated closest to the F2 axis (which represents 27.43% of the variables). Thus the two populations differ distinctly with regard to the variation of their DNA content.

DNA and total protein is not significant ($r=0.21$) for a threshold of p 1%, which corresponds to $r=0.4487$.

It will be noted that there is no correlation between total protein content and incorporation of ^{14}C into either PCA or HCl extracts ($r=-0.255$ and -0.109 respectively).

The bulk of the variance according to the F1 axis (47.29%) corresponds to ^{14}C-AA-HCl and to ^{3}H-TdR (Fig. 6).

In addition it can be seen in Fig. 7 that P(5) diminishes from day 1 to day 8, with a sharp decline between the first and second day.

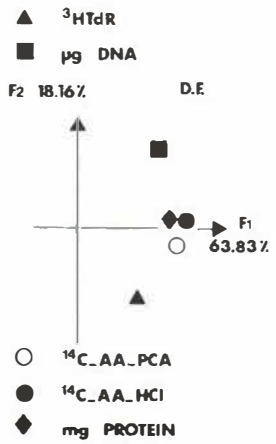

Fig. 4. Analysis of DF. The three variables representing protein synthesis (^{14}C-AA-PCA, ^{14}C-AA-HCl, and mg protein) are closest to the F1 axis which represents 63.83% of the variance. This indicates that DF populations have a strong tendency to (and thus are best characterized by) protein synthesis.

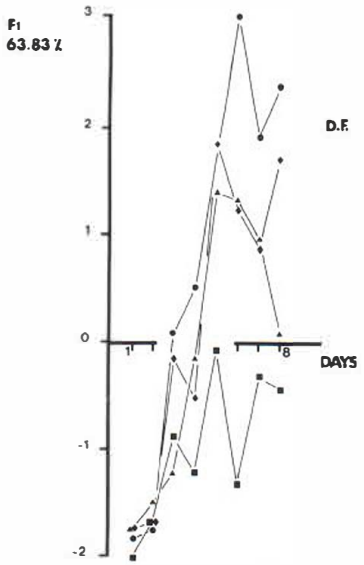

Fig. 5. Evolution of the five parameters P(5) in DF is expressed in arbitrary units along the F1 axis according to time. There is a fairly regular increase with time.

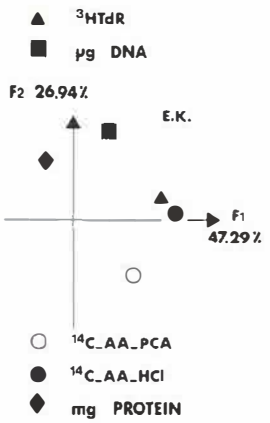

Fig. 6. Analysis of EK. The variables which are located closest to the F1 axis (representing 47.29% of the variance) are ^{3}H-TdR and ^{14}C-AA-HCl. This indicates that EK populations are best characterized by thymidine uptake and highly organized protein synthesis.

INTERPRETATION

1) *Study of correlations in the analysis of whole data* suggests that cell density can be grossly estimated independently by measuring total DNA or total protein contents. However, analysis of EK will show that this applies only to analysis of whole data (see below).

2) *Analysis of whole data of EK and DF populations* (Fig. 3) shows that the major parameter of discrimination between the two cultures is the amount of DNA. This means that the main element of appreciation is the number of nucleated cells at any given time.

3) *Analysis of DF* (Fig. 4) indicates that this population is essentially characterizable by its tendency to synthesize proteins. This applies to poorly organized proteins (AA-PCA) as well as to more highly organized proteins (AA-HCl).

As regards the variations of P(5) the shape of the curve (Fig. 5) tends to suggest a rather slow start in synthesis activity (1 to 3 days) which then plateaus from the 5th day on.

4) *Analysis of EK* (Fig. 6) yields several items of information:

(a) The fact that there is correlation between ^{14}C-AA-HCl and ^{3}H-thymidine and no correlation between ^{14}C-AA-PCA and ^{3}H-thymidine suggests that cells are engaged preferentially into the synthesis of highly organized proteins.

(b) Since the correlation between total DNA and total protein is not significant, it is not acceptable to express cell numbers independently on the basis of DNA or total protein content of the culture.

(c) The lack of correlation between protein synthesis and protein content requires explanation (see Discussion).

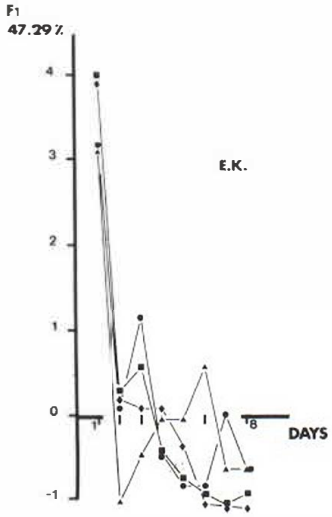

Fig. 7. Evolution of the five parameters P(5) in EK is expressed in arbitrary units along the F1 axis according to time. There is a sharp decline between the first and the second day and little change thereafter.

(d) The variations of P(5) according to time indicate that after a short period of 1 to 2 days during which the cell population is oriented toward cell multiplication and highly organized protein synthesis, there is a dramatic decrease in cell metabolism, as if the cells were in a state preceding death.

DISCUSSION

The first point of interest of the present study is that the characterization of EK versus DF cultures is easily feasible when using the statistical method of principal component analysis. In addition, since the amount of DNA is the major point of distinction between DF and EK and since DF are essentially characterizable by their tendency to synthesize proteins, it seems that temporal incorporation of labelled amino acids and thymidine should suffice to characterize EK and DF respectively. Such a simplification is of some import in pharmacological testing.

The second point is the apparent discrepancy between theoretical predictions and experimental data as regards the relation between DNA and protein content in EK culture.

As stated in the introduction, the amount of proteins contained in the EK cell fraction should theoretically be directly proportional to the number of cells, whereas, in DF, this need not necessarily be so. It is clear from Fig. 2 that in DF the number of nucleated cells (expressed as μg DNA) and the amount of proteins in the cell fraction (expressed as mg protein) increase in parallel throughout the duration of culture. By contrast, in EK (Fig. 1) the amount of cellular DNA decreases with time, whereas the protein content remains fairly stable. Furthermore, on statistical grounds it is stated (see paragraph Interpretation 4*b*) that in EK, cell numbers cannot be expressed independently on the basis of DNA or total protein content of the culture. Thus, the conclusions of our experiments are not in agreement with the theory.

However, this opposition is only apparent. Indeed, when the number of cells is estimated on the basis of the total DNA content of the culture, it is assumed that all cells are nucleated. This is the case in DF cultures. Since maturation of EK in vivo includes loss of nuclei, it may be hypothesized that if, in vitro, the curve of μg DNA decreases (but not that of mg protein) it is because loss of nuclei also occurs in culture.

Additional evidence in favour of the maturation of EK in culture comes from the comparison of protein content with protein synthesis.

As shown in Fig. 1 the mg protein curve remains practically flat, which means that there is no increase in the protein content of the cell fraction, whereas the curve of amino acid incorporation reveals that the synthesis of proteins continues to take place throughout the period of the culture, even though it is lower from day 2 to 8 than on day 1. Also, on statistical grounds there is no correlation between total protein content and protein synthesis (see paragraph Interpretation 4*c*). As stated above, one explanation could be that a proportion of keratinocytes mature in culture. Indeed, in addition to the loss of nuclei, maturation of epidermal keratinocytes in vivo is accompanied by desquamation, i.e. the shedding of cornified cells. If maturation had occurred in culture, a certain number of matured cells would have flaked off into the culture fluid, and, since the estimate of total protein content was made on the cell fraction after washing the cultures, the protein of these flaked-off cells would not have been measured.

Thus, the apparent discrepancy between experimental evidence and theoretical considerations seems to reflect both the loss of nuclei and the "desquamation" in vitro, i.e. maturation of keratinocytes in culture.

The final point is that in paragraph 4*d* of Interpretation, which states that EK cells are in a state preceding death.

This interpretation cannot be accepted in view of the data given in the sixth paper of the present series which showed that mitotic activity started at day 6 and was even higher at day 8. This so-called 'state preceding death', as pinpointed by the statistician, probably corresponds to the block in phase G2 which was noted in the sixth paper quoted above, which block lasted from day 2 to day 6, i.e. much longer than normal (1). Thus, EK cells in culture pass through a non-physiologic phase of evolution which finds its reflection in the principal component analysis.

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M. Prunieras, M.D.
Laboratory on Human Skin Tumors
Fondation Ophthalmologique Adolphe de Rothschild
29, rue Manin
F-75019 Paris
France