Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear

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Summary

The stria vascularis of the mammalian cochlea is composed primarily of three types of cells. Marginal cells line the lumen of the cochlear duct and are of epithelial origin. Basal cells also form a continuous layer and they may be mesodermal or derived from the neural crest. Intermediate cells are melanocyte-like cells, presumably derived from the neural crest, and are scattered between the marginal and basal cell layers. The marginal cells form extensive interdigitations with the basal and intermediate cells in the normal adult stria. The stria also contains a rich supply of blood vessels.

We investigated the role of melanocytes in the stria vascularis by studying its development in a mouse mutant, viable dominant spotting, which is known to have a primary neural crest defect leading to an absence of recognisable melanocytes in the skin. Melanocytes were not found in the stria of most of the mutants examined, and from about 6 days of age onwards a reduced amount of interdigitation amongst the cells of the stria was observed. These ultrastructural anomalies were associated with stria dysfunction. In the normal adult mammal, the stria produces an endocochlear potential (EP), a resting dc potential in the endolymph in the cochlear duct, which in mice is normally about +100 mV. In our control mice, EP rose to adult levels between 6 and 16 days after birth. In most of the mutants we studied, EP was close to zero at all ages from 6 to 20 days.

Melanocyte-like cells appear to be vital for normal stria vascularis development and function. They may be necessary to facilitate the normal process of interdigitation between marginal and basal cell processes at a particular stage during development, and the lack of adequate interdigitation in the mutants may be the cause of their strial dysfunction. Alternatively, melanocytes may have some direct, essential role in the production of an EP by the stria. Melanocytes may be important both for normal strial development and for the production of the EP. We believe this is the clearest demonstration yet of a role for migratory melanocytes other than their role in pigmentation.

Key words: Melanocytes, inner ear, stria vascularis, viable dominant spotting mutant.

Introduction

The stria vascularis of the mammalian cochlea is an interesting structure for studying tissue interactions during development because it receives contributions from several different cell types. The adult stria is composed of three main cell types. The marginal cells form a continuous layer along the lumenal surface (facing the endolymph in scala media) and are of epithelial origin. The basal cells also form a continuous layer, next to the spiral ligament and cochlear wall, and the origin of these cells is not known: they may be either mesodermal or derived from the neural crest. There are also intermediate cells, some of which are melanocyte-like cells which are presumed to be of neural crest origin; these are found scattered around the stria between the marginal and basal cell layers. The stria also contains a rich supply of blood vessels. During normal development, the marginal cell layer changes from being a simple cuboidal epithelium on a basal lamina to having an extremely complex array of processes which interdigitate extensively with the basal and intermediate cells. As a result of this interdigitation, in the adult only about 3% of the total marginal cell membrane area faces the lumen, the remaining 97% forming the basolateral surface (Rodriguez-Echandia and Burgos, 1965). The basal lamina is lost during this process of development (Hilding, 1969; Kikuchi and Hilding, 1972; Hilding and Ginzberg, 1977; Thorn and Schinko, 1985).

Another useful feature of the stria vascularis for developmental studies is that its function is reasonably well-defined. It produces the endocochlear potential (EP), a potential difference measured in the endolymph of scala media with reference to earth (Tasaki and Spyropoulos, 1959; Marcus, 1986). In the mouse, the EP is usually about +100 mV (e.g. Steel and Bock, 1980; 1983a). The stria is probably also mainly responsible for producing the endolymph, an unusual extracellular fluid which has a high K+ and low Na+...
cochlear hair-cell function. The presence of an EP is important for cochlear hair-cell function.

Mammals with spotting mutations affecting the coat (such as white cats, Dalmatian dogs, white mink, Waardenburg's syndrome in humans, etc) often show associated hearing impairment (Steel and Bock, 1983b). One reason for this association might be that melanocytes are important in cochlear function, although no specific role for them has yet been established (Marcus, 1986). The white areas of the coats of animals with spotting mutations show no evidence of recognisable melanocytes (e.g. Silvers, 1979), and this spotting has been found to extend to the inner ear in a number of mutants (Deol, 1970a). In the mutant described here, viable dominant spotting ($W^v/W^v$), no melanocytes could be identified in the stria of adults by transmission electron microscopy (Steel et al. 1987), and no pigment clumps could be found in surface preparations of striae of most of the mutants, indicating that there were no melanocytes present (Moorjani et al. 1989). Viable dominant spotting mutants are profoundly hearing-impaired and their EP is usually close to zero (Steel et al. 1987). The striae in the young adults used in the previous study were abnormally thin, particularly the marginal cell layer, and there was an abnormally distinct boundary between the marginal and the basal cell layers. The dysfunction of the stria, indicated by low or absent EP, correlates well with its appearance, because between 10 and 20% of $W^v/W^v$ mutants partially escape the effect of the mutation and have a small EP, or an EP of nearly normal magnitude, measured in the basal turn. This small group of mutants have a stria with a normal histological appearance in the basal turn, and pigment clumps can be seen in the basal turn in surface preparations, although the stria in the apical turns of the cochlea always looks abnormal in these mutants. An earlier study of this mutant by Deol (1970b) also reported that the only pigmented regions of the stria were the basal turns in those few mutants in which the basal turn stria looked histologically normal.

The main purpose of the study reported here was to discover whether the structural and functional abnormalities of the stria in adults are the result of degenerative changes, or a consequence of anomalous development. We examined mice at various postnatal ages by light and transmission electron microscopy. In animals aged 6 days or more, EPs were also measured to permit correlation of the structure with function. Several more specific questions were also addressed. One of these was whether the unusually distinct boundary between the marginal and the basal cell layers in the mutant striae might be due to an abnormally long persistence of the basal lamina. Another question was whether the marginal cells were abnormal throughout development, because the marginal cells seemed to be particularly affected in the adult mutant striae. This was surprising because the marginal cells are of epithelial origin and are not derived from the neural crest, while the primary anomaly in dominant spotting mice lies in the neural crest tissue, from which most melanocytes are derived, rather than in a host tissue defect (see Mayer, 1970; Mayer and Green, 1968). Presumably, the neural crest cell that would, in a normal animal, become melanocytes either fail to migrate normally, or migrate to their normal positions and then die, or fail to differentiate into a recognisable form in these mutants. The $c$-kit gene, which encodes a transmembrane protein tyrosine kinase receptor, is a candidate gene for the $W$ locus (Chabot et al. 1988). Mutations at the $W$ locus also lead to abnormal gametogenesis and erythropoiesis, and these pleiotropic effects appear to be linked by an abnormality of cell proliferation and/or migration early in development.

Our main findings were that EPs were close to zero in most of the mutants at all stages of development studied, and that marginal cells appeared to develop normally at first in the mutants.

Materials and methods

The viable dominant spotting mouse mutant is maintained in our laboratory on a heterogeneous genetic background, and mutants ($W^v/W^v$), heterozygotes ($W^v/+)$ and controls (+/+ can be readily distinguished in segregating litters by their coat colours (Steel et al. 1987). Newborn mice were more difficult to classify by external appearance, and so blood smears were taken when the mice were used for fixing. The mean red blood cell sizes were used as an aid to classification, because $W^v/W^v$ mice show macrocytic anaemia (Russell, 1970). The cochleae of mutants and their littermate controls (+/+ were examined within 24 hours of birth and at 2, 3, 4, 5, 6, 8, 10, 13, 16 and 20 days after birth. In mice aged 6 days and older, the EP was recorded before fixation. The mouse was anaesthetized with 0.01 ml gm⁻¹ body weight of 20% urethane, a tracheal cannula was inserted, and the bulla opened to reveal the cochlea. A small hole was made in the bony wall of the cochlea over the basal turn scala media, and a micropipette electrode filled with 150m² KCl was advanced through the hole and through the spiral ligament of the lateral wall of the cochlea using micromanipulators. The site of the recording was selected at random from the scalae tympani and media. A glass micropipette electrode containing 150 mOsm KCl was advanced through the hole and through the spiral ligament of the lateral wall of the cochlea.

![Graph showing the progressive increase in the endocochlear potential in control (+/+ mice with age (open squares), while all but three of the mutants (Wv/Wv) show EPs close to zero (filled circles).](image-url)
Fig. 2. (A) +/+ newborn. A melanocyte (mel) showing a number of melanosomes, with two cell processes from a melanocyte shown to the left. A basolateral process from a marginal cell is also shown (mar) with its characteristic proliferation of membranes and a basal lamina (bl) beneath. The figure is oriented so that the lumen is towards the top of the page in this and all subsequent figures. Inset drawing shows distribution of cell types: stippled areas represent melanocyte processes, and unshaded areas are basal or marginal cells. Scale bar=5 μm. (B) +/+ 2 days. Enlarged melanosome in an intermediate cell (ic), which is closely associated with a blood vessel (bv) in the stria. Scale bar=1 μm.
Fig. 3. (A) W^c/W^c, 2 days, apical turn. The future marginal cells form a simple cuboidal epithelium upon a basal lamina, and show dark staining. The basolateral membranes of the marginal cells are proliferating. The mesodermal cells below the basal lamina are loosely packed at this stage. Inset drawing shows distribution of cell types. sm, scala media. Scale bar=5 \mu m. (B) W^c/W^c, 2 days, apical turn. An enlargement from the top left corner of A showing the marginal cells (mar), basal lamina (bl) and scala media (sm). Scale bar=5 \mu m. (C) +/-, 4 days, extreme apex. An equivalent stage of development in a control, showing a similar stage of marginal cell differentiation as in the mutant. Scale bar=5 \mu m.
**Fig. 4.** \(W^+/W^+\), 4 days, lower apical turn. A similar appearance is seen in controls. Scale bar=5 \(\mu m\). (A) The marginal cells (darkly stained) are extending large processes in the mesodermal cell layer (paler staining cells), and there are only small pockets of basal lamina remaining (see B). The mesodermal cells are more compactly organised at this stage, and basal (bc) cell nuclei are becoming flatter. Inset drawing shows distribution of cell types. (B) An enlargement from the top left corner of A. Small pockets of basal lamina (bl) remain at this stage.
The cochleas were fixed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M-sodium phosphate buffer for 2-4 h at 4°C, after opening the round and oval windows. 1% tannic acid was added to the fixative for some specimens to enhance the preservation of the basal lamina. After post-fixation in 1% osmium tetroxide, the specimens were decalcified in EDTA, dehydrated, block-stained with phosphotungstic acid and uranyl acetate, and embedded in Araldite resin.

Serial one micron-thick sections were collected through the region of the hole made by the EP-recording electrode, until the hole could be positively located in toluidine-blue-stained sections by light microscopy. EP recordings from mice that had a potential close to zero were included in the data presented in Fig. 1 only if the hole made by the electrode was found to pass into an open scala media, indicating that the electrode had been correctly positioned. This was an important control because a potential difference close to zero could also be obtained if the electrode had passed into the scala vestibuli or scala tympani by mistake. After locating the hole, the block was trimmed further if necessary to give mid-modiolar semi-thin sections, which were examined by light microscopy. Thin sections were taken from selected regions and examined using a Philips EM300 or 410 transmission electron microscope. A total of 33 Wv/Wv and 30 +/+ mice were examined by light microscopy, and thin sections from various turns were examined from 16 Wv/Wv and 16 +/+ specimens, including at least one animal at each of the ages used.

Results

The EP recordings are plotted as a function of age in Fig. 1. In control mice, the EP rose steadily from 6 days onwards, reaching a mature level at around 13–16 days. Most of the mutants, on the other hand, had EP recordings near zero at all stages of development studied. Three out of eighteen mutants showed small EPs, and this proportion (17%) of Wv/Wv mice which partially escape the effects of the mutation is in line with previously reported data (e.g. Steel, 1989).

Melanocyte-like cells were not found in any of the mutant strias studied, although the basal turn strias of the three mutants with small EPs have not been included in this investigation. In the controls, melanocytes, identified primarily by the presence of melanosomes (Fig. 2), were particularly noticeable in the younger specimens, but they were not observed in every section, presumably because the cells are normally fairly thinly spread in the stria of the mouse. Intermediate cell nuclei were found in the intermediate zone of the stria in control mice (Fig. 3), but nuclei are only occasionally observed in this region of the stria of mutants. These nuclei probably represent the non-melanocyte intermediate cells which have recently been observed in normal chinchillas and cats (Wright and Lee, 1988; Conlee et al. 1988).

Apart from the absence of melanosomes, there was a

![Fig. 5. (A) +/+, 6 days, basal turn. (B) W/W, 6 days, basal turn. Subtle differences in the amount of interdigitation between marginal and basal cells can be seen at this stage, with the mutants showing less interaction and more clumping together of marginal cell processes. The marginal cell nuclei are becoming flatter at this stage, and shallow indentations appear on the endolymphatic surface, in both mutants and controls. Intermediate cell nuclei (ic) are shown in this example of a control stria. Scale bar=5 μm.](image-url)
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Similar pattern of development in mutants and controls up to about 6 days after birth. There is a gradient in development from base to apex, so several stages of differentiation could be seen within one cochlea. The earliest stage we saw is illustrated in Fig. 3. The marginal cells appear as a simple cuboidal epithelium on a continuous basal lamina with microvilli on their luminal surface and show proliferation of their basolateral membranes to varying degrees. The cells below the basal lamina are loosely packed, and the nuclei of both these cells and the marginal cells are large and slightly irregular in shape. Blood vessels are present among the cells below the basal lamina. The next stage of development is marked by the presence of large cell processes emerging from the marginal cells down into the lower cell layers (Fig. 4). The basal lamina is disintegrating and is seen only in small pockets below the marginal cells, or within the marginal cell body (Fig. 4). The cells below the marginal cells now appear to be more compacted, and the basal cell nuclei are becoming flattened and oriented parallel to the surface of the stria.

The next stage is characterized by the complete loss of the basal lamina and further extension and growth of the marginal cell processes. These is a progressive increase in the amount of interdigitation between marginal cell processes and processes from the other cells below. The marginal cell nuclei flatten and the microvilli on the luminal surface are reduced in size and number. The luminal surface of the stria shows shallow indentations, with ridges at the junctions between marginal cells (Fig. 5). Basal cells continue to become elongated, forming the lower boundary of the stria, and tight junctions are formed between them. Tight junctions also appear to form normally between adjacent marginal cells near the luminal surface. All of these features can be seen in both mutants and controls up to 6 days of age.

The major difference between mutants and controls in the process of differentiation can be seen at about 6 days after birth, when the extent of the interdigitation between marginal and basal cell processes appears to be limited in mutants (Fig. 5). The marginal cells of mutants probably have as many individual cell processes as controls, but the processes appear to remain clumped together in blocks, rather than spreading out and interleaving with basal cell processes, as happens in controls. This is more clearly seen in Fig. 6, in which only a few small pale-staining basal cell processes can be seen amongst the darker-staining marginal cell processes of the mutant. The clumping together of the marginal cell processes in the mutants gives the appearance of an unusually distinct boundary between the marginal cell and basal cell layers, which can also be seen by light microscopy. This boundary becomes even more distinct at later stages of development (Fig. 7). The marginal cells in mutants up to about 20 days appear to be differentiating normally, except for the

Fig. 6. (A) +/+, 13 days, basal turn. (B) W/W, 13 days, lower apical turn. The difference in the amount of interdigitation is most clearly seen in these illustrations. The control stria looks fully mature, but in the mutants the individual marginal cell processes are clumped together and show little interdigitation with basal cell processes. Junctional complexes between basal cells are evident in both mutants and controls. Scale bar=5 μm.
failure to interdigitate normally. In adult mutants from about 6 weeks onwards, the marginal cells have very few cell processes (Fig. 8).

The part of the stria nearest to Reissner's membrane attachment showed a different abnormality in several of the 8- and 10-day-old mutants examined. In these unusual regions, a basal lamina is seen below the marginal cells, and the cells beneath look like loosely packed mesenchymal cells, while in the adjacent region there is no basal lamina and the cells below are compacted like normal basal cells at this stage (Fig. 9).

Finally, one mutant and one control aged 4 days were fixed and the strias removed and cryo-sectioned prior to staining with anti-laminin antibody. The basal lamina around the strial blood vessels reacted with the antibody, but there was no evidence of a continuous basal lamina persisting between the marginal and the basal cell layers at this age in either mutant or control (G. P. Richardson: personal communication).

Discussion

The resting potential in scala media in most of the mutants studies was close to zero, which suggests that EP never develops in this group of mice, rather than the lack of an EP being due to degeneration of the stria after it has begun to function. There are two possible explanations for this. The most likely is that the stria vascularis is unable to generate the EP; reasons for this are discussed later. The second possibility is that there may be an abnormally low resistance of the boundaries of scala media which prevents any potential difference between endolymph and ground being maintained. This explanation seems extremely unlikely, because in these young mutants there are no apparent abnormalities of the cells bordering scala media, and tight junctions between them appear to develop normally.

EP is believed to be generated primarily by the marginal cells using Na, K-ATPase pumps in their basolateral membranes (Offner et al. 1987; Marcus, 1986). However, a primary marginal cell defect seems unlikely because marginal cells appear to differentiate normally in the mutants in early development, and also the other pleiotropic defects in dominant spotting mice are all found in mesodermal rather than epithelial cells. The failure of the marginal cells to produce an EP
suggests that they are dependant upon some form of interaction with other strial cells. The role of the basal and intermediate cells in strial function is not well-defined, although they may be involved with the circulation of potassium. The potassium in endolymph is derived primarily from perilymph rather than from blood (Marcus, 1986 for review) and the most likely circulation route is via the spiral ligament and basal cells of the stria. An interruption in potassium circulation might also lead to a failure in EP production.

Although the mechanism of strial dysfunction requires further investigation, the present study clearly indicates that the primary cause of the dysfunction is an absence of melanocytes. All other cell types are present in the stria vascularis of mutants and appear to develop normally at first, including marginal cells, basal cells, capillaries, and even occasionally non-melanocyte intermediate cells. Previously, melanocytes have not been thought generally to be vital for EP production (e.g. Marcus, 1986; Offner et al. 1987), but our study indicates that they are important. They may have some direct, essential role in the production of an EP by the stria in the mature cochlea. Alternatively, melanocytes may be necessary at a particular stage of development to facilitate the normal differentiation of the other strial cells. For example, the reduced amount of interdigitation between marginal and basal cells suggests that the relationship between these two cell types is not normal in mutants, so melanocytes may normally stimulate this interaction. The abnormal relationship between marginal and basal cells in the mutants might, in turn, cause strial dysfunction. Melanocytes might influence the distribution of pumps and channels or junctional complexes in the membranes of other strial cells, leading to dysfunction. A requirement for melanocytes at a critical stage of development only would perhaps explain why different mammals and different strains show a wide variation in the number and distribution of melanocytes in the adult stria. Melanocytes might, of course, be important both for normal strial development and for the production of the EP.

It is not yet clear what aspect of the melanocyte is important for its role in the stria vascularis. Melanocyte function in the stria is not dependent upon its ability to produce melanin, because there is no evidence that the striae of albino animals, which contain amelanotic melanocytes, are dysfunctional (Bock and Steel, 1984). One feature that we investigated in the mutant stria was the basal lamina, because this structure has been shown to act as a filter in some situations (Farquhar, 1981) and, in other tissues, it has been suggested that mesenchymal cells may be responsible for breaking down the lamina (Bernfield et al. 1984). In the normal stria, the basal lamina below the marginal cells is known to become progressively disrupted during development until the only basal lamina in the stria is found around the capillaries (Hilding, 1969; Hilding and Ginzberg, 1977; Kikuchi and Hilding, 1972; Thorn...
and Schinko, 1985). Our hypothesis was that melanocytes might normally contribute to basal lamina degradation, and a persistent lamina in mutants might inhibit the free flow of ions through the stria. However, in most cases, we found no evidence that the basal lamina remained intact in the mutants for any longer than it did in the controls, suggesting that melanocytes are not necessary for the breakdown of the basal lamina in the stria.

We found some evidence that a close association with basal cells is necessary for basal lamina breakdown. In those 8- and 10-day-old mutants that showed a fluid-filled space below the marginal cell layer, a basal lamina was observed. It followed the lower boundary of the clump of marginal cell processes, separating the cells from the fluid, and it disappeared at the point where the marginal cells were once more in close contact with compacted mesenchymal cells. There are two possible explanations for this. Firstly, the basal lamina may never have been resorbed during development. This would indicate that close contact with compacted mesenchymal cells is necessary for the breakdown of the

Fig. 9. W/W, 10 days upper apical turn. Scale bar=5 μm. (A) The stria vascularis near its attachment to Reissner’s membrane, which is out of the field of view on the left. This unusual appearance occurred in several 8- and 10-day-old mutants. On the left, a basal lamina is seen below the marginal cells, and the cells below look like loosely packed mesenchymal cells. On the right, there is no basal lamina and the cells below are compacted like normal basal cells at this stage. Scale bar=5μm. (B) An enlargement of a similar area to that shown in A, to show the presence of a basal lamina below the marginal cells.
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References


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