Induction of follicle formation and hair growth by vibrissa dermal papillae implanted into rat ear wounds: vibrissa-type fibres are specified

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Summary

Adult vibrissa follicle dermal papillae have the capacity to induce hair growth and follicle formation when associated with epidermis from various sources. However, the range of conditions under which hair follicle induction will take place has not been established. The question of whether or not the adult papilla carries information to impose fibre-type specificity has also not been fully answered. This study describes how the implantation of isolated papillae into small incisional cuts on the rat ear pinna resulted in the subsequent emergence of abnormally large hair fibres from the wound sites. Many of these hairs were found to display vibrissa-type characteristics. Histological observations indicated that the papillae had interacted with the edges of the wound epidermis to produce new, and particularly large follicles, while immunohistochemical staining revealed that early follicle construction was accompanied by a profusion of the basement membrane constituents laminin and type IV collagen in the subjacent dermis. These findings show that adult rat papillae retain the capacity, as displayed by embryonic dermis, to determine vibrissa specificity in induced follicles.

Key words: vibrissa, dermal papilla, hair growth, induction, wounding, epidermis, extracellular matrix.

Introduction

The development of skin appendages such as feather and hair follicles relies on interactions between the epidermis and dermis. In embryonic development, a sequential exchange of information between these elements underpins a complex series of morphogenetic processes culminating in the formation of adult follicle structures. Many of the spatial, temporal and directional parameters described by these two-way communications have been elucidated in a detailed series of heterotypic recombination experiments (reviews: Wessells, 1967; Kollar, 1972; Sengel, 1976, 1986 and Dhouailly, 1977a). One general finding from these studies is that the origin of the dermal component determines the site-, size- and region-specific distribution pattern of induced appendages. More specifically, concerning hair follicles, recombinations of mouse upper lip dermis with epidermis from different body sites can induce vibrissa follicle formation (Dhouailly, 1977b). Dermal-epidermal interactions persist in adult skin and skin appendages and are demonstrably important for fibre production and cyclic activities in hair follicles. The use of large rat vibrissa follicles to isolate dermal and epidermal follicular components for transplantation experiments was pioneered by Cohen (1961, 1965). Then, as part of a series of experiments that revealed the dermal influence in adult follicle interactions, Oliver (1966a,b) showed that the dermal papilla was crucial for hair growth maintenance. The same author went on to demonstrate that isolated papillae stimulated hair growth when associated with epidermis at the base of inactivated follicles (Oliver, 1967). This result implied that the dermal papilla might be the instructive force for fibre initiation at the start of each normal hair growth cycle.

In later work, papillae were shown to have inductive properties comparable with those of embryonic appendage mesenchyme. Isolated papillae induced follicle formation when they were combined with afollicular scrotal sac epidermis, or ear epidermis, and implanted into pockets in rat ear pinnae (Oliver, 1970). Histological evidence of large, fibre-forming follicles suggested that “vibrissa-type” appendages would have been produced but, because the experiment was of limited duration, it was not possible to say whether the inductive information carried by the papillae included the capacity to specify fibre type. One attempt to answer this question by combining adult rat vibrissa papillae with embryonic mouse epidermis (Pisansarakit and Moore, 1986) resulted in the formation of follicles of indeterminate type. This problem of “tract specificity” was originally addressed by Cohen (1965). He suggested that isolated papillae implanted into ear dermis induced follicles, but that these assumed local size and character as a result
of the host dermis exerting regulatory influences. Subsequently, both Cohen (1969) and Oliver (1970) discounted this idea because isolated vibrissa papillae implanted into dermis away from epidermis retain a discrete identity, but do not induce follicle formation (Oliver, 1970).

In the present work, dermal papillae from rat vibrissa follicles were introduced into incisional wounds in the rat ear, to investigate whether dermal papillae would interact with wound epidermis. It was shown that newly formed large follicles at the operational sites produced hairs much larger than those typical of the ear - the majority with vibrissa fibre characteristics. Histological and immunohistochemical observations were used to follow the interactive events.

Materials and methods

Isolation of dermal papillae

Intact dermal papillae were obtained from inbred PVGC rat vibrissa follicles as previously described (Oliver, 1967; Jahoda and Oliver, 1981). Briefly, the bases of whisker follicles were dissected from the mystacial pads of freshly killed adult rats into Minimal Essential Medium (MEM; Gibco, Paisley). Under a dissecting microscope (×16), the outer collagen capsule of each bulb was everted using sharpened watchmakers forceps, and the epidermal matrix component removed. The exposed dermal papilla was then cleaned of adherent material, cut from its point of attachment at the level of the basal stalk, and transferred to fresh MEM for operational use. As a further precaution against epidermal contamination, some dissected papillae were maintained in MEM at 4°C for 24 hours prior to a final cleaning and implantation. This step was found to facilitate removal of any adherent epidermis.

Host site preparation and implantation procedure

Since the procedure involved a very minor operation, adult rats aged between 3 and 12 months were generally sedated with ether. Some animals were anaesthetised by intramuscular injection with 0.4 ml of hypnorm (Janssen Pharmaceuticals Ltd) and then intraperitoneally with 0.5 ml of valium (Roche Products Ltd). Each ear designated for operational use was depilated with Immac (Whitehall laboratories, London), and then cleaned with 70% ethanol, followed by sterile saline solution. The ear was then supported at right angles to the head by loosely clamped artery forceps attached to the ear tip, all supported by a wad of cotton absorbent cotton wool to arrest bleeding.

Isolated papillae were then transferred into each cut with watchmakers forceps. Papillae continued to be pushed into the incision site until it was filled. The final number put in depended on the size and depth of the cut, but it usually ranged from 8 to 16. No attempt was made to sew the wound at the end of each operation, as a blood clot was normally seen covering the region within a few minutes. Control incisions were made on the opposite ear and then left without the introduction of papillae. Normally only one incision was made per ear. Twenty four papilla implantations, and twenty control procedures were carried out.

Observation and microscopic examination of fibres

Ears were examined at regular intervals, and hair growth on and around the incision site was monitored. Between 26 days and 9 months postoperatively, half of the experimental animals were killed and their wound sites biopsied for histological observation. Sometimes a photographic record of fibres was obtained at this stage. Some hairs were also cut off for light microscopy and/or scanning electron microscopy. Size, shape and medullary structure were used as criteria to establish if fibres were vibrissa-like. Vibrissae take on the shape of an extended cone, and become progressively wider in diameter from tip to base. They also possess a central medulla which is open, or hollow, unlike the medullae of most large body hairs which is striated or ladderedin appearance.

To follow the events taking place just after implantation twelve animals were killed at intervals from 1 hour to 3 weeks postoperatively, and the wound sites fixed for histology, or snap frozen for immunohistochemical analysis.

Histology

For histological purposes, wound areas were fixed in formal saline, dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin wax. Serial sections of 8 µm were then cut, and stained with a combination of Weigert’s haematoxylin, Alcian blue, and Curtis’s Ponceau S.

Immunohistochemistry

Polyclonal antibodies to fibronectin, laminin and type IV collagen, all raised in rabbits, were obtained from the Institute Pasteur, Lyon, France.

For immunohistochemistry, specimens were embedded in a water-based mounting fluid (Tissue tek III, Miles Scientific), inside aluminium holders, and snap frozen over liquid nitrogen. Sections of 6 µm were cut on a cryostat (Reichert) at −20°C, air dried and immunolabeled at room temperature using the indirect method. Sections were immersed for 30 minutes in the primary antibody solutions in phosphate-buffered saline at pH 7.4 (dilutions anti-fibronectin 1:30, anti-laminin 1:30, and anti-type IV collagen 1:40). The second fluorosothiocyanate (FITC)-labelled goat anti-rabbit Ig globulin (Wellcome diagnostics) antibody, containing 70 μg/ml of Evans blue counterstain was applied under the same conditions, and the sections mounted in buffered glycerin or citifluor (Agar aids) embedding medium.

Control procedures included the use of preimmune sera instead of the primary antibody, and conjugated second antibody alone. Fluorescence was absent in all control material.

Photography

Sections were photographed with a Zeiss ICM 405 inverted microscope equipped with epi-illumination for fluorescence observations, using Kodak panatomic X, or tungsten 160 colour transparency film.

Results

Macroscopic observations

Postoperative scrutiny of wound sites from around 20 days revealed the emergence of hair fibres that were easily distinguishable from the small local hairs by their much larger
size and variable direction of growth. Subsequent observations revealed clusters of long hairs, which were always restricted to the line of the wound scar, and often included both pigmented and unpigmented specimens (Fig. 1). After 4 to 8 weeks, sixteen of the twenty-four papilla implantations had produced at least one large fibre, and up to eleven big hairs were visible in one instance - although four or five were more regularly found. The longest single hair was 14 mm in length. Scar tissue was distinguishable at control incision sites, where only minor abnormalities in fibre growth were occasionally observed, and no long hairs seen.

Extended observation of experimental sites did not establish whether individual hairs were growing cyclically. Fibres were visible up to 12 months postoperatively, but an overall reduction in the number of long hairs from any given implantation site was evident over time.

Microscopic examination of a number of the large hairs revealed that they belonged to one of two categories. Most fibres (pigmented and unpigmented) increased in diameter from their tips to their bases. The others had a thin tip and a wider midregion, but then tapered and became narrower basally, at the skin surface (Figs 1,2). Of the former cone-shaped hairs, many had an open central medulla along all or part of their length which is typical of vibrissa follicle fibres (Fig. 3A), while others had no visible medulla at all (Fig. 3B). Scanning electron microscopy confirmed the presence of a medullary opening. The long fibres, which showed a narrowing of diameter towards their bases, revealed a more variable but generally striated or punctate medullary arrangement, corresponding to that seen in the largest fibres of the body pelage, the monotrichs and the awls (Dry, 1926; Priestly, 1966).

**Histology and immunohistochemistry**
Follicles found within the papilla implantation wound site were frequently aligned through different planes and this made longitudinal sectioning difficult. However, in specimens biopsied between 4 and 6 weeks, there was a striking difference between the size of the induced follicles, and adjacent ear follicles. In the wound area, some atypically large follicles had pelage-shaped lower bulbs, larger than adjacent ear appendages, but similar to them in respect of the angle in which they descended into the dermis. Other follicle bulbs had vibrissa-sized papillae containing many more cells than are usually seen in ear follicle papillae (Figs 4,5). Some of these large papillae had a pear shape typical of vibrissa follicles, except that the normal elongated apex was absent (Fig. 4). Many of their bulbs were found close to the wound epidermis, and the follicles often appeared to run at a shallow angle to the skin surface. Often, although a large bulb was visible, it did not incorporate all of the papillary material in the immediate vicinity, since distinctive clusters of dermal papilla cells were seen close by, or joined up with, papilla cells at the base of the follicle (Fig. 5). There was no evidence that the largest follicles had formed the thick collagenous capsule characteristic of sinus follicles, however many newly formed follicles had a substantial external capillary network running externally along their length (Fig. 6). Some new follicles had complex bulbar forms which incorporated multiple papillae (Fig. 7).

When sectioned longitudinally, many of the big follicles displayed an asymmetrical bump as a result of enlargement of outer root sheath on one side; and a dumbbell shape produced by a constriction, or narrowing, just above the level of the bulb (Fig. 8).

The large number of dermal papillae that did not interact with epidermis to form follicular structures were easily visible histologically as discrete dermal cell aggregates. Sometimes the dark papilla cell nuclei were tightly packed together with no extracellular matrix. Alternatively, they

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**Fig. 1.** A clump of long straight hairs visible on an ear wound region following papilla implantation. The largest of the fibres is unpigmented.

**Fig. 2.** The cut bases of three long fibres from an experimental site. Two have a broad diameter, while the third (arrowed) has narrowed down from a wide mid-length region. (×20)

**Fig. 3.** Medulla structure in two broad based, vibrissa-type experimental hairs showing (A) an open medulla (×130), (B) no medullary space (×55).
displayed lighter coloured, rounder nuclei and were associated with voluminous Alcian-blue-stained extracellular matrix (Fig. 9). Some papillae appeared to have loose cells in one region and closely associated cells in another. Dark pigment was also seen around many papilla cell clusters (Fig. 10). This was presumably released from papilla implants obtained from follicles with active melanocytes. Papilla induced angiogenesis was suggested by the large network of small blood vessels seen around implanted papillae, whether in isolation or associated with the basal region of newly formed follicles. This phenomenon was emphasised by the intense staining of papilla-associated vasculature by laminin and type IV collagen antibodies (Fig. 11).

Among implant specimens sectioned more than three months postoperatively, there were some large follicles growing hair (anagen) and others with club fibres but whose bulbs were not making hair (telogen) (Fig. 12). At 6 months, one group of five follicles was found to be in synchronous telogen (Fig. 13); however, this was not a universal pattern as later biopsies contained anagen and telogen follicles side by side (Fig. 14). Isolated dermal papillae were still visible as discrete balls of cells within the dermis after 10 months.
Histology of wound areas some days after operation revealed several examples of follicle construction. Papilla clumps were associated with downward projections of epidermal cells (Fig. 15), or found directly below an epidermal column (Fig. 16). Most commonly, papillae were seen between epidermal downgrowths near the surface of the skin - papilla cells possibly trapped by the wound edges as they came together (Fig. 17). Immunohistochemistry showed that the enclosed papilla always marked strongly for laminin and type IV collagen, with particularly bright marking of capillaries, and an unusual line of non-vascular granular staining, which descended straight down from the enclosed papilla cells into the dermis (Figs 18,19). As well as capillaries, small nerve branches were sometimes visible along the same line. Fibronectin marking was intense all through the wound dermis.

Examination of control incisions revealed only established elements of skin wound healing. Early on there was strong fibronectin marking throughout the dermis. Around the periphery of the wound, many follicles appeared to be in a telogen-like state, or somewhat distorted, but the central wound region was initially bare. At several months, the wound scar was smaller, presumably as a result of some contraction.

Discussion

It is well established that isolated dermal papillae from mature whisker follicles can be associated with skin epidermis from various sites and initiate follicle morphogenesis and fibre production (Oliver, 1980). In the present study, as a result of vibrissa dermal papilla association with wound epidermis, it appeared that not only large but specifically vibrissa-type fibres were produced. Vibrissa follicles have several specialised features thought to be associated with their sensory function, including a thick collagenous outer capsule, blood sinuses and the ringwulst. Although the largest follicles seen after implantation lacked these peripheral components, when newly formed, they had an hour glass shape which is typical of developing vibrissa but not pelage follicles (Dhouailly, 1977b). It is not possible to distinguish between vibrissa and pelage fibres by analysis of their keratin composition (Delorme, 1989). However, the criteria employed to establish vibrissa fibre type, size, thickness, medulla structure and elongated cone shape were all shown by hairs growing from the wound sites. The latter feature is particularly revealing, since it provides information about how the fibres grow. The elongated cone shape of the whisker represents a continuous increase in fibre productivity until just before growth ceases. By contrast, the narrowing of the largest coat hair fibres from approximately mid-length (Dry, 1926; Priestly, 1966) must represent a gradual slow-down of fibre formation towards the end of each growth cycle. The important point shown by this study is that the adult whisker dermal papilla retains the information needed to determine hair fibre type as well as follicle size, and that this property is manifested in adult induction. In other words, the regional specificity of embryonic dermis is maintained and is highly localised in the adult follicle.

In relation to embryonic skin appendage development, the present results are consistent with recombination experiments in which the dermis has been shown to determine the site, distribution pattern and size of appendages, as well as their species-specific morphology (Sengel, 1986). In mouse embryos, the choice between the formation of whisker or pelage follicles resides in the dermis, and the results of recombination experiments involving dermis and epidermis of the upper lip and dorsal region led Dhouailly (1977b) to conclude that in some respects the development of vibrissa follicles might predominate over pelage type. Sengel (1986) suggests that information for appendage formation is a multistep process and, in the current work, a group of papilla cells react with wound epithelium to produce a hair follicle, followed by a fibre that is characteristic of the dermis of origin. Some follicle formation appeared to start with papillae interacting with the edges of the wound epithelium to produce structures that were near to the skin surface and at different angles to each other and the prevailing local ear follicles. This reinforced the point that completely new follicle structures had been made. However, much wound epithelium is believed to derive from hair follicle epidermis (Eisen et al., 1955; Krawczyk, 1971; Pang et al., 1978). Therefore, it can be argued that papillae that are combined with follicle-derived wound epidermis have to provide less of an inductive influence to
stimulate new follicle production, than if they had to interact with a follicular epidermis. However, this does not alter the fact that a different type of fibre was produced.

The current finding that large follicles and hairs persisted over a period of many months argues strongly against local skin dermis exerting a controlling action over follicular dermis (Cohen, 1965), although longer term modulatory influences cannot be excluded. The fact that long-term biopsies contained follicles in anagen suggested that cyclic hair production was taking place at the level of individual structures. The observation that at different biopsy times some isolated papillae had an anagen-like appearance and extracellular content, while others were telogen-like, gives support to the idea put forward by Oliver (1980) that papillae might continue with an intrinsic, hair cycle-related rhythm, even when separated from epidermis.

In this work, compared with previous induction experiments involving more sophisticated transplantation protocols (Oliver, 1970; Pisarsarakit and Moore, 1986), emergent fibres were produced more rapidly, and greater numbers of complete follicular structures were formed. One reason may be because in the current work papillae were interacting with epidermis at the skin surface, as they do in embryonic development, rather than deeper down. In the present experiment, growth factors such as PDGF, FGF and TGFβ will have been produced as part of the wound repair process (for reviews see Huang et al., 1988; Fox, 1988; and Assoian, 1988), but these would also have been present with previous protocols. In developing skin, a high density of fibronectin, the presence of glycosaminoglycans and rarified fibrous collagen are considered to be conditions that stimulate morphogenesis and histogenic changes (Sengel, 1986). The wound sites used in this work initially created a “space” that was almost free of fibrous collagen, but that was filled with fibronectin, and glycosaminoglycans and proteoglycans in the papillae themselves. Thus the region was provided with a labile extracellular milieu, within which morphogenesis would be facilitated, and follicles could develop and extend. A parallel to this is described in embryonic wound healing (Whitby and Ferguson, 1991), where a loose wound scaffolding rich in glycosaminoglycans, proteoglycans and glycoproteins is thought to facilitate cell movement, compared with a denser matrix found in adult wounds.

**Fig. 15.** Papilla cells (p) associated with epidermal projections (e) in an implant site at 16 days. A degree of organization has taken place in the basal layer of the epidermis. (×230)

**Fig. 16.** Transverse section through a wound region at 16 days postoperatively. Follicles are seen to either side of a central broad epidermal peg (e), which has a dermal condensation immediately below it, and a larger ball of papilla cells directly underneath this (×90).

**Fig. 17.** Closely associated dermal papilla cells in between downward projections of wound epidermis at 14 days (×90).

**Fig. 18.** Another group of papilla cells inside wound epidermis after 19 days. Massive amounts of marked extracellular material are visible within the papilla cluster, and descending straight down into the dermis. Note that this association appears at the thickest region of wound epidermis, and that there are no normal appendages locally. (×195)

**Fig. 19.** Papilla material enclosed within epidermal downgrowths 18 days after wounding labelled for type IV collagen. Brightly stained capillaries descend directly from the structure, and a small nerve branch could also be traced to this area. Substantial amounts of loose extracellular material can also be seen projecting down into the dermis, and granular staining is present in the papilla. (×118)
The presence of basement membrane components underneath papillae during the early stages of follicle formation is not described in embryonic follicle development (Mauger et al., 1987), and has no clear parallels in the normal adult hair growth cycle (Couchman and Gibson, 1985). However, in adult whisker follicle experimental regeneration (Jahoda et al., 1992), the same basement membrane components are found in the follicle mesenchyme or dermal sheath cells that are remodelling a new papilla.

In conclusion, this work extends understanding of the inductive powers of the vibrissa follicle dermal papilla and provides a simple method of studying dermal-epidermal interactions in a wound context.

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References


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Fig. 4. A region of an ear wound that has produced big fibres, showing one large follicle with a pear-shaped and Alcian blue-stained dermal papilla. The blue marking is indicative of glycosaminoglycan production. Another large follicle, which has been cut transversely, is visible at a shallow plane to the skin surface, and a cluster of unassociated dermal papilla cells (arrow) is positioned deeper in the dermis. (×70)

Fig. 5. The base of a large experimental follicle, where not all of the whisker papilla cells have been enclosed by the epidermal component. Note the blue staining of these loose papilla cells and the shallow depth and unusual orientation of this follicle, and the one to its right. (×70)

Fig. 6. A large follicle 25 days after wounding. Vasculature runs down its length just external to the follicular dermis. Note that the basal stalk and lower dermal sheath contain Alcian blue stained extracellular matrix and patches of pigment, suggestive of a dermal papilla origin. (×112)