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Analysis of Epidermal Growth

Tape Stripping of Skin and Explant Culture

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An explant culture model of epidermal cell growth is outlined. Based on sequential measurements of the radius of the outgrowth around the explant, the paper describes two phases of growth. In the first phase, growth depends on migration of cells out from the explant and on their subsequent proliferation by mitosis. This period occurs during the first week after explantation. At a certain point in time, migration ceases and during the following phase a linear increase in log cell number takes place. A mathematical analysis of the growth is outlined and necessary parameters described. In the system, migration and proliferation rates are determined and the latter related to cell kinetic parameters. This model of epidermal cell growth is used to describe the explant culture of pig skin after activating the skin in vivo by tape stripping. This leads to a mitotic burst which in the explants is shown by augmented migration of cells from the explant, when compared with control explants. The migration rate was no different from that of controls. The duration of migration was prolonged in the experimental group. The proliferation rate observed in these outgrowths was similar. Cell cycle time was 53 and 43 h in control and stripped skin cultures, respectively. An analysis of the model is given. *Key words: Cell kinetics; Experimental model.* (Received January 10, 1988.)

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The explant culture has been utilized as a model of keratinocyte growth. Recently some of its characteristics have been dealt with (1–3). This paper summarizes and extends the information on this model. The stripping of the corneocyte layers prior to explantation of pig skin is used to exemplify the potential of the present model.

MATERIAL AND METHODS

Domestic pigs 5–8 weeks old were used to obtain the dorsal skin for explant culture (1). Skin slices 0.3 mm thick taken with a Castroviejo dermatome were cut into 1 mm square pieces then plated in Falcon dishes, 35 mm in diameter. They were placed in the open to dry for about 10 min before 2 ml incubation medium per dish was introduced. The incubation medium consisted of RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Fungizone®) from Gibco (Grand Island, New York, NY, USA, No. 320-1825, 200-6290, 600-5070 or 5340). The pH of the medium was kept at 7.6 but increased towards 7.9 during readings of the cultures. The medium was not changed during the period of primary culture except for the kinetic study.

Kinetic study. At the end of the incubation 75 nN [^{3}H]thymidine (spec. act. 6.7 Ci/mmol, 0.5 µCi/ml, New England Nuclear, Boston, Mass., USA) was used for autoradiography. The labelling period was 3 days. The cultures were fixed in absolute ethanol, rinsed in saline and processed through dipping in Kodak NTB-3 emulsion, exposed in the dark for 2–3 weeks, developed and finally stained in hematoxylin and eosin.

Readings. The living cultures were read daily at room temperature in an inverted Nikon phase-contrast microscope at 100×. The time when reading was done was noted. Generally, the cultures were outside the incubator for less than 5 min. When the experiment was finished each culture with growth along all sides or at three sides of the original explant was taken for analysis. These were fixed

and stained in H & E. Readings of the fixed and stained cell sheets included all cells of the outgrowth. The whole sheet was also divided into small squares, the number of which was counted. A large sample of such squares was counted for total number of cells excluding those with pyknosis or keratinization. The cell to area ratio was 10.26×10^{-4} cells/μm^2 (1).

Characterization of growth

Outgrowth of the cultures is composed of two different processes: 1) migration of germinative cells from the explant, and 2) proliferation of these cells within the outgrowth. The whole process is essentially determined by the following parameters:

First phase of growth during which emigration of cells and their subsequent divisions occur:
a) the duration of the migratory phase expressed as its half-life, $T_{0.5}$ days;
b) the migration rate constant k_m given as number of migrating cells at the peak of migration expressed as the logarithm of number of cells per day.

Second phase of growth during which only proliferation is present.
a) number of cells of the outgrowth, both the total number (n_i cells at time t_i) and the portion deducted as the total number of migrated cells, m_e;
b) the proliferation rate constant (in phase 2), k_2, given as number of new cells formed per day and expressed as the logarithm of number of cells per day.

The values of these parameters were obtained from each culture after serial measures of the outgrowth during the period of culture. In a semilogarithmic plot ($\ln n_i$ vs. day t_i) number of cells increased rapidly during the first growth phase and were followed during the second phase by a slower but constant rate (2, 3).

The measured point in time in which the growth curve changed its slope to a constant but lower value compared with the preceding readings was designated the *breakpoint*. In order to determine this point mathematically, the following procedure was used. The linear regression of log total cell number versus time in the second phase of the growth was ascertained by addition of measured points ($\ln n_i$; t_i) starting from the end of the experiment (e.g. $\ln n_e$; t_e) going backwards in time. On addition of each new point (at times t_{e-j}, $j=1,2,\ldots$) the test was made to decide whether the added point was part of the linear regression or not. A *t*-test with $p<0.05$ decided whether the point belonged to regression formed by the points taken from t_{e-j} to t_e or not. If this was not the case, the point was not included and the one thereafter designated the breakpoint.

Analysis

Cell number. The maximum distance (radius) perpendicularly from the margin of the explant out to the edge of the outgrowth (R_i µm) was measured. A relationship was shown between counted total cell number and the length of the radius when cells with signs of pyknosis and keratinization were excluded (1). The number of cells (n_i) at time t_i followed the expression

$$\ln n_i = -5.23+2.74\ln R_i-0.09(\ln R_i)^2 \quad \text{eq. (1)}$$

Proliferation. The proliferation rate constant in phase 2, k_2, was obtained from each culture by calculating the slope of the growth curve (coefficient of linear regression). It is known (1) that this factor depends on the growth fraction (a) and the cell cycle time (t_c), since

$$k_2 = \ln(1+a)t_c \quad \text{eq. (2)}$$

The growth fraction was taken as the ratio of labelled keratinocytes after long-labelling with [^{3}H]thymidine to all cells in the outgrowth except those cells exhibiting signs of pyknosis and keratinization.

Breakpoint was evaluated as indicated above. An algorithm was constructed to perform this task by computer (not shown but available on request).

Migration. The cumulative number of migrated cells (m_i) at time t_i was calculated from

$$\ln m_i = \ln n_i - k_2(t_i-t_0) \quad \text{eq. (3)}$$

where n_i is the number of cells at time t_i. The total time of migration is defined in parentheses, where t_0 is the measured point just prior to the start of outgrowth of the particular explant culture (2, 3).

Total number of migrated cells (m_e) was obtained from eq. (3) for t_i equal to the breakpoint. It has been shown that the proliferation rate constant of the first growth phase is equal to or somewhat larger than that of the second phase (3). This may affect the value obtained for m_e as calculated from eq. (3). Furthermore, we do not know precisely when migrated cells start to divide. As a consequence

thereof, migration calculated in eq. (3) is an estimate somewhere in between an upper and lower limit of its true value.

After being normalized, the series of m_i-values follows the normal cumulative distribution (2). The end-oint was arbitrarily set to 0.99 when m_i by this assumption equals m_e. If the distribution is $\Phi(z_i)$ and is a function of z_i, then it has the ordinate

$$\Phi(z_i) = 0.99\ m_i/m_e \qquad \text{eq. (4)}$$

and the abscissa z_i. The latter may be expressed by

$$z_i = (t_i - T_{0.5})/s \qquad \text{eq. (5)}$$

where $T_{0.5}$ is the half-life of migration in days and s its standard deviation as proved earlier (2). The regression of z_i on t_i was calculated for each group of cultures. Its coefficients are $-T_{0.5}/s$ and $1/s$. The value of z_i is obtained from the cumulative normal distribution for the corresponding ordinate value.

The migration rate constant varies during the course of migration and peaks at time $T_{0.5}$ when the distribution has its inflexion point. The value in this point was also deduced (see 2) and, after logarithmic conversion, found to be

$$k_m = \sqrt{(2/\pi s^2)} \qquad \text{eq. (6)}$$

Statistical treatment

The means were used for comparisons. They were obtained from one or several groups of cultures after summarizing data obtained from individual cultures. The error of the mean was calculated from analysis of covariance or variance. In each culture of an experiment the regressions $\ln n_i$ on t_i in phase 2 and z_i on t_i (eqs. (4–5)) in phase 1 were calculated. For the complete experiment the regression of the individual values of k_2 on $\ln m_e$ was also calculated (Fig. 1*b*).

Analyses of covariance were used to test the differences among means and slopes (4). For tests of means the procedure utilizes the difference in mean height between parallel regression lines. To do so, an adjustment of the means (the dependent variable of the regression; ordinate) is performed according to Snedecor & Cochran (4). The values compared are taken at the point on the abscissa which is indicated by the mean of the independent variable of the two groups under comparison. The adjusted means for $\ln n$ and z were calculated for the particular comparison and it is indicated by the prefix a, e.g. ${}_an$ and ${}_az$.

The adjusted mean of $T_{0.5}$ in a treatment group (say X of the two in question) was obtained by an algebraic operation. Firstly, the covariance of z_i on t_i from the two experiments were obtained. The adjusted mean of z in the particular group ${}_az(X)$ and the slope of the common regression (b_{com}) were determined. Finally, the common $T_{0.5}$ (indicated by $T_{0.5,com}$) for the two groups was derived from eq. (5) inserting the coefficients from the common regression. Then, the adjusted mean of the half-life of migration in group X was found by

$${}_aT_{0.5}(X) = T_{0.5,com} - {}_az(X)/b_{com} \qquad \text{eq. (7)}$$

In duplicated experiments, growth curves might vary to some extent. In the statistical treatment, data were therefore combined and the complete set used to determine the common regression. In comparisons between treatments, such regressions were used to test the difference between means. For combined experiments with different sets of readings in time, moving averages were computed, ln n for each day calculated and summarized to get the mean of the composite growth curve for each day. Its error was taken from the variance of common regression and expressed as a percentage of the mean as obtained from the analysis of covariance of original data.

RESULTS

The results of 60 cultures from stripped skin and 69 from control skin are summarized in Table I. The long-labelling with [^{3}H]thymidine was performed in 12 and 22 of these cultures, respectively. One of the controls was lost during the technical procedure. The total number of migrated cells and total number of cells in the outgrowth were increased by stripping, but not the migration rate constant. The proliferation rate constant, k_2, varied among cultures but was similar in stripped skin when compared with control (see Fig. 1*a*, *b*). The result was similar in the minor group of long-labelled cultures, Table I. Correlation

existed between k_2 and $\ln n$ at the time of the breakpoint in both groups, $r>0.87$, $p<0.01$. Likewise, correlation was present between k_2 and $\ln m_e$ (Fig. 1*b*).

The growth fractions obtained by long-labelling were 45.2 and 45.8% for stripped and control groups, respectively. The cell cycle time was determined according to eq. (2) for each of the labelled cultures. The means and standard errors were 43±2 and 53±2 h for stripped and control groups, respectively, $p<0.01$, $t=4.2$, d.f. 31. When the reciprocal t_c was plotted against $\ln m_e$ (Fig. 1*c*), the two curves did not differ in height or slope ($p>0.10$, $F=2.19$, d.f. 1/30).

DISCUSSION

The method

Total cell number. This study utilizes the measurement of the radius of the outgrowth to assess total number of cells in the outgrowth. Care was taken not to include clearly differentiated cells (2). Various ways were tried before finding the relationship expressed by eq. (1). We know that the cell size varies during culture, smaller cell diameters are present in the beginning of the outgrowth as a sign of a larger quantity of cycling cells. Furthermore, the outgrowth comprises cells which are in motion (migration or otherwise) and other in place (including preparation for mitosis). The details of these processes are only roughly ascertained by the analysis given.

The logarithmic term of the cell number in eq. (1) may indicate that it increases exponentially. In the span for about 1–2 weeks during phase 2 this increase in the logarithm of cell number is straight linear as a proof thereof. In each culture the coefficient of correlation between logarithmic cell numbers and time is above 0.9. This fact underlines the validity of this procedure. It enables easy measurement of the proliferation rate in this phase.

Table I. *Growth parameters in explant cultures originating from stripped and normal skin of pigs*

Means and SEM are given. The parameters are migration and proliferation rate constants (k_m, k_2; logarithm of number of new cells formed per day), half-life of migration ($T_{0.5}$; days) and total number of cells due to migration (m_e, ${}_{a}m_e$) and total number of cells (n, ${}_{a}n$). Comparisons between striped and control explants are indicated by ratios of adjusted means (indicated by the prefix "$_a$") as outlined in the text

Material	No. of cultures	Phase parameters k_m	k_2	$T_{0.5}$	m_e	n day 7
Stripped	60	1.05	0.184	3.05	1 880	7 070
±SEM		0.05	0.009	0.08	150	120
Control	69	1.02	0.187	2.80	1 220	4 710
±SEM		0.04	0.009	0.07	100	70
Ratios of means of stripped and control group:						
	No.	${}_{a}k_m$	k_2	${}_{a}T_{0.5}$	m_e	${}_{a}n$
Total material	60 vs. 69	1.03°	0.98°	1.08**	1.54**	1.51**
Autoradiographic m.	12 vs. 22	0.91°	0.72°	1.17**	2.32**	1.60**[d]

° $p>0.05$, * $p<0.05$, ** $p<0.005$ as obtained from analyses of covariance. Values indicated by a trailing [d] are p-values obtained with the notice that there is a significant difference in the slope of the regressions whose means are compared. The difference between m_e was tested by t-test.

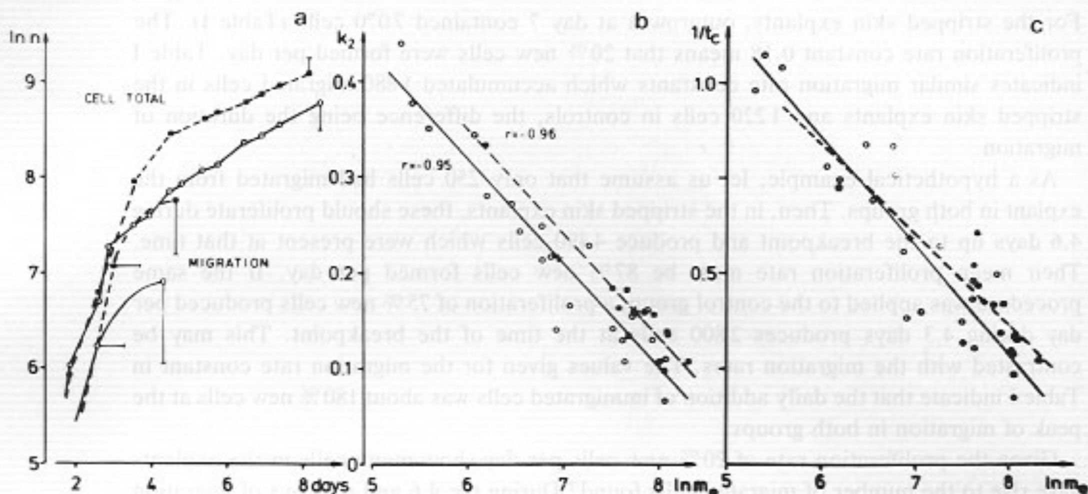

Fig. 1. (*a*) Growth curves of explant cultures from pig skin. The upper two curves represent total numbers of cells from stripped skin (■---■) and control skin (○—○). The lower two curves show the calculated migration. Vertical range bars indicate standard deviations of the means of total cell number ($\ln n$) and total number of migrated cells ($\ln m_e$). The horizontal bars indicate standard deviation of half-life of migration ($T_{0.5}$). (*b*) The relationship between the proliferation rate constants and total number of migrated cells (eq. (3)). The correlation coefficients were above 0.9 and both significant ($p<0.001$). The difference between the adjusted means of the regression of k_2 on $\ln m_e$ in the two groups was significant ($p<0.005$). The slope of the straight lines equals the reciprocal of the mean of the total time of migration. (*c*) The relationship between reciprocal cell cycle times and total number of migrated cells. The correlation coefficients of these regressions were below -0.86 and both significant ($p<0.01$). The difference between the adjusted means of $1/t_c$ in the two groups was not significant.

The breakpoint was sought by a statistical approach. The precision of its estimate may depend on how narrow-spaced individual measurements were made. Up to three measurements per day were chosen in an attempt to more precisely define the point. It was found in the evaluation of the whole experiment, however, that the most important factor for a consistent result was to increase the number of cultures in the particular group rather than make more than one observation per day.

The identification of the breakpoint for each culture is important, since different parameters influence growth in the two phases. Authors using explant culture should be aware of this condition.

The proliferation rate constant. Exponential growth was found during the second growth phase. This was expected to occur in healthy expanding cultures. Variation in the proliferation rate constant was present among individual cultures. Correlation existed between k_2 and $\ln n_i$ at time of the breakpoint as well as between k_2 and $\ln m_e$ (Fig. 1*b*). Both comparisons indicate a diminished proliferation rate constant when migration is increased and the total cell number becomes larger. It may be noted, however, that in the experiment with long-labelling of these cultures, the whole cell sheet contained labelled cells. The density of the labelling varied, with some portions more heavily labelled than others (1). The marginal portion of the outgrowth always had a high proportion of labelled cells.

Migration and migration rate constant. These parameters were calculated under the assumption that the proliferation rate constant did not vary during the period of culture.

For the stripped skin explants, outgrowth at day 7 contained 7070 cells (Table I). The proliferation rate constant 0.18 means that 20% new cells were formed per day. Table I indicates similar migration rate constants which accumulated 1880 migrated cells in the stripped skin explants and 1220 cells in controls, the difference being the duration of migration.

As a hypothetical example, let us assume that only 250 cells had migrated from the explant in both groups. Then, in the stripped skin explants, these should proliferate during 4.6 days up to the breakpoint and produce 4490 cells which were present at that time. Their mean proliferation rate must be 87% new cells formed per day. If the same procedure was applied to the control group, a proliferation of 75% new cells produced per day during 4.3 days produces 2800 cells at the time of the breakpoint. This may be contrasted with the migration rates. The values given for the migration rate constant in Table I indicate that the daily addition of immigrated cells was about 180% new cells at the peak of migration in both groups.

Given the proliferation rate of 20% new cells per day, how many cells in the explants gave rise to the number of migrated cells found? During the 4.6 and 4.3 days of migration in stripped and control cultures, respectively, the stripped skin has to activate 790 cells which proliferate by adding 20% new cells per day and in controls the corresponding number would be 520 cells in order to produce the number of migrated cells as being present as immigrants during the 5th day of culture. This indicates that stripping induced 50% more cells to migrate compared with control level. It is probable that both the stripping procedure and the treatment of the tissue during the preparation for culture induce mitotic signals to the explanted keratinocytes. The point being that stripping influences cellular behaviour and the model can detect it by the migration parameters.

The graph in Fig. 1*b* represents eq. (3) which is rearranged to display the relationship between k_2 and $\ln m_e$. The curves for k_2 vs. $\ln n$ recorded at the breakpoint look the same. The slope of the curves indicates the reciprocal of the time of total migration and the intercept on the abscissa the logarithm of total number of cells at the time of the breakpoint. The plot normalizes cultures, starting at different times after explanation and displays the variation in the proliferation rate constants. The relationship to the curves in Fig. 1*c* may be noted.

Experimental findings

Migration. It varied between the two groups (Table I). It is known that a mitotic burst occurs in the epidermis after stripping (5). In the present experiment it is conveyed in vitro. As can been seen in Table I, the migration rate constant did not vary between the groups, but the half-life of migration was prolonged and total number of migrated cells increased.

Proliferation. The proliferation rate constants were comparable in the two groups, which indicates that the proliferation signal induced in the stripped skin was not conveyed into a sustained increase in mitotic activity within the outgrowth. In contrast to migration, the constraints of the culture conditions did overtake the in vivo discrepancy.

The estimated cycle time differed between the groups. However, when the regressions of reciprocal cell cycle time on the logarithm of the total number of migrated cells (Fig. 1*c*) or total number of cells at time of the breakpoint between the stripped and control groups were compared, they did not differ.

Feasibility

The explant culture model is expanded by the present analysis. The system may be used as a screening device for factors affecting migration and growth of epidermal cells. Additional

methods for analysis of cell functions as cell cycle kinetics and cell metabolism should be suggested in situations where the growth parameters show variation.

The limitations of the model should also be recognized. The evaluation of the relationship between the proliferation rate constant and total number of migrated cells is important since the latter may concern effects of growth factors within the explant. It is possible that the first growth phase reflects interactions between epidermal and dermal factors in the explant which induce cell movement and subsequent mitosis. The migration signal may therefore be a more important variable to evaluate. One may surmise that the migration rate constant and the half-life of migration should be judged carefully in this respect. The proliferation rate constant is an important basis for the estimation of these parameters. It also reflects the constraints of the culture condition. In this sense it is valuable in the evaluation of factors for culture growth.

Another limiting factor is the change in the outgrowth itself. In small outgrowths, most cells (including those which divide) are still in the basal position. As the culture grows, a greater area of the outgrowth is multilayered and starts to show keratinization and desquamation. Cultures followed for more than 10–14 days using either the original culture medium throughout or renewing it, show the same growth pattern with the leading edge slowly decreasing in lateral expansion. In large cultures there is still vertical growth, as shown by the presence of DNA-synthesizing cells in all regions of the sheet (1). It is concluded from our observations that the present system is useful during the first 10–14 days of culture.

Factors which govern senescence are of importance and are not controlled by the model which excludes them. During the first week of phase 2 it is uncommon to find cultures which have a curvilinear growth curve when $\ln n_i$ is plotted against t_i. The model is no good if this situation occurs. In that situation, variables of radial and vertical growth must be included in the model. Other systems have also been suggested as counting the number of desquamating cells (6). It is not applicable in the present system, since the original explant is able to desquamate corneocytes.

Knowing the limitation of the explant culture, this model for epidermal growth is straight- forward, the technique simple and reproducible conditions can be obtained when the additional analysis presented herein utilized.

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