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The fate of medial edge epithelial cells during palatal fusion *in vitro*: an analysis by DiI labelling and confocal microscopy

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Summary

Fusion of bilateral shelves, to form the definitive mammalian secondary palate, is critically dependent on removal of the medial edge cells that constitute the midline epithelial seam. Conflicting views suggest that programmed apoptotic death or epithelial-mesenchymal transformation of these cells is predominantly involved. Due in part to the potentially ambiguous interpretation of static images and the notable absence of fate mapping studies, the process by which this is achieved has, however, remained mechanistically equivocal.

Using an *in vitro* mouse model, we have selectively labelled palatal epithelia with DiI and examined the fate of medial edge epithelial (MEE) cells during palatal fusion by localisation using a combination of conventional histology and confocal laser scanning microscopy (CLSM). In dynamic studies using CLSM, we have made repetitive observations of the same palatal cultures in time-course investigations.

Our results concurred with the established morphological criteria of seam degeneration; however, they provided no evidence of MEE cell death or transformation. Instead we report that MEE cells migrate nasally and orally out of the seam and are recruited into, and constitute, epithelial triangles on both the oral and nasal aspects of the palate. Subsequently these cells become incorporated into the oral and nasal epithelia on the surface of the palate. We hypothesize an alternative method of seam degeneration *in vivo* which largely conserves the MEE population by recruiting it into the nasal and oral epithelia.

Key words: palatal fusion, medial edge epithelial cells, DiI, confocal laser scanning microscopy.

Introduction

Morphogenesis of the mammalian secondary palate is characterised by the growth, reorientation and union of apposing bilateral palatal shelves to form a continuous structure which separates the oral and nasal cavities (Pourtois, 1972; Greene and Pratt, 1976; Ferguson, 1988). Simultaneously the palatal epithelium undergoes three regionally distinct developmental fates specified by an inductive epithelial-mesenchymal interaction (Ferguson and Honig, 1984; Sharpe and Ferguson, 1988). On the nasal aspect the palatal epithelium differentiates into ciliated, pseudostratified columnar cells whereas on the oral aspect it differentiates into a stratified squamous epithelial phenotype. Medial edge epithelial cells, which constitute the newly formed midline seam, are removed to accomplish palatal fusion by permitting merging and continuity of the core mesenchymal component of both palatal shelves.

Mammalian palatogenesis has most commonly been studied using rodent models. In culture, paired apposing palatal shelves undergo normal *in vivo* patterns of differentiation including seam formation and degeneration (Tyler and Koch, 1975; Ferguson et al., 1984; Shiota et al., 1990). Both *in vitro* and *in vivo* studies have been responsible for a long-standing dogma, that disappearance of seam MEE cells is a result of classical programmed apoptotic cell death (Shapiro and Sweney, 1969; Clarke, 1990). The evidence, based largely on the ultrastructural localisation of apoptotic bodies and autophagic vacuoles in a number of midline seam cells, has always been problematical: in part due to the limited sampling of TEM studies; in part due to suggestions that different anteroposterior regions of the seam may degenerate differently (Smiley, 1975); in part due to the lack of any evidence of cellular debris or phagocytic activity at any stage of seam degeneration and in part by the histochemical demonstration of metabolic activity within the so-called apoptotic cells (Gartner et al., 1978a). Recently, seam degeneration *in vivo* has, alternatively, been attributed to transformation of basal MEE cells to mesenchyme (Fitchett and

Hay, 1989); however, despite extensive morphological characterisation of seam disruption *in vivo* at both the gross cellular (Waterman et al., 1973; Gartner et al., 1978b) and ultrastructural levels (Farbman, 1968; Hayward, 1969; Smiley, 1970) the precise mechanism of degeneration remains equivocal.

We have recently reported the appearance of a distinct differentiated migratory medial edge phenotype in isolated embryonic murine palatal epithelial cultures (Carette et al., 1991). In the light of this observation, the work presented here was undertaken to determine the fate of MEE cells during embryonic mouse palatal fusion *in vitro*. Our study includes the application of confocal laser scanning fluorescence microscopy (CLSFM) coupled with the use of the carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate (DiI) as a lineage marker.

Confocal microscopy (for review see Shotton, 1989) is a relatively new optical microscopic technique, which for our purposes offers significant advantages over conventional microscopy, including improved resolution, enhanced contrast and a reduction of out-of-focus interference. Most importantly, the ability of the confocal microscope to optically section living specimens (Van Spronsen et al., 1989; Rojanasakul et al., 1990) obviates the necessity of chemical fixation, hence reducing artifacts caused by tissue processing. In addition, CLSFM can be less damaging to living cells than conventional epifluorescence microscopy and therefore confers the ability to study temporal phenomena by repetitive observation during time-course studies (Zhang et al., 1990).

The fluorescent, hydrophobic and lipid soluble, carbocyanine dye, DiI, is rapidly and irreversibly incorporated into the cell membranes of cells that it contacts. The dye does not spread from labelled to unlabelled cells (Honig and Hume, 1989), has no reported adverse effects on labelled cell survival (Honig and Hume, 1986) and remains visible for several population doublings (Korn and Downie, 1989). DiI is therefore an ideal candidate marker for studies of cell migration and cell lineage (Korn and Downie, 1989; Serbedzija et al., 1990; Pomeranz et al., 1991). The dye is also intensely fluorescent and fades more slowly than fluorescein or rhodamine fluorescence, making it amenable to repetitive optical visualisation by CLSFM in time-course studies.

We believe this study represents the first reported "fate map" analysis of MEE cell behaviour during palatal fusion using a dynamic experimental system. Results reveal a hitherto unsuspected method of seam degeneration which may have important implications for the development of other embryonic structures.

Materials and methods

Tissues

Following overnight mating, pregnant female mice (strain MF1) were killed by chloroform overdose on day 13 of gestation (day of vaginal plug considered day 0). The embryo complement was asceptically removed and transferred to sterile Dulbeccos modification of Eagles medium and Hams F12 (DMEM/F12, 1:1 volume) where they were individually staged according to morphological criteria (Theiler, 1972). Left and right palatal shelves were separately dissected from the decapitated heads of Theiler stage 21 embryos and pooled.

DiI labelling

Palatal epithelia were labelled by immersing whole palates in a 25 μg/ml solution of DiI (Molecular Probes, Eugene, OR, 2.5 mg/ml in 100% ethanol stock diluted 1:100 v/v in DMEM/F12 plus 10% donor calf serum (DCS)). Incubation was for two hours at 37°C. This was followed by three washes (5 minutes each) with DMEM/F12 to remove residual dye.

Organ culture

Palatal shelves were cultured in random pairs, comprising a left and right palate, of which both (paired labelled cultures) or one (mixed labelled culture) member was DiI labelled. The nasal and oral surfaces of each palate were identified and pairs cultured with either both nasal or both oral surfaces uppermost (due to the limitation of the depth of optical sectioning and the disruption of the epithelium contacting the filter during culture) in equal numbers (Fig. 1). Medial edges were apposed and aligned in an anterior-posterior direction to simulate the *in vivo* orientation. The culture technique used was that described by Ferguson et al., (1984). Cultures were placed on Millipore filters (0.22 μm) on top of stainless steel grids over 500 μl of medium in Falcon organ culture dishes. The medium used was Eagles MEM (pH 7.4) supplemented with ascorbic acid, glycine, glutamine, HEPES buffer and 1% antibiotic/antimycotic (Gibco). Cell death control cultures additionally contained sodium azide (1%). Cultures were maintained in a 37°C, 100% humidity, air environment and medium replaced every 48 hours.

Epithelial sheet culture

Palatal epithelial sheets were cultured according to the technique of Carette et al., (1991). Briefly, epithelial sheets were separated from DiI-labelled palates by incubation in trypsin (Difco) 0.5% w/v in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS), pH 7.4 at 37°C for 1 hour after which time trypsin activity was reversed with soybean trypsin inhibitor (Sigma), 1% w/v in DMEM/F12. After washing with DMEM/F12 (3× 5 minutes) epithelial sheets were removed from the underlying mesenchyme using electrolytically sharpened tungsten needles and explanted onto a laminin substratum under sterile agarose (1.25% in water) overlays preequilibrated with serum-free DMEM/F12. Following overnight attachment, agarose overlays were removed and the cultures supplemented with fresh medium. The epithelial sheets were removed for analysis after 24 and 48 hours and examined, unfixed, by phase-contrast microscopy using a Labovert microscope and subsequently on a Leitz Dialux Microscope by incident light fluorescence using a rhodamine filter set.

Histology

Paired labelled cultures were removed for histological examination after 2 hours (day 0), 24 hours (day 1), 48 hours (day 2) and 120 hours (day 5). The cultures were embedded in OCT freezing compound and frozen on the surface of a liquid nitrogen cooled metal block. The cultures were sectioned (7 μm) transversely (Fig. 1C, D) using a Leitz Cryostat and mounted in 2.5% 1,4, diazabicyclo-2,2,2octane (DABCO)

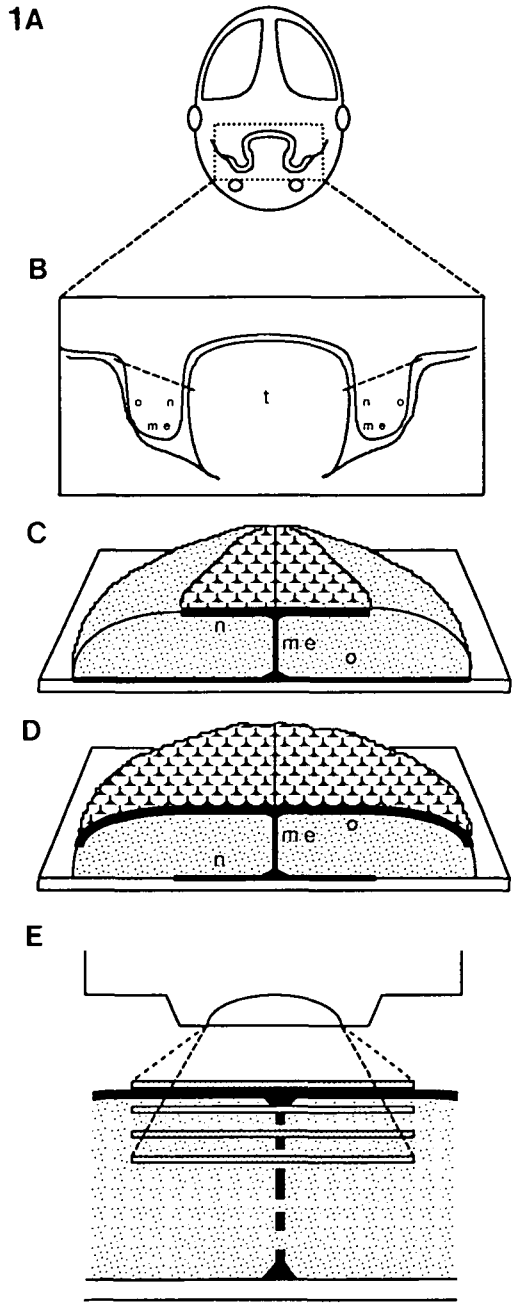

Fig. 1. Schematic representation of the source, culture and histological/confocal analysis of paired, whole palate organ cultures. Bilateral palatal shelves growing vertically down the sides of the tongue (t) were excised (dotted lines) from the heads of embryonic day 13 mice (A,B). Isolated palatal shelves were bounded by epithelium on the future nasal aspect (n), by more extensive epithelium on the future oral aspect (o) and medial edge (me) epithelium at the palatal tip (B). Paired palates (shown in transverse section) were explanted onto millipore filters (medial edges apposing) with either both nasal (C) or both oral (D) surfaces uppermost. Confocal imaging in a horizontal plane was performed at sequential depth increments in the region of the midline epithelial seam (E).

anti-fading aqueous mountant. Slides were viewed within half an hour on a Leitz-Dialux microscope by incident light fluorescence using a rhodamine filter set and subsequently using phase-contrast optics.

Confocal analysis

Cultures were moistened with a drop of medium, placed on a glass slide, and viewed directly using an argon-ion-laser scanning confocal system (model MRC-600, Bio-Rad, Microscience division, Oxford), under microcomputer control, attached to a Zeiss IIRS microscope. The ×16 plano objective lens used (N.A.,0.4) was positioned above the midline seam fusion zone and sequential optical sections collected (from the surface downwards) under computer control at 20 μm intervals with the fine focus of the microscope automatically stepped with a microstepping motor (Fig. 1). Cultures were sectioned to a depth of 140 μm beyond which tissue penetration by the laser was limiting. This value represented approximately one third of the total depth of cultures which averaged 400 μm. Full-power argon ion excitatory light, operating at 514 nm wavelength, was reduced in intensity by a neutral density filter (ND=1) to 10% to minimise cell damage and bleaching of the fluorophore. The aperture size of the confocal pinhole was maintained at a standardised setting for all observations.

Static analyses

Representative paired-labelled, mixed-labelled and azide-treated cultures were analysed after 2 hours (day 0), 24 hours (day 1), 48 hours (day 2) and 120 hours (day 5). Cultures were viewed once and discarded. Frame averaging (Kalman) was used to improve the signal:noise ratio.

Dynamic analyses

Paired labelled and mixed cultures were viewed after 8, 16, 24 and 40 hours. Analysis of the same culture was made at successive time points. Optical sectioning was performed using a single scan direct imaging mode to minimise exposure to potentially detrimental radiation.

Photography

Confocal images were recorded by photographing the high-resolution monitor directly with a 35 mm Nikon camera. Conventional fluorescence, phase-contrast and confocal images were all recorded on Ilford Technical Pan black and white film rated at 100 ASA from which prints were made.

Results

Histology

Inspection of representative cryosections using phase-contrast optics revealed that palatal fusion occurred, or was occurring, in all cultures examined as judged by established morphological criteria. At day 0, the midline seam was intact and two cell layers thick. Early disruption was observed at day 1 as evidenced by the presence of epithelial islands within the seam. Epithelial triangles were formed on both the oral and nasal aspects of each culture. By day 2, epithelial triangles were increasingly prominent and few epithelial cells were observed in the seam. By day 5, palate mesenchyme was continuous across the palatal fusion zone.

Fluorescence

Day 0 cultures exhibited specific and uniform DiI labelling of the palatal epithelium with additional limited uptake of dye by mesenchymal cells at the cut ends of each palate. Leakage of dye is known to occur in sectioned material when membrane integrity is disrupted. This artifact was minimised by viewing cultures within half an hour of sectioning. In day 0 cultures, the 2-cell layer epithelial seam was intensely labelled and extended through the depth of the culture from the oral to the nasal aspect (Fig. 2A)

On day 1 of culture, DiI was intensely localised within forming epithelial triangles and in discrete epithelial islands throughout the seam (Fig. 2B,C). There was no evidence of any labelled cells within the neighbouring mesenchyme.

On day 2, DiI labelling was absent from the middle of the degenerating midline epithelial seam. DiI labelling was, however, very intense within the epithelial triangles on both the oral and nasal aspects of the palate (Fig. 2D). Surface epithelia were less intensely labelled than the epithelial triangles with the exception of short stretches of intensely labelled epithelium on the surface adjacent to the zone of the midline epithelial seam (Fig. 2E). No dye could be detected within the mesenchyme anywhere in the vicinity of the midline epithelial seam.

On day 5, dye was almost completely absent from the entire depth of the midline epithelial seam zone and adjacent palatal mesenchyme. Labelling was restricted to the surface epithelia and a small subjacent area in the midline region and was conspicuously more intense in surface areas adjacent to the midline seam zone (Fig. 2F).

Dye localisations were similar in paired palates cultured with either oral or nasal surfaces uppermost.

Epithelial sheet culture

Whole epithelial sheets were cultured from labelled palates randomly selected from the experimental pool. The objective was to confirm labelling and investigate patterns of dye distribution during culture, in particular the noticeably higher intensity of dye in the seam and epithelial triangles of organ cultures. After 48 hours the palatal epithelia had differentiated into oral, nasal and medial edge phenotypes (Carette et al., 1991, Fig. 3A). DiI labelling was weak and punctate in oral and nasal epithelial cells (Fig. 3B); however, MEE cells were intensely labelled as were migratory cells of medial edge origin towards the periphery of explants (Fig. 3B).

Confocal analysis

Confocal images were collected as a series of eight sections starting initially at the palatal surface and then subsequently at consecutive 20 μm increments of penetration into the tissue (Fig. 1E). Images presented in Figs 4 and 5 are sequences of alternate optical sections representing successive depth increments of 40 μm. The discrete and restricted cellular localisation of the dye emphasised the specificity of DiI labelling in fresh living tissue. Dye retention in MEE cells, confirmed by the epithelial sheet cultures, was used as an aid to image interpretation. Brightly labelled epithelial cells, particularly in later time point cultures, were inferred to be of medial edge origin.

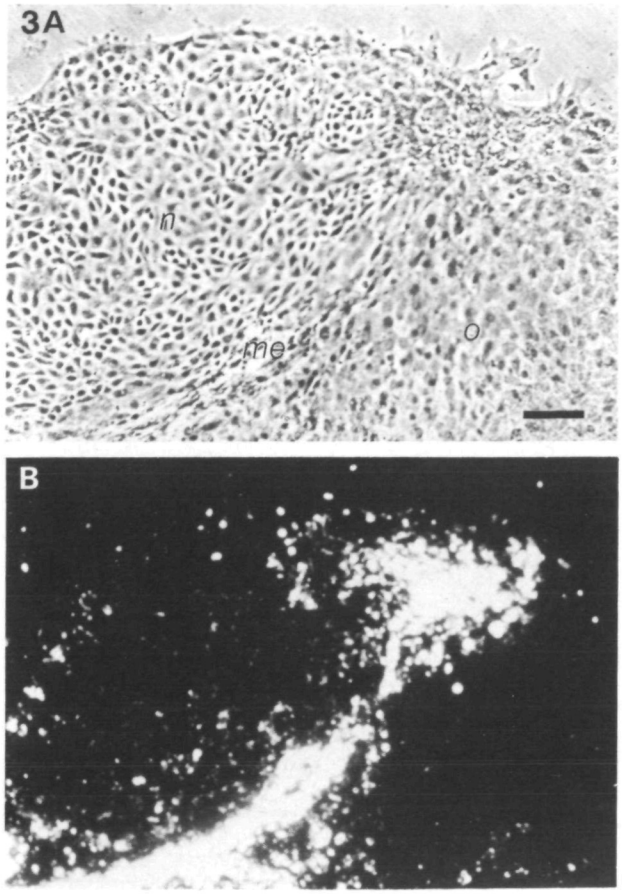

Fig. 3. DiI labelling in the differentiated oral (o), medial edge (me) and nasal (n) cell phenotypes of an isolated epithelial sheet cultured for 48 hours. Paired phase (A) and fluorescence (B) micrographs. DiI labelling was intense in medial edge epithelial cells and migratory cells of medial edge origin at the periphery of the explant whereas it was diffuse and punctate in oral and nasal epithelial cells. Bar is 75 μm.

Static analyses

Static confocal images complemented the histological survey, providing artifact-free optical sectioning in a plane perpendicular to that of cryosectioned material.

Day 0. 2 hours after explant, surface epithelium (Fig. 4A) and medial edge seam epithelium (Fig. 4B,C,D) exhibited intense labelling with dye.

Day 1. After 24 hours, a broad intense band of labelled cells was observed at the surface along the line of the seam (Fig. 4E). Below the surface, a prominent line of intensely labelled cells were visible which had decreased in width and showed evidence of disruption (Fig. 4F). Deeper within the tissue, increasing disruption of the seam was evident (Fig. 4G,H). Dye localisation was, however, restricted to the narrow line of the epithelial seam. No labelling was observed at any level within the adjacent mesenchyme.

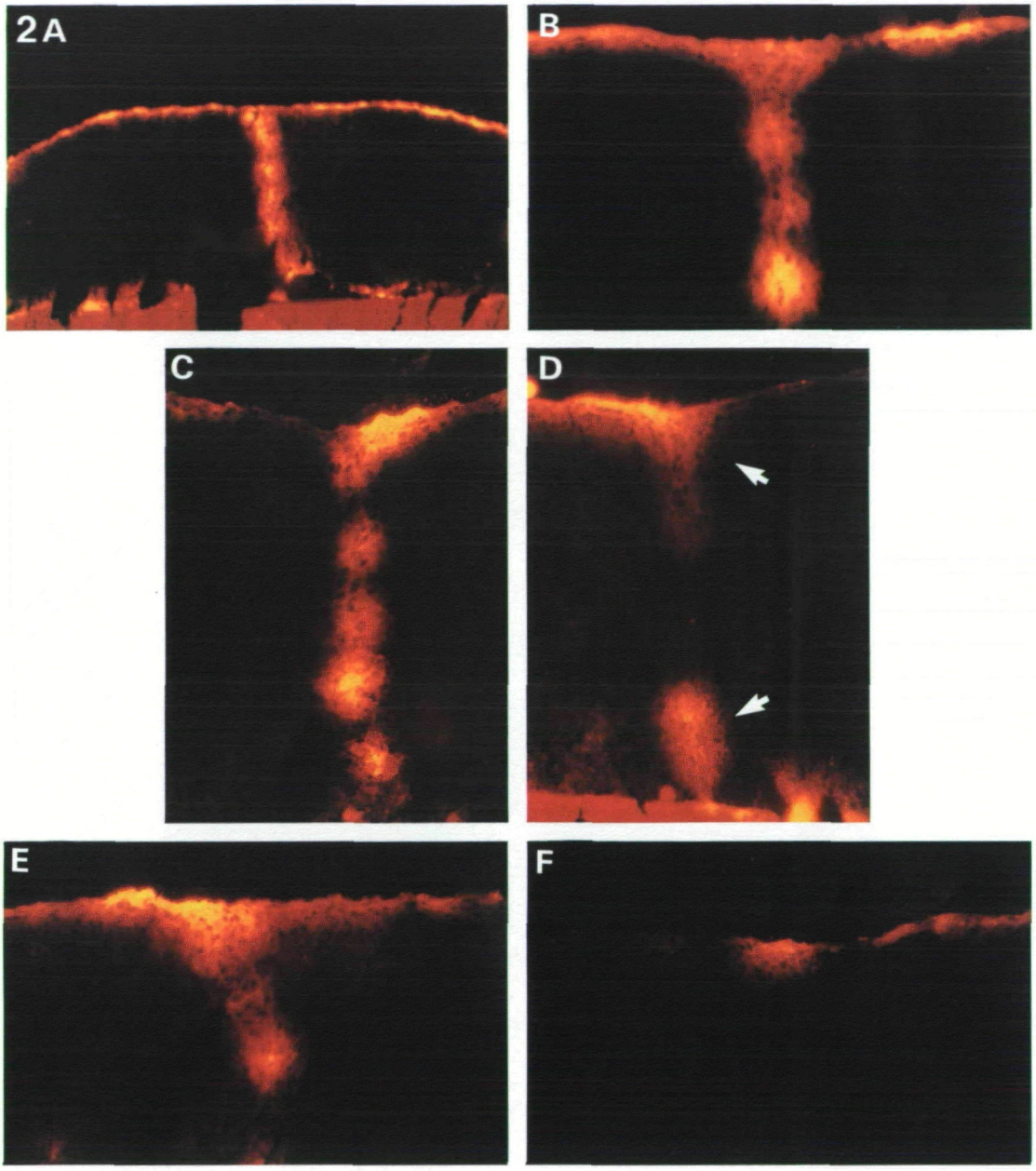

Fig. 2. Time-course analysis of the degenerating epithelial seam in transverse histological sections of DiI-labelled paired palatal organ cultures. On day 0 the surface epithelia and medial edge cells throughout the entire depth of the seam were intensely labelled with DiI (A). After 24 hours (day 1) labelling was observed in epithelial triangles and in discrete islands indicative of the onset of seam degeneration (B,C). After 48 hours (day 2) labelling was intense in the epithelial triangles on the oral and nasal aspects of the culture (arrowed) but absent from the mid-seam region and surrounding mesenchyme (D). Labelled cells in the epithelial triangles were frequently more intense than the adjacent surface epithelium (E). After 120 hours (day 5) labelled cells were restricted to the surface epithelium and a subjacent region in the midline area (E).

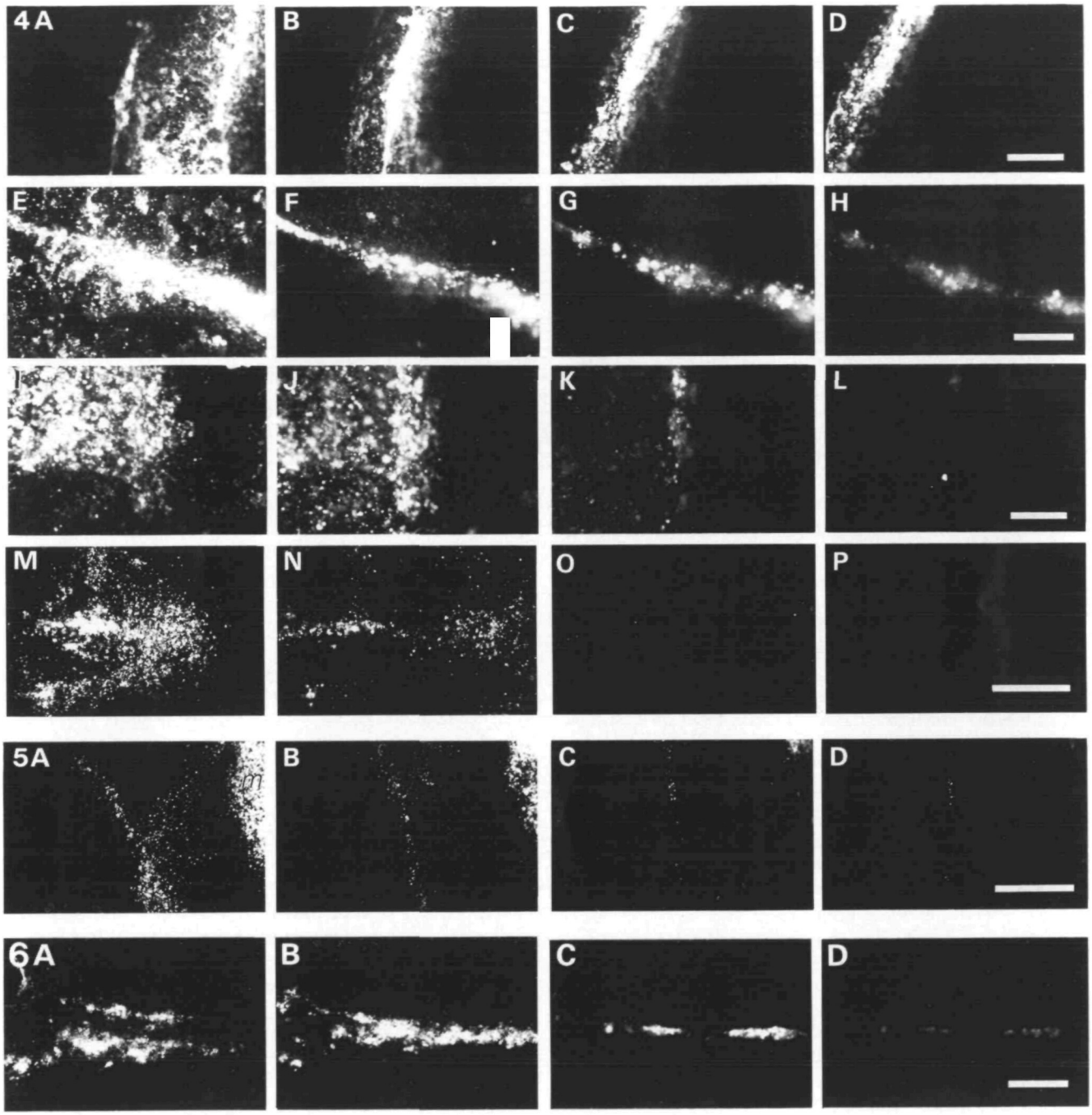

Fig. 4. Confocal images of DiI-labelled paired palate organ cultures collected at the surface (A,E,I,M) and further sequential 40 μm depth increments (40 μm - B,F,J,N; 80 μm - C,G,K,O; 120 μm - D,H,L,P) at the onset of culture (A-D), after 24 hours (E-H), 48 hours (I-L) and 120 hours (M-P) of culture. (A-D) The surface and midline epithelium were intensely labelled. (E-H) Band of intense staining at the surface along the line of the midline seam. Other surface cells exhibited diffuse and punctate staining (E). Deeper within the culture the seam became broken but without labelling in the mesenchyme (F,G,H). (I-L) Intense surface labelling was more widespread (I). The epithelial triangles labelled heavily (J). Labelling within the seam was diminished and there was no labelling in the mesenchyme (K,L). (M-P) Intensely labelled cells were restricted to the surface (4M) and immediately subjacent regions along the line of the seam (4N). Deeper, the seam and mesenchyme were unlabelled (O-P). Bars in D,H,L are 100 μm. Bar in P is 200 μm.

Fig. 5. Confocal images of DiI mixed labelled paired palate organ cultures after 48 hours. (A) Labelled cells at the palatal surface were restricted to the originally labelled palate. The bright area (m) in the top right-hand area of the micrograph is exposed, labelled palatal mesenchyme. (B-D) Scattered labelled cells within the epithelial seam, but none in the mesenchyme. Bar is 50 μm.

Fig. 6. (A-D) Confocal images of azide-treated DiI-labelled palatal cultures after 120 hours. Intensely labelled cells persisted in the seam (compare with untreated cultures after 120 hours [Fig. 4M-P]). Bar is 100 μm.

Day 2. The labelling of the surface epithelium was greatly diminished, with the exception of areas of intense fluorescence in close proximity to the degenerating seam (Fig. 4I). Below the surface, labelled cells accumulated in the nasal and oral epithelial triangles whilst location of the seam was still emphasised by a broken line of labelled cells (Fig. 4J). Deeper within the palates, the labelling rapidly diminished (Fig. 4K). At 120 μm no labelled cells were detectable within the seam or adjacent mesenchyme (Fig. 4L)

Day 5. By day 5 brightly labelled cells were observed at the surface of the cultures adjacent to the underlying midline epithelial seam (Fig. 5M). A few remaining labelled cells were also evident just below the surface (Fig. 5N). Labelling was not detected deeper within the cultures (Fig. 4O,P).

Mixed cultures. In mixed palatal cultures, the labelled cell complement was halved, allowing better resolution and the potential to detect movement of cells from the labelled to the unlabelled palatal shelf. The patterns of labelling were similar to those in paired labelled cultures at the corresponding stages (Fig. 5A,B,C,D). Brightly labelled surface cells were restricted to the originally labelled palate of the pair (Fig. 5A) and there was no evidence for movement of cells from labelled to unlabelled palates.

Azide-treated cultures. Control azide-treated cultures enabled the appearance and distribution of dye in the seam to be assessed when the constituent cells were killed. Labelling was observed throughout the depth of the seam examined at all time points up to and including day 5 (Fig. 6A-D) although the intensity of label diminished with increasing depth at later time points. Dye was however restricted to the seam with little or no leakage into adjacent tissue.

Dynamic analyses

By contrast to single time point static analysis, after which cultures were discarded, dynamic analyses involved repetitive observation of the same specific region of fusing palates at successive time points over 40 hours. The distribution of dye in both paired and mixed labelled cultures correlated closely to that observed in static analyses at the corresponding times. In paired labelled cultures, surface labelling became progressively more intense (Fig. 7A,E,I,M) whereas simultaneously labelling diminished within the epithelial seam (Fig. 7C,G,K,O,D,H,L,P).

Discussion

In the present study, we have examined the fate of medial edge epithelial cells during embryonic mouse palatal fusion *in vitro*. This has been achieved by selectively labelling palatal epithelial cells with a lipophilic membrane probe (DiI), and subsequently localising labelled seam (medial edge) cells by a combination of conventional histology and confocal microscopy. As a result of dynamic time-course analyses, we have presented a series of confocal images, which we believe portray the movement of MEE cells during seam degeneration. Our interpretation of these images, substantiated by a complementary histological survey, postulates a new mechanism of seam disruption *in vivo*. This new interpretation suggests that most, if not all, MEE seam cells migrate orally and nasally into the epithelial triangles and subsequently into the epithelium covering the nasal and oral surfaces of the palate.

Before commencing this study we made extensive histological and ultrastructural investigations of the morphology of seam degeneration in MF1 embryonic mice *in vivo* and *in vitro*. These data are not included as the findings document that palate development in this mouse strain is identical to that reported previously in the literature (see earlier citations). As assessed at the gross cellular level, seam degeneration progresses by thinning of the midline epithelial cell layers, disruption of the basal lamina and formation of epithelial islands. At the nasal and oral surfaces of the palate, the seam expands into prominent triangular areas (Smiley and Dixon, 1968) which together with epithelial remnants in the seam disappear as mesenchymal confluency is achieved.

Additional criteria have been cited to support programmed cell death or epithelial-mesenchymal transformation which represent the currently favoured, albeit conflicting theories purporting to explain the mechanism of MEE cell removal from the midline palatal seam.

Cessation of DNA synthesis in MEE cells 24-36 hours prior to palatal shelf contact *in vivo* (Hudson and Shapiro, 1973) is regarded as the first indication of the imminent terminal differentiation (apoptotic death) of these cells. It should, however, be noted that labelling protocols used in these studies would not differentiate between cessation of DNA synthesis (and imminent death) in the MEE cells and the differentiation of the medial edge epithelium into a population of slowly cycling cells (also present as a population of adult epithelia (Potten and Loeffler, 1990)). In addition, the appearance of lysosomes (Mato et al., 1966), mitochondrial swelling (Sweeny and Shapiro, 1970), and electron-dense granular (lysosomal) structures (Mato et al., 1967) specifically within the MEE cells during seam degeneration is believed to be evidence of the lysosomally mediated autolytic breakdown of these cells. Macrophage migration and subsequent phagocytosis of degenerating epithelial cells has also been reported (Koziol and Steffek, 1969).

Recently, Fitchett and Hay (1989) suggested that the predominant mechanism of midline palatal seam disruption is by transformation of MEE cells into mesenchyme cells and their subsequent recruitment into the palatal mesenchyme. In support of this theory, they present a series of electron micrographs and evidence of a shift in the intermediate filament (IF) profile of medial edge cells from cytokeratin to

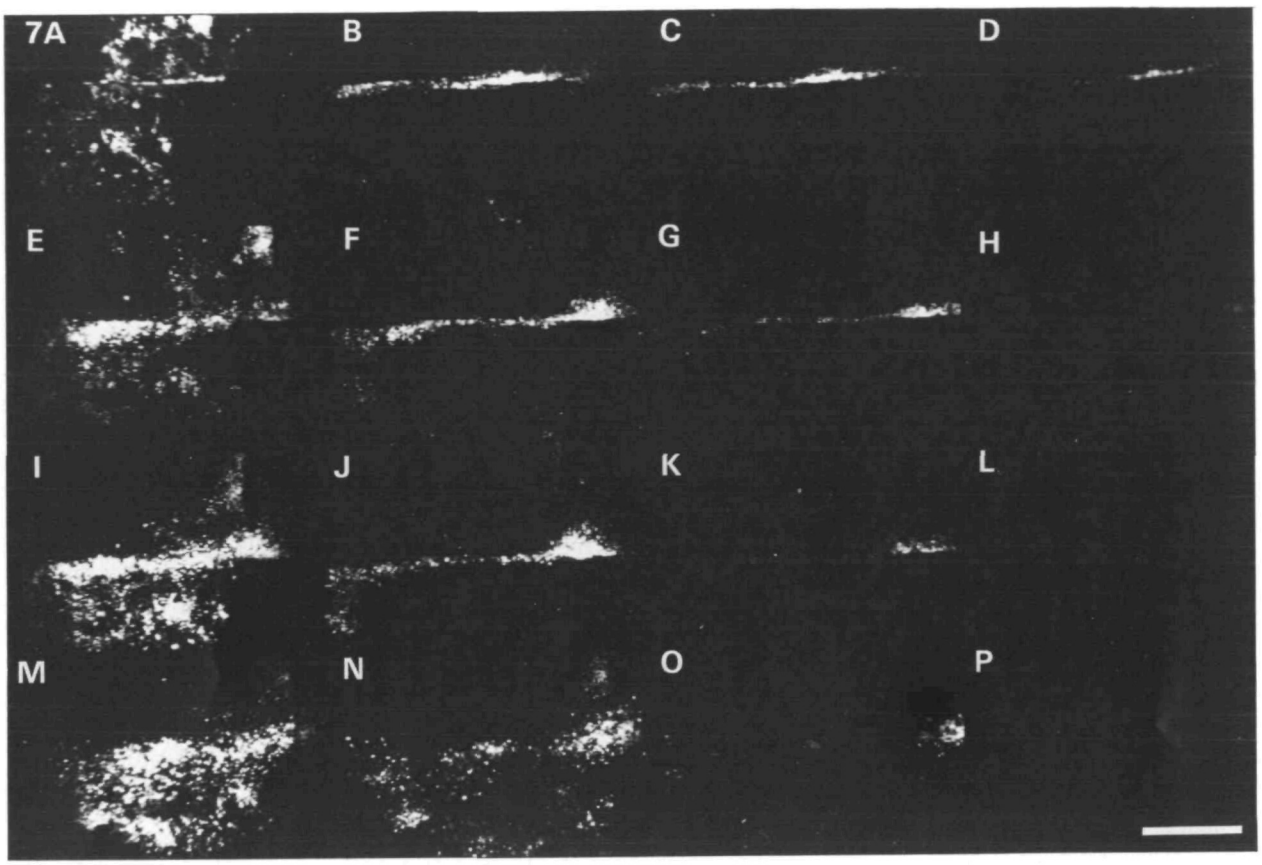

Fig. 7. Temporal progression of seam degeneration illustrated by images obtained from 'dynamic' confocal analysis of a single DiI-labelled paired palatal culture. Images were collected at the surface (A,E,I,M) and at further sequential depth increments of 40 μm (40 μm - B,F,J,N; 80 μm - C,G,K,O; 120 μm - D,H,L,P) after 8 hours culture (A-D), after 16 hours culture (E-H), after 24 hours culture (I-L) and 40 hours culture (M-P). Note the appearance of intensely labelled cells at the surface (A,E,I,M) and the gradual disappearance of labelled cells from the midline seam (C,G,K,O; D,H,L,P) with time. Bar is 200 μm.

vimentin. The absence of cytokeratin-positive cells in the seam area during advanced stages of degeneration was interpreted as evidence of a complete transition of the IF profile of these transformed MEE seam cells which are proposed to become vimentin positive and indistinguishable from their surrounding palate mesenchymal counterparts (Fitchett and Hay, 1989). Suprisingly, no attempt was made to study the ultimate fate of these cells. Palatal fusion, was therefore postulated to involve epithelial-mesenchymal transformation as allegedly occurs in several other developmental systems eg. cornea, heart valves, Mullerian duct (Bernanke and Markwald, 1982; Greenburg and Hay, 1986, 1988; Trelstad et al., 1982).

Both the programmed cell death, and in particular the epithelial-mesenchymal transformation theory of seam degeneration rely on the potentially ambiguous interpretation of a dynamic event from a series of static images. As part of our survey using CLSFM, however, we have followed the temporal progression of seam degeneration by making successive observations of the same region of a fusion zone in single paired palate organ cultures. Our images of medial edge epithelial cell disappearance (histological and confocal) concur with the recognised morpholgical criteria for seam degeneration. However, the shifting patterns of distribution of dye-labelled cells within the degenerating seam provide no evidence for either cell death or epithelial-mesenchymal transformation. This can be inferred from the absence of dye or labelled cells within the seam area or adjacent mesenchyme at an advanced stage of seam degeneration. We would expect residual dye in the seam area as a result of extensive cell death, which occurred in azide-treated control cultures. Similarly we would anticipate epithelial-mesenchymally transformed cells to retain dye and be visible within the mesenchyme.

Rather, our results indicate that a conserved migratory phenotype is adopted by medial edge cells. These cells migrate orally and nasally to be recruited into, and constitute, the epithelial triangles on both the oral and nasal aspects of the palate. They subsequently become incorporated into the surface epithelia of the nasal and oral aspects of the palate of origin with little or no cross migration. We propose, therefore, a previously unrecognised significance of the epithelial

triangles: that they comprise a transiently migratory population of epithelial cells en route from the medial edge epithelial seam to the surface of the palate. Interestingly, both the epithelial triangles and the cells of the epithelial seam during disruption represent an area of expression of TGFβ-3 transcripts (Fitzpatrick et al., 1990) and localisation of TGFα (Dixon et al., 1991), Insulin-like growth factor II and acidic and basic fibroblast growth factor (Sharpe, Thompsett and Ferguson, unpublished). Such growth factors have been causally implicated, in other systems, in epithelial migration (Vales et al., 1990).

Much of the evidence for MEE cell death during palatal fusion has been obtained from *in vitro* studies. Fitchett and Hay (1989) proposed that the cell death observed *in vitro* is considerably greater than that *in vivo* as a result of the death of trapped peridermal cells which are normally sloughed off during palatal reorientation *in vivo*. We, however, have found no evidence for trapping of peridermal cells using our culture system.

Migratory behaviour of medial edge cells was not a surprise. We have previously reported the appearance of a distinct migratory medial edge phenotype in whole isolated palatal epithelial sheets cultured *in vitro* (Carette et al., 1991). These cells, like their *in vivo* counterparts (Dixon et al., unpublished data), express vimentin (unpublished observation), but retain expression of a distinct cytokeratin profile (Carette et al., 1991). Coexpression of cytokeratin and vimentin IFs in epithelial cells has been previously reported (Ramaekers et al., 1983; Viebahn et al., 1988) and may reflect migratory functionality in these cells. Clearly, the evidence of Fitchett and Hay (1989) that MEE cells transform to mesenchyme, as indicated by their loss of cytokeratin intermediate filaments, is based on inadequate sampling with non specific cytokeratin antibodies.

In reconciling past and present observations, we propose that accumulation of electron dense lysosomal bodies in MEE cells (interpreted in the past as a hallmark of death) may be indicative of increased intracellular and extracellular remodelling by degradative enzymes, rather than death. MEE cells are bioactive and remodel their intermediate filament (cytokeratin) profile, whilst migratory and invasive cells may use lysosomal enzymes to degrade surrounding structures during their movement (Goldfarb and Liotta, 1986; Liotta et al., 1986; Tryggvason et al., 1987).

In day 2 and day 5 cultures, MEE cells invariably appeared brighter and more clearly labelled with DiI than both the oral and nasal epithelial cells. This phenomonen, which was utilised as an aid to interpretation of images, may be a result of DiI dilution in dividing oral and nasal cells but not in post mitotic MEE cells. The preferential uptake and/or retention of dye by the MEE cells as a consequence of possessing abundant lipophilic inclusions may alternatively, or in addition, be a contributory factor and could similarly account for the preferential uptake of neutral red dye by MEE cells in previous studies (Ferguson et al., 1984). The more intense and persistent labelling of MEE cells was confirmed in DiI labelled epithelial sheet cultures which showed a greater degree of dye retention in the MEE cells and the migratory cells of medial edge origin than in the oral or nasal cells.

Azide-treated cultures clearly exhibited retention and persistence of label in the seam when compared with untreated cultures at the corresponding time points. However, labelling diminished with depth, particularly at later time points. A likely explanation for this observation is the time taken for the azide to kill all the cells.

The present *in vitro* study addresses the fate of the MEE cells in the posterior three quarters of the palate, where the bilateral shelves fuse only with each other. *In vivo* the anterior quarter of the mouse palate also fuses with the nasal septum on its nasal aspect. As the nasal septum fusion is excluded from our cultures, we cannot comment on the fate of the MEE cells in this region. It would, however, be interesting to determine the fate of the nasal epithelial triangle in this fusion region. This study has clearly demonstrated the fate of the MEE cells in the majority of the fusing palate, where the cells migrate into the oral and nasal triangles and are subsequently recruited into the oral and nasal epithelia. Whether one calls this transient cell population "transitory migratory epithelia" or "transitory migratory mesenchyme" is largely a question of semantics and depends upon ones' definition of an epithelial or mesenchyme cell. However, there is perhaps an analogy between the events in palatal fusion and the formation of primary mesenchyme (Hay, 1982). In the latter, epithelial cells move through the primitive streak and become primary mesenchyme cells which subsequently aggregate to form epithelial somites.

This mechanism of palatal closure probably also facilitates the strength of palatal fusion (to resist the muscular forces of the tongue, cheeks etc. Ferguson, 1988): initially established by firm epithelial cell adhesions and desmosomes and later strengthened by coordinated MEE cell migration and mesenchymal infilling at different stages of advancement along the anterior-posterior length of the palate. Intuitively, this would appear stronger than a zone of epithelial cell death down the midline of the recently formed palate, which might be more susceptible to post-fusion dehiscence. There is no evidence (apart from poorly preserved human abortuses) for post-fusion rupture as a significant cause of cleft palate in any experimental system (Ferguson, 1988).

It is worthy to note that palatal closure in crocodilians is achieved by massive migrations of medial edge epithelial cells and their recruitment onto the oral and nasal aspects of the closing palate (Ferguson, 1981, 1984). Consequently, our findings indicate that the mechanisms of palatal closure in different vertebrates may not be as phylogenetically diverse as previously thought.

Finally this study emphasises the importance of dynamic tracing studies to elucidate cell fate during development. The transient migration of epithelial

cells, as opposed to their persistent transformation to mesenchyme, may be a mechanism operational in other developing structures.

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