

STUDIES ON GUINEA PIG SKIN CELL CULTURES

III. Minimum Cell Numbers for Establishment

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Abstract. The number of Leucinaminopeptidase positive cells (dermal fibroblasts) contaminating epidermal cell suspensions was found to be 0.08%. The minimum cell number required to establish a culture was about 5×10^5 and 4×10^3 for epidermal and dermal cells respectively. Therefore, when an epidermal suspension of 5×10^5 cells is seeded to grow epidermal keratinocytes, the number of dermal elements which contaminate the epidermal seed lot is around 400. This is ten times below the minimum cell number of 4 000 required to establish a culture of dermal fibroblasts. In addition, it was found that the tissue culture medium in which epidermal cells have grown (spent medium) had no effect upon the growth ability of dermal fibroblasts. It is concluded that these numerical studies lend support to the assumption made in previous papers of this series that long-term cultures of fibroblast-like cells can be established from adult guinea pig ear skin, without being overgrown by dermal fibroblasts.

In the first paper of this series (6), it has been shown that separate cultures of epidermal keratinocytes and dermal fibroblasts can be established from one single piece of guinea pig ear skin. In primary culture, these two kinds of cells are easily characterized on the basis of ultrastructural as well as cytochemical criteria. With the electron microscope, epidermal keratinocytes have fibre desmosome complexes that dermal fibroblasts have not. And, using the cytochemical test of Nachlas et al. (5), for Leucinaminopeptidase (LNase), epidermal keratinocytes are negative whereas dermal fibroblasts react positively. When one proceeds from primary cultures to long-term cultures propagated through serial subculture transfers, the clean distinction between these two cell types becomes more difficult to ascertain. The main difficulty arises from the fact that during the process of splitting epidermis from dermis, fibroblasts are detached from the dermis and are admixed to the epidermal suspension. Since dermal fibroblasts surpass epidermal keratinocytes in growth ability, as shown by tritiated thymidine

incorporation (6), there is a definite possibility that the latter cell cultures will be eventually replaced by the former.

In fact, it has been shown in previous studies that long-term cultures derived from adult guinea pig ear epidermis are composed of fibroblast-like cells which have no detectable desmosomes and, hence, can hardly be distinguished from dermal fibroblasts on morphological grounds alone (3). However, it has also been shown that: (a) epidermis derived fibroblast-like cells react negatively for LNase (4, 2, 6); (b) all dermis derived fibroblast-like cells react positively; (c) LNase positive guinea pig cells do not become negative during subsequent tissue culture processing (6); and, (d) positive cells do not become negative when co-cultured with LNase negative cells (4). In view of the above data, it has been proposed that LNase negative epidermis derived fibroblast-like cell cultures are composed of "dedifferentiated" keratinocytes and not of dermal fibroblasts which would have overgrown epidermal keratinocytes.

Because of its theoretical and practical import, this assumption was considered worth additional support. In the present study the problem was approached from a different angle. When cells are explanted in vitro from a given tissue, there is a critical number under which no culture can be established. Following this line, we have sought the minimum numbers of epidermal and dermal cells, respectively, needed to establish a culture and we have counted the number of fibroblasts actually present in epidermal cell suspensions.

It will be shown that these numerical studies support the view that long-term cultures of fibroblast-like cells can be established from adult guinea pig epidermis, which are not necessarily composed of overgrowing fibroblasts.

Table I. Number of fibroblasts (LNase +) contaminating epidermal (LNase -) suspensions

Expt. no.	Number of cells	
	LNase +	LNase -
1	1	1 208
2	0	1 171
3	1	1 219
4	1	1 403
5	0	1 115
6	1	1 094
7	2	1 053
8	1	1 061
9	1	1 128
Total	8	10 452

MATERIALS AND METHODS

The animals used in this study were adult male guinea pigs of a tricolor outbred stock weighing 250-350 g and maintained on regular diet.

The cell suspensions, both epidermal and dermal, were prepared according to Regnier et al. (6). The leucinaminopeptidase (LNase) cytochemical test was performed according to Jacquemont & Prunieras (4). Cell counts were made with the use of a hemocytometer (Burker).

The cells were grown on 12 × 32 mm coverslips in flattened tubes (Leighton) with Eagle's BME (1955) supplemented with 10% preheated calf serum. All cell numbers given in this paper refer to above defined coverslips cultures, unless otherwise stated.

RESULTS

Number of fibroblasts in epidermal suspensions

Epidermal cell suspensions were stained for LNase and positive cells were counted as fibroblasts. At

least one thousand cells were counted each time. The results of nine counts are given in Table I.

One can calculate from this table that the number of LNase-positive cells present in epidermal cell suspensions is about 0.08%.

Minimum cell numbers required to establish a culture

The establishment of a culture begins at the time primary culture vessels are saturated with actively growing cells, thus requiring subculturing. Therefore, the saturation of primary coverslip cultures was taken in this study, as a prerequisite criterion for establishment.

Fig. 1 shows how many cells had to be seeded in order to reach saturation in 22 days or more. One can see in the left part of the figure that 10 000 dermal cells regularly yielded saturated cultures within 18 days, whereas 1 000 such cells consistently failed to do so.

In this latter case, some cell colonies could be seen after a week or so, but these colonies remained scarce and showed evidence of degeneration (vacuolization) after about 15 days of culture.

Further experiments using seed lots of 2, 4, 6 and 8 thousand cells have shown that 4 000 is the minimum cell number needed to obtain actively growing cell colonies (without vacuolization) that will reach saturation after 22 days of culture.

On the right side of Fig. 1, it can be seen that 500 000 epidermal cells were required to establish a culture in 3 weeks. This means that at least 100 times

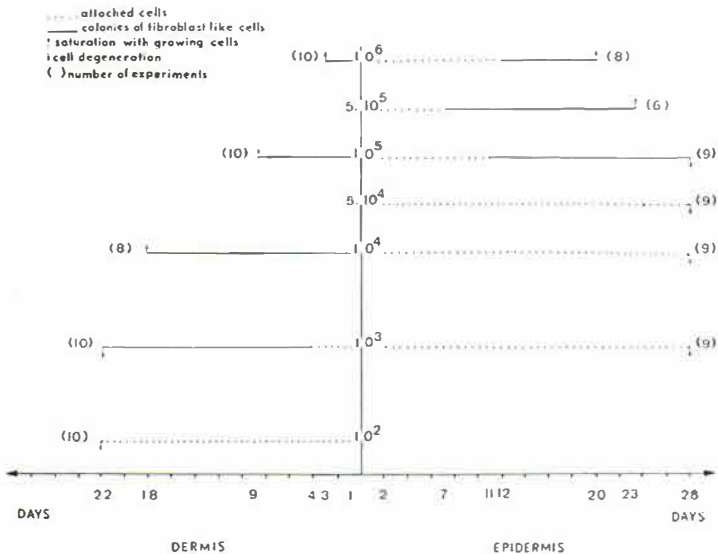


Fig. 1. This figure summarizes a total of 98 experiments. The number of seeded cells appears on the vertical scale from 10² to 10⁶. One can see the evolution of dermal (*left*) and epidermal (*right*) cultures, according to the number of seeded cells. Cells first attached (---) to the coverslip and second develop into fibroblast-like colonies (—), except for dermal cells which start forming colonies from the first day in cultures of 10⁴ cells or more. These colonies either reach saturation (↑) or degenerate (↓), as for cultures started with 10³ dermal and 10⁵ epidermal cells.

more epidermal cells than dermal fibroblasts must be used to achieve the same goal.

Effect of epidermal culture "spent" medium of dermal fibroblasts

To test the effect of epidermal cell culture "spent" medium on dermal fibroblasts, the following experiment was performed in triplicate.

Epidermal cell cultures were started by seeding 3.5×10^6 cells in Falcon plastic 30 culture vessels. They were fed with fresh tissue culture medium (TCM) and constituted the source of "spent" medium.

One day later dermal fibroblasts were seeded on coverslips in Leighton tubes at a concentration of 10^3 and 10^5 cells per tube. They were grown from the very first minute in TC "spent" medium since the cell suspension itself was made in "spent" TCM.

As controls, sister cultures were grown in normal TCM.

As expected from Fig. 1, control cultures started with 10^3 cells did not grow, whereas those seeded with 10^5 yielded saturated cultures within 9 days.

As regards cultures grown in "spent" epidermal TCM, no differences could be detected on comparison with control cultures, in terms of cell morphology and growth ability, since no culture could be established from seed lots of 10^3 cells whereas seed lots of 10^5 cells yielded consistent confluent cultures in less than 9 days.

Estimation of LNase + versus LNase - colonies in primary culture of epidermal cells

To further investigate the growth capacity of dermal fibroblasts admixed to epidermal cells in the seed cell suspension, the number of LNase-positive (fibroblasts) versus negative (keratinocytes) colonies was counted at various intervals in actively growing primary cultures.

The results of seven counts are given in Table II.

One can see in this table that in three instances no LNase-positive colonies were recorded. Of the four other cultures, the amount of positive versus negative varies from 1/22 to 9/19.

DISCUSSION

The number of LNase-positive cells in the epidermal cell suspension is, in this study, 0.08%. This compares favorably with recent data by Fritsch & Diem who found one positive per 1 200 negative, working on the same animal system (1).

Table II. Number of LNase + versus LNase - colonies in primary cultures of epidermal cell suspensions

Expt. no.	Days in culture	No. colonies	No. positive	No. negative
1	13	3	0	3
2	18	12	0	12
3	19	23	1	22
4	22	103	26	77
5	24	20	0	20
6	25	15	3	12
7	28	28	9	19

It is interesting to note that these latter authors, like us, reported that their subcultures derived from epidermal suspensions were consistently LNase-negative (2, 4, 6). This seems to be easily explained in view of results given in Fig. 1.

Indeed, the minimum number of cells needed to establish a culture from epidermal suspensions is about 5×10^5 . This means, knowing that the fibroblast contamination level is of the order of 0.08% that each time one seeds an epidermal suspension of 5×10^5 cells one seeds at the same time about 400 fibroblasts. Now, the minimum number of dermal fibroblasts needed to establish a culture amounts to 4 000, i.e. ten times more.

One may hypothesize that epidermal keratinocytes release "something" in the medium which increases the growth capacity of dermal fibroblasts.

As shown in this paper, the "spent" medium in which epidermal cells were grown did not exhibit any particular enhancing effect on the growth of dermal fibroblasts. It seems logical, therefore, to conclude that fibroblast-like cultures derived from epidermal suspensions are not overgrown by dermal fibroblasts because these latter cells are not sufficiently numerous to do so.

However, the estimation of the number of fibroblasts (present in epidermal suspensions) depends upon the quality of the cell suspension. This means that according to how well dispersed the cell suspension is, the accuracy of the counts will be good or poor. Indeed, in the epidermal cell suspensions there always exists a certain number of cell clumps (the origin of which may be dermal) and this number depends finally on the skilfulness of the one who prepares the suspensions.

As a reflexion of this, one can understand Table II given in this paper. Obviously, in experiments 1, 2

and 5, no LNase-positive colony having developed after 13, 18 and 24 days of culture, respectively, one can expect that the future cell lines will be LNase-negative. As regards exp. 3, only one colony is positive and 22 negative after 19 days in vitro. It is very likely that the number of fibroblasts (LNase +) seeded along with the epidermal cell suspension was enough for a colony to grow but will not be sufficient to establish an LNase-positive culture. This can be inferred from the lower left part of Fig. 1 which shows that when fibroblasts number about 1 000 they are sufficiently numerous to form some colonies, but these colonies are scarce and degenerate before a culture can be established.

As regards experiments 4, 6 and 7 they could well represent instances in which the number of counted LNase-positive cells was undervalued because of an especially high content of cell clumps. As a result, these three cultures can actually be overgrown by dermal fibroblasts.

In conclusion, this numerical study lends support to the assumption that long-term cultures of epidermal keratinocytes can be established without being overgrown by dermal fibroblasts. But, depending upon how carefully the epidermal suspension is prepared, the possibility of having an LNase-positive culture (developing from an abnormally high level of contamination with dermal fibroblasts) does exist and is documented in the present study.

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