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Vibrissa dermal papilla cell aggregative behaviour *in vivo* and *in vitro*

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SUMMARY

Parallel cultures of adult rat vibrissa dermal papilla cells and skin fibroblasts revealed differences between the two cell types with respect to a number of criteria. In particular the dermal papilla cells demonstrated a distinctive single cell morphology, and at confluence formed cell aggregates radically different from regular fibroblast multilayering and patterning. This finding confirmed repeated observations of papilla cell clumping in short-term culture. The dermal papilla cells which are mitotically quiescent *in situ* were also shown to have a lower proliferative capacity than the skin fibroblasts. The affinity shown by papilla cells towards each other in culture reflected the behaviour demonstrated by isolated dermal papillae transplanted into ear dermis and into the collagenous capsule of the vibrissa follicle. In the absence of epidermal contact the papilla cells remained as recognizable rounded aggregates for the experimental period of up to nine months. Synthesis of extracellular material typical of that seen *in situ* was observed, particularly during the first weeks following transplantation. The collective behaviour of the dermal papilla cells revealed in this study may be significant for the morphogenetic activity of the papilla, and for papilla size during the hair cycle. It may also reflect the retention of embryonic-like properties by the dermal component of adult hair follicles.

INTRODUCTION

It is frequently suggested that the dermal papilla is important for the production of hair, and much of the experimental evidence for dermal papilla involvement in hair growth mechanisms derives from work on the vibrissa follicle, a system first employed by Cohen (1961). Subsequently Oliver (1966*a,b*) demonstrated that following removal of the dermal papilla, or up to one third of the follicle base, hair growth ceased, and was not resumed until after the formation of a new papilla. Dermal papillae implanted into the superficial halves of cut vibrissa follicles also induced hair growth (Oliver, 1967), and when associated with glabrous ear and scrotal sac epidermis, papillae were able to induce the formation of follicular structures and then hair fibres (Oliver, 1970). Wounding of the follicle bulb, and particularly the dermal papilla component (Jahoda and Oliver in preparation) has been shown to increase hair length, and the duration

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of the hair cycle. Thus the dermal papilla is implicated in many facets of hair growth control.

The dermal papilla cell population is established early on during follicle development as a condensed aggregation of mesenchymal cells, and Wessels & Roessner (1965), demonstrated early cessation of mitotic activity in the developing papilla. This trait is probably maintained in the fibroblast-like cell population of postembryonic papillae (Pierard & de la Brassinne, 1975), nevertheless these cells have a number of intriguing properties which distinguish them from other skin fibroblasts. During the course of the hair cycle they undergo changes in cell morphology, cell contact, and in the synthesis of extracellular material (Young, 1980). Moreover in ultrastructural observations of human hair papillae Hashimoto & Shibizaki (1976) described specific intercellular junctions, and reported the presence of a basal lamina-type structure surrounding individual papilla cells. The same authors pointed to the morphological similarities between papilla cells in adult follicles, and those seen during follicle development (Hashimoto, 1970).

Clean separation of vibrissa follicle components has allowed a discrete dermal papilla cell population to be grown in culture for the first time (Jahoda & Oliver, 1981). This paper describes work designed to examine some papilla cell characteristics in comparison with skin fibroblasts. In particular an aggregative phenomenon displayed in both short and long-term papilla cell culture is related to the behaviour of isolated papillae transplanted into ear dermis, or through the follicle capsule wall. The importance of this property for papilla function during the hair cycle, and the nature of the papilla cell population are examined.

MATERIALS AND METHODS

Isolation of dermal papillae

Vibrissa follicles were dissected from the upper lip region of inbred hooded PVGC rats (colony Dundee University), with ages ranging from 2–6 months. Discrete dermal papillae were obtained by first removing the lower region of anagen follicles (i.e. these producing a hair) from the mystacial pad musculature (Cohen, 1961). Parallel cuts were then made on either side of the enveloping follicle capsule which was then inverted to expose the dermal papilla. This was then cleared of surrounding epidermal elements before being excised just above the level of the dermal stalk. All the above procedures were performed under a low-power (×20) stereoscopic microscope, and papilla isolation was carried out in TC 199 medium (Gibco).

In vivo *implantation*

Using standard operation procedures (Oliver, 1970), discrete dermal papillae were implanted (a) into rat ear dermis and (b) through the collagen capsule of otherwise intact whisker follicles. In method (a) papillae were introduced into ear dermis parallel to and between the skin surface and the ear cartilage. In (b) the follicle capsule was pierced with a sharp tungsten needle about a third of the way up from the base. Papillae were then pushed through the capsule wall using watchmakers forceps, and in towards the hair shaft. Observations were based on 42 papillae recovered from 14 rats in series (a), and 34 papillae from 13 follicles in 7 rats in series (b). For histological processing implant specimens were fixed in formol saline, serially sectioned at 8 μm, and stained in a combination of Weigert's haematoxylin, Curtis's Ponceau S, and alcian blue. The latter strain is indicative of glycosaminoglycans and stains dermal papillae blue-green in anagen follicles.

Cell culture establishment and maintenance

Dermal papilla cell cultures established from isolated papilla explants in Cruickshank tissue-culture chambers (Sterilin) were used for short-term behavioural studies. Cell numbers were then substantially increased by serial transfer of cells into 35 mm plastic Petri dishes (Sterilin), from which 25 cm flasks (Nunc) were initiated.

For comparative observations skin fibroblasts were obtained from rats of the same age and sex as those used to provide dermal papillae. Animals were killed and a patch of dorsal skin, previously shaved and swabbed with alcohol, was removed and minced into small pieces. These were grown squashed under small round coverslips in 35 mm Petri dishes, three to four explants to each dish. When sufficient numbers of cells had formed a monolayer outgrowth the coverslips and central explants were removed, and cells lifted and inoculated into flasks.

The culture medium consisted of Eagle's Minimal Essential Medium (MEM), containing 1 % L-glutamine (20 mM), 15–20 % foetal bovine calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml), all components from Gibco. Both cell types were maintained at 37 °C in an atmosphere of 5 % CO_2–95 % air. The medium was changed every three days, and on reaching confluence cells were routinely subcultured. Cell dissociation was obtained using a 0·25 % trypsin and 0·02 % EDTA solution (Gibco).

All comparative studies between the two cell types were carried out at the same cell passage number for each population.

Comparison of dermal papilla and skin fibroblast cells

Morphology and behaviour

Petri dishes containing small glass coverslips on the bottom were seeded with single cell suspensions of 4×10^4 cells for each dish. Twelve dishes were inoculated, six with either cell type. At intervals from 24 h coverslips were removed from one of each of the paired series of dishes, washed, fixed and stained with haematoxylin and eosin before being mounted in DPX on glass slides. The experiment was repeated.

Population growth

Equal numbers of 35 mm Petri dishes were inoculated with papilla and skin fibroblasts, at a seeding density of 2×10^4 cells/ml. After 24 h and at successive intervals, three dishes from each source were harvested and cell numbers counted with a haemocytometer, using ten replicate counts for each dish. The culture medium was changed immediately after the first sampling, and thereafter every two days throughout the experiment.

The results were displayed graphically, and mean population doubling times were estimated for the period of most rapid growth (between 1 and 3 days). The times were estimated by:

$$\text{Population doubling time} \qquad \text{PDT} = \frac{\text{hours of growth}}{\text{number of divisions}}$$

where the number of divisions = $(\log_{10}N_1 - \log_{10}N_o)/\log_{10}2$

N_o = Initial number of cells.

N_1 = Cell number at a subsequent time.

Final cell numbers were arbitrarily measured at day 13.

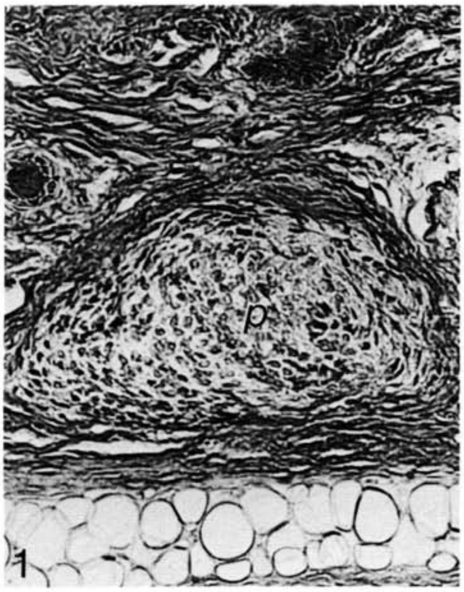
[^{3}H]thymidine incorporation

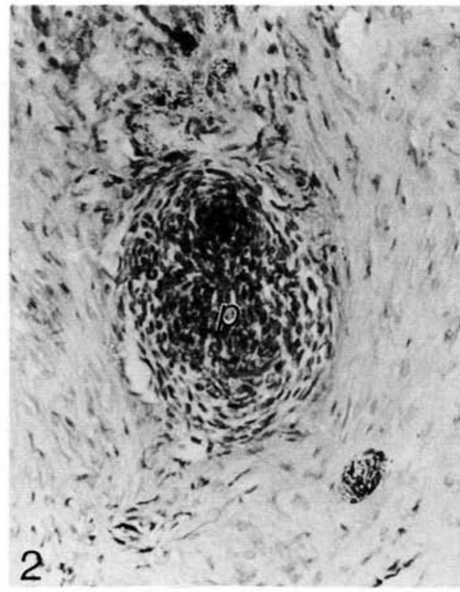
For each cell type three dishes were seeded with 2×10^4 cells. After a 24 h settling period the medium was pipetted off, and fresh medium containing 4 μCi/ml [^{3}H]thymidine (specific activity 45 Ci/mMol; Radiochemical centre Amersham) was added. The dishes were incubated for 4 h, the labelled medium removed, and each dish rinsed once with PBS/EDTA (0·2 mg/ml) solution. Cells were then scraped off with a rubber policeman in 0·5 ml of the same solution, mixed with 0·05 ml of sodium lauryl sulphate detergent, and incubated for 20 min at 60 °C. Aliquots of 0·05 ml were then spotted out onto 2×3 cm filter paper rectangles, two replicates for each sample. The paper strips were then washed in cold 5 % trichloroacetic acid (TCA) for 20 min, 0·5 M HCL for 10 min, and ethanol for 5 min and dried overnight. Each rectangle was placed on a plastic scintillation vial, shaken with 4·2 ml scintillation fluid, and the radioactivity counted for 10 min on a Packard Tri-Carb 2660 scintillation system.

RESULTS

Papilla implantation

Transplanted dermal papillae free of epidermal associations displayed a consistent appearance, both in ear dermis (Fig. 1) and in the intracapsular region (Fig. 2). In each case implants were visible as discrete and easily recognizable entities, consisting of more or less rounded aggregations of cells. While these cell





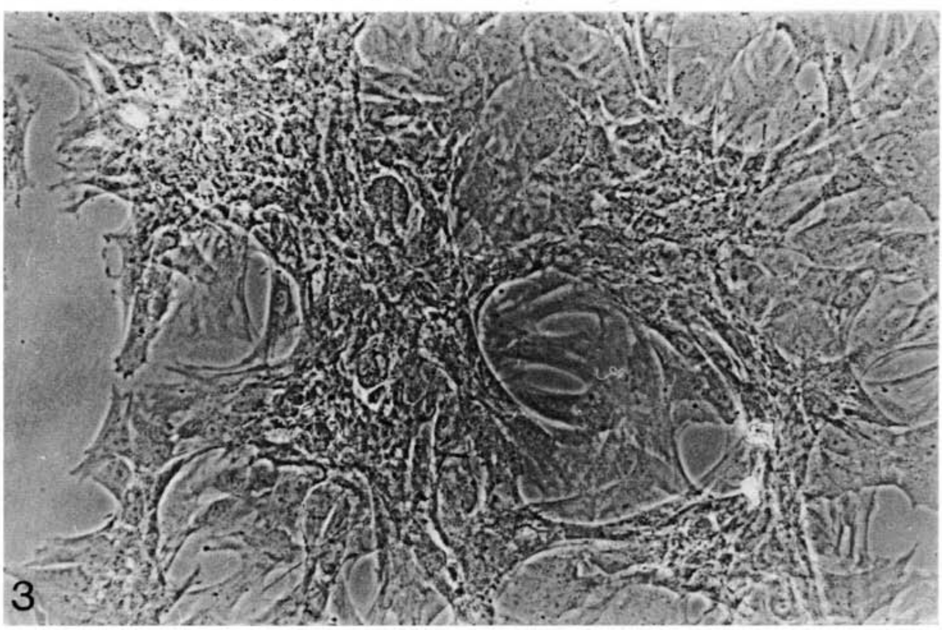


Fig. 1. Dermal papilla (p) 5 weeks after implantation into ear dermis. The extracellular matrix is positively stained with alcian blue (×220).

Fig. 2. Dermal papilla (p) 9 months after implantation into the follicle capsule. Note the condensed nature of the cell nuclei, and lack of extracellular material (×220).

Fig. 3. Dermal papilla cell aggregates in explant culture (×220 phase contrast).

clusters frequently revealed a reduction in size when compared with anagen papillae *in situ*, this was apparently due to compaction associated with a reduction in cell cytoplasm and intercellular materials, rather than a decline in cell numbers. Thus at their most condensed state, the displaced papilla cells resembled markedly the condition of papillae during the telogen (or non hair producing) stage of the hair growth cycle. Of considerable interest was the observation that at shorter biopsy times in particular (1 to 6 weeks), several specimens revealed alcian blue staining, characteristic of this cell population during the active phases of hair growth.

Papillae recovered at the longest biopsy times (9 to 10 months) often showed a spiral-like configuration, with the central body of cells remaining highly compact, and those towards the periphery being increasingly widely separated. In the latter instances intercellular spaces were filled with collagen fibres.

One papilla implanted into a follicle capsule was found to have been incorporated into outer root sheath, and was alcian blue positive, however, no extra hair fibre was being produced. Several other papillae were found encapsulated in the follicle wall, and one was observed exterior to a follicle. Of the total papillary material some 14 % of the ear implants and 24 % of the intracapsular implants were not recovered.

Cell culture observations

In explant culture and during early subculture passages, dermal papilla cells showed a number of behavioural idiosyncrasies when compared with fibroblasts from a number of other sources. These differences included an apparently slow rate of cell spreading and of cell multiplication. Moreover the papilla cells revealed a highly characteristic flattened morphology, particularly at the edge of outgrowths, and in cultures of low cell density. Importantly, irrespective of cell morphology the papilla cells revealed a tendency to form cell aggregates, some of which had a certain regularity of pattern (Fig. 3).

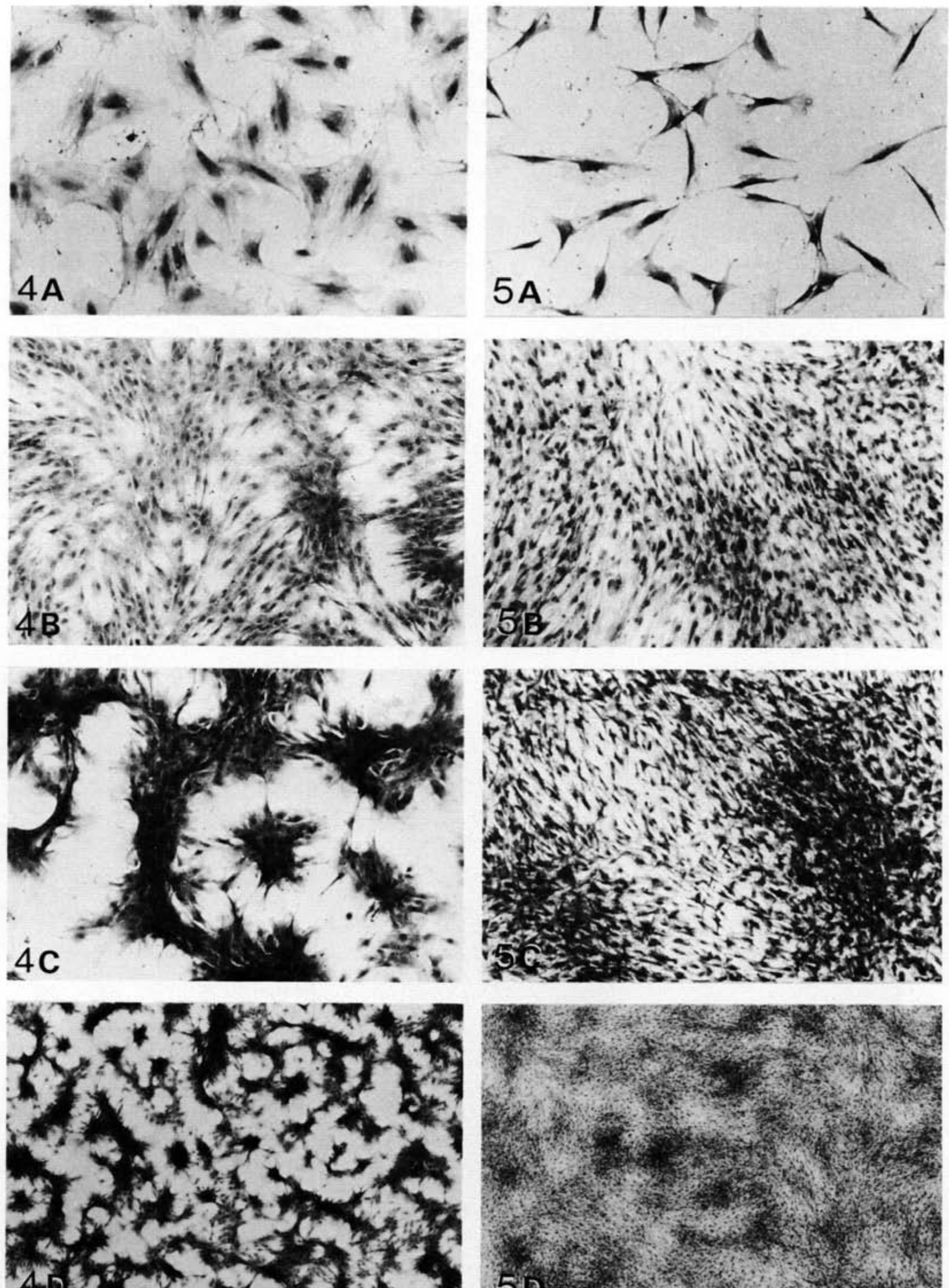
Comparative studies

Morphology and behaviour

Observation of the two cell populations after a settling period of 24 h revealed clear morphological differences. Papilla cells in the stationary state were broader

Fig. 4. Single cell morphology and social behaviour of dermal papilla cells. A. Isolated cells showing flattened, striated cytoplasm and multiple cell projections (×110). B. At confluence, groups of cells starting to form clumps (×50). C. Dermal papilla cell aggregates with areas of uncovered substrate in between (×50). D. Low power view of the papilla cell aggregates (×15).

Fig. 5. Single cell morphology and social behaviour of the skin fibroblasts. A. Isolated cells, generally bipolar with narrower cytoplasm and fewer cell projections. B. Post confluent piling up of the skin fibroblasts has started (×50). C. Well defined regions of multilayered cells at the stationary phase of culture (×50). D. Low power view showing the overall typical fibroblast-like patchwork cell pattern (×15). (Note that the paired pictures in Figs 4 and 5 are not matched with respect to length of time in culture. Rather, they are intended to represent equivalent stages in colonization of the culture substrate).



Figs 4 and 5

with a large surface area, and numerous cell processes extending from the edge of striated cytoplasm (Fig. 4A). Once again this conformed with previous papilla cell culture observations. The skin fibroblasts were manifestly narrower, with a more-densely stained central spine, and fewer cytoplasmic projections (Fig. 5A). Both populations were relatively homogeneous looking.

The distinctive dissimilarities in cell morphology were maintained through the subsequent growth phases. However it was the social behaviour of the two cell types which was particularly revealing. With increasing cell numbers the skin fibroblasts immediately started to demonstrate curved parallel alignments, and assembled at confluence with the same configuration. Subsequently the skin fibroblasts started to multilayer (Fig. 5B), eventually producing an orthodox patchwork pattern (Elsdale & Bard, 1972) covering all of the substrate (Figs 5C, D).

By comparison, the dermal papilla cells revealed an initial tendency to align. However, concomitant with, or before attainment of the monolayer configuration, groups of papilla cells began to pull apart and form aggregates (Fig. 4B). These groupings eventually became isolated clumps, with areas of uncovered substratum in between (Figs 4C, D). On close examination these clumps were seen to be a combination of rounded cell clusters and more elaborate ridge shaped structures. Moreover the papilla cells retained recognizable morphological characteristics, and in particular a multiplicity of cellular projections which were seen emanating from the aggregates (Fig. 6).

Replicate experiments with the same and other papilla cell sources revealed

Fig. 6. Papilla cell aggregate with numerous cell processes still in evidence (×220).

constant manifestation of this aggregation phenomenon, although the degree to which the substratum became uncovered was variable.

Population growth

Typical growth curves of the two cell types are shown in Fig. 7. The first sample

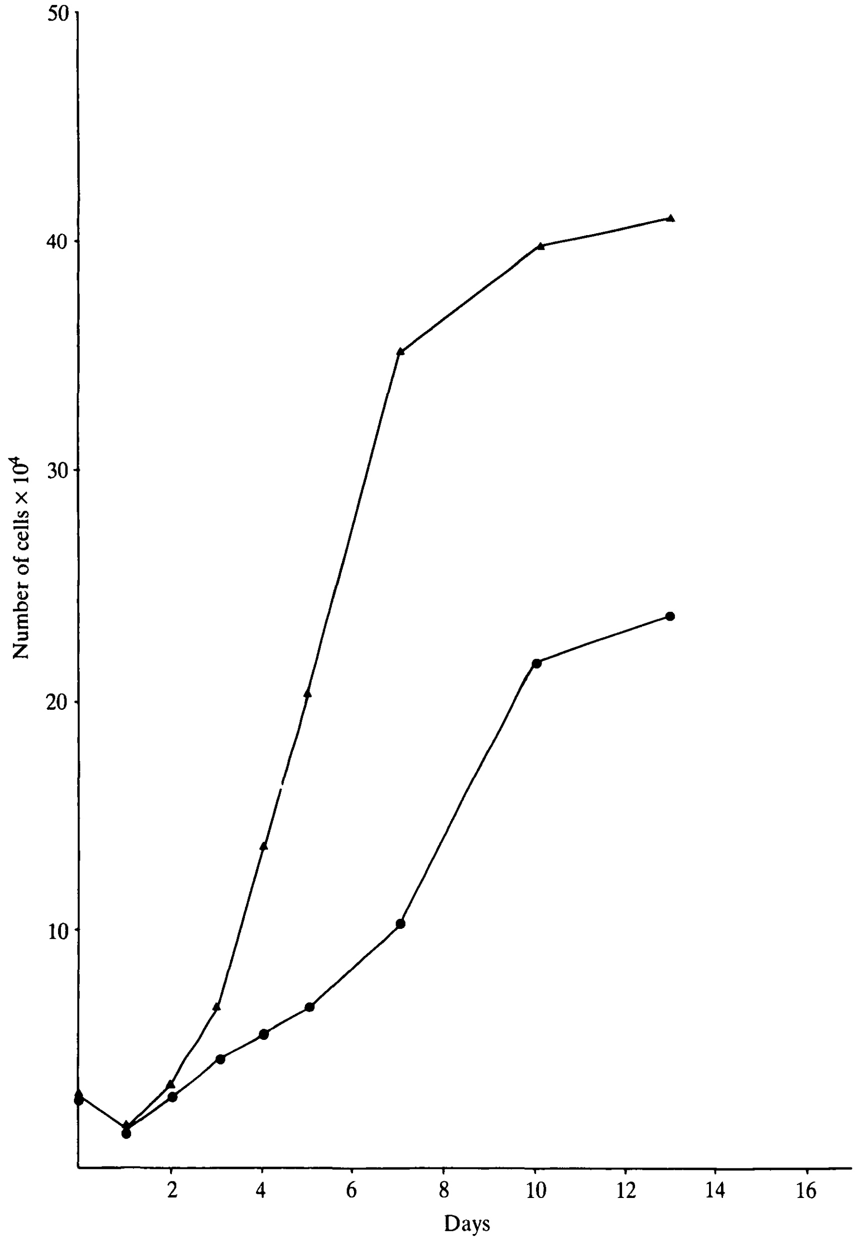


Fig. 7. Growth curves of dermal papilla cells ●, and skin fibroblasts ▲. Each point represents the mean value of three cell counts.

Table 1. *Growth characteristics of dermal papilla cells and skin fibroblasts in culture*

	Final population number ($\times 10^4$) after 13 days	Population doubling time (hours)	Mean [^{3}H]thymidine incorporation (disintegrations per minute ± S.E.M.)
Skin fibroblasts	41·3	23·4	4035·3 ± 301·9
Dermal papilla cells	23·9	39·1	1071·2 ± 27·1

Population doubling times were obtained between 24 and 72 h in culture, and [^{3}H]thymidine uptake was measured after 24 h.

count taken at 24 h revealed that equivalent numbers of cells had settled from either source. Subsequent effects were therefore not due to differences in success of cell attachment. The skin fibroblasts then commenced a phase of rapid proliferation and between 24 and 72 h in culture their population doubling time was considerably shorter than for the slower growing dermal papilla cells (Table 1). Clear cut differences between the two cell sources were also evident for the final cell number estimates after 13 days in culture. In this case there were nearly twice as many skin fibroblasts as dermal papilla cells (Table 1).

[^{3}H]thymidine incorporation

A parallel result to the above was obtained after [^{3}H]thymidine incorporation by the two cell populations 24 h after being seeded into culture dishes. Mean uptake of label by the skin fibroblasts was nearly four times that for the dermal papilla cells (Table 1).

DISCUSSION

Papilla implantation

Transplantation of isolated dermal papillae has revealed that they retain a distinct aggregative property and collective morphology which distinguishes them from surrounding fibroblasts. The same long term phenomenon has been observed incidentally by Young (1977), when attempting multiple papilla grafts, and by Jahoda (1982), following displacement of papillary material during follicle wounding experiments. The non recovery of a number of implants could be interpreted as being due to cell dispersal, however as the proportion of lost specimens did not increase with longer biopsy times, alternative explanations such as papilla necrosis, loss of papillae from wound scabs, or their displacement from the area of histological examination are considered more plausible. The absence of new hair follicle structures in the biopsy material stresses the requirement for direct association, or at least extremely close contact, between dermal and epidermal components before morphogenetic activity can occur.

That papilla cells remain as discrete structural entities when free from the influence of follicular epidermis can be logically equated to their role during the hair cycle where they provide the basis for repeated morphological reconstruction of the lower follicle. Thus at telogen when fibre production is arrested, and the dermal papilla is not encapsulated by an epidermal matrix, this collective phenomenon prevents papilla cell loss, and assures the retention of a stable structure for the renewal of follicle morphology. Failing this, the hair cycle would involve the repeated loss and recruitment of the dermal papilla component, a process mistakenly postulated by some early workers (Dry, 1926; Wolbach, 1951). Similarly, those follicle types which undergo major follicle shortening at catagen, and subsequent elongation during the early anagen phase of growth (not including vibrissae, Young & Oliver, 1976) would be particularly susceptible to papilla cell loss during these cell movements without this property.

The morphological stability displayed by the isolated papillae suggests that for the vibrissa follicle at least, papilla size is maintained by an intrinsic factor, and not by the epidermal element. This finding therefore lends weight to the view of Van Scott, Ekel & Auerbach (1963), that papilla size controls epidermal matrix volume, rather than the reverse. Evidence from embryonic recombination experiments suggests that dermal components are dominant in determining the size of integumentary derivatives (Sengel, 1976) and this rule applies to vibrissa follicles (Kollar, 1970). With the initiation of each new adult hair cycle the characteristics of the hair bulb, including the size of the epidermal matrix, become established. Therefore if, as is frequently suggested (e.g. Straile, 1969), reorganization of the lower follicle during the early anagen phase of the adult cycle largely resembles embryonic follicle development, then the dermal papilla would logically be responsible for establishing the size of the epidermal component.

Cell culture

Differences between fibroblast-like cells have been noted since the early days of cell culture (Parker, 1932). More recently Conrad, Hart & Chen (1977) demonstrated that fibroblast-like cells from three connective tissue sources could be distinguished by individual morphology and social behaviour criteria. In adult human skin Harper & Grove (1979) showed proliferative differences between fibroblasts derived from reticular and papillary layers, and Azzarone & Macieira-Coelho (1982) confirmed and extended this finding to reveal intralayer proliferative heterogeneity dependent on initial cell seeding densities. Thus the results of the present work are consistent with the concept of differences among fibroblast-like populations in the skin. However Tajima & Pinnell (1981) have demonstrated that fibroblasts from different skin layers, while displaying proliferative dissimilarities, were almost identical with respect to collagen synthesis. The importance of the unusual and distinctive behaviour displayed by dermal papilla cells over long periods in culture lies in the possibility that it can be directly related to the functional activities of the dermal papilla *in vivo*, and in particular to the previously discussed phenomenon.

Mesenchymal cell aggregations are now recognized to be vital stages in a whole variety of developmental systems, not least in avian and mammalian skin where they play a crucial role in feather and hair development respectively. Wessels (1967) considers the mechanisms by which dermal condensations arise to be a problem of general significance to embryologists, however the basis for this behaviour has not been determined. The importance of maintaining dermal cell condensations during mouse vibrissa morphogenesis was demonstrated by Jacobson (1966). Where X-irradiation of embryos resulted in the irreversible loss of vibrissae, the effect on the papilla cells was one of disruption and dispersal rather than destruction. If the comparisons drawn between adult and embryonic papillae (Hashimoto & Shibazaki, 1976), and between embryonic follicle formation and the postnatal hair cycle are valid, then dermal papilla cell aggregations may provide an adult model for the study of normal transient mesenchymal condensation behaviour. Interestingly, the two-dimensional structures, and sometimes the patterns produced by papilla cell aggregates, were reminiscent of those seen during the embryonic development of skin appendages, supporting the idea that cells with a predisposition to aggregate might produce ordered structures automatically once a given cell density is attained (Sengel, 1976).

Work is now in progress to examine the nature of this aggregative phenomenon, together with other properties of dermal papilla cells. Among the characteristics of these cells which can now be compared with other skin fibroblasts in culture are their synthetic activities (e.g. collagen and glycosaminoglycans), and their behaviour on a variety of different substrates including three-dimensional collagen gels. It will also be possible to examine the interaction of dermal papilla cells both with skin fibroblasts and, importantly, when associated with epidermal cell sources. A major limitation of most cell culture systems lies in the inability to relate the activities of cells in culture to their *in situ* function, and the importance of the present system lies in the possibility of reimplanting cultured papilla cells, and testing their capacity to induce hair growth. This procedure has been successfully carried out in short-term cultures (in preparation) and the stage at which this property is lost is being examined. Nevertheless, the vital point is that it will be possible to examine all cellular activities before and after the loss of functional capacity, and thus perhaps gain an insight into the undoubted role of the dermal papilla in the hair growth process.

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