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Original Article

Rapid disappearance of the medial epithelial seam during palatal fusion occurs by multifocal breakdown that is preceded by expression of α smooth muscle actin in the epithelium

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ABSTRACT Breakdown of the medial epithelial seam (MES) is essential to allow bridging of the mesenchyme during palatal fusion. Evidence exists for three mechanisms for this breakdown that are incompatible at the level of individual cells in the seam. To determine if breakdown of the seam was regionally restricted, 3-dimensional reconstructions were generated using volume rendering software from 1 μ serial sections in the sagittal plane of rat palates fixed during the process of fusion. The earliest break detected in electron micrographs was cell separation and in reconstructions was a discrete defect, with a rounded outline, nearer to the nasal than to the oral margin of the seam. Further breakdown produced a pattern of rounded defects along the nasal margin of the seam resulting in interconnected columns of cells preferentially attached to the oral epithelium. Computer generated slicing of reconstructed seams showed that groups of cells evident in cross-sections as islands at this stage of breakdown of the MES could be artifacts. Unequivocal islands of epithelial cells formed later in fusion had a rounded outline, an incomplete basal lamina and a halo of cells containing phagocytosed apoptotic debris. The pattern of breakdown indicated that the MES breaks down under tension. Laser confocal microscopy of sections and whole-mounts of palates demonstrated α-smooth muscle actin preferentially localized in the epithelial cells of the palatal shelves immediately before and during formation of the seam. Expression in epithelial cells of the isoform of actin normally restricted to smooth muscle cells engaged in tonic contraction supported an interpretation that the epithelial cells of the seam may be capable of generating tension during the palatal fusion event.

KEY WORDS: *palatal fusion, 3-dimensional reconstruction, epithelium, α smooth muscle actin, epithelial-mesenchymal transition, laser confocal microscopy*

Introduction

A crucial process in the formation of the maxilla during craniofacial development is the midline fusion of the palatal shelves, where dispersal of the midline epithelial seam (MES) is required for mesenchymal confluence (Ferguson, 1988; Shuler, 1995). The MES is formed by integration of the medial edge epithelium (MEE) on each of the palatal shelves following contact of the shelves in the midline. A substantial body of evidence has been accumulated for at least three mechanisms as potentially responsible for the breakdown of the MES; epithelial-mesenchymal transformation (EMT) (Fitchett and Hay, 1989; Shuler *et al.*, 1991), programmed cell death (apoptosis) (Mori *et al.*, 1994; Taniguchi *et al.*, 1995; Yano *et al.*, 1996) and migration of the cells of the seam into the epithelium of the developing palate (Carette and Ferguson, 1992). Precisely when and within which cells of the epithelial seam these mechanisms are active becomes an important question because, operationally, any one of them effectively excludes the other two as a possible fate for any individual cell. On the other hand, these individual mechanisms are not mutually exclusive if each mechanism

Abbreviations used in this paper: MEE, medial edge epithelium; MES, medial epithelial seam; EMT, epithelial-mesenchymal transition; dpc, days post-coitum; PBS, phosphate-buffered saline; FCS, fetal calf serum; RT, room temperature; TGFβ, transforming growth factor beta.

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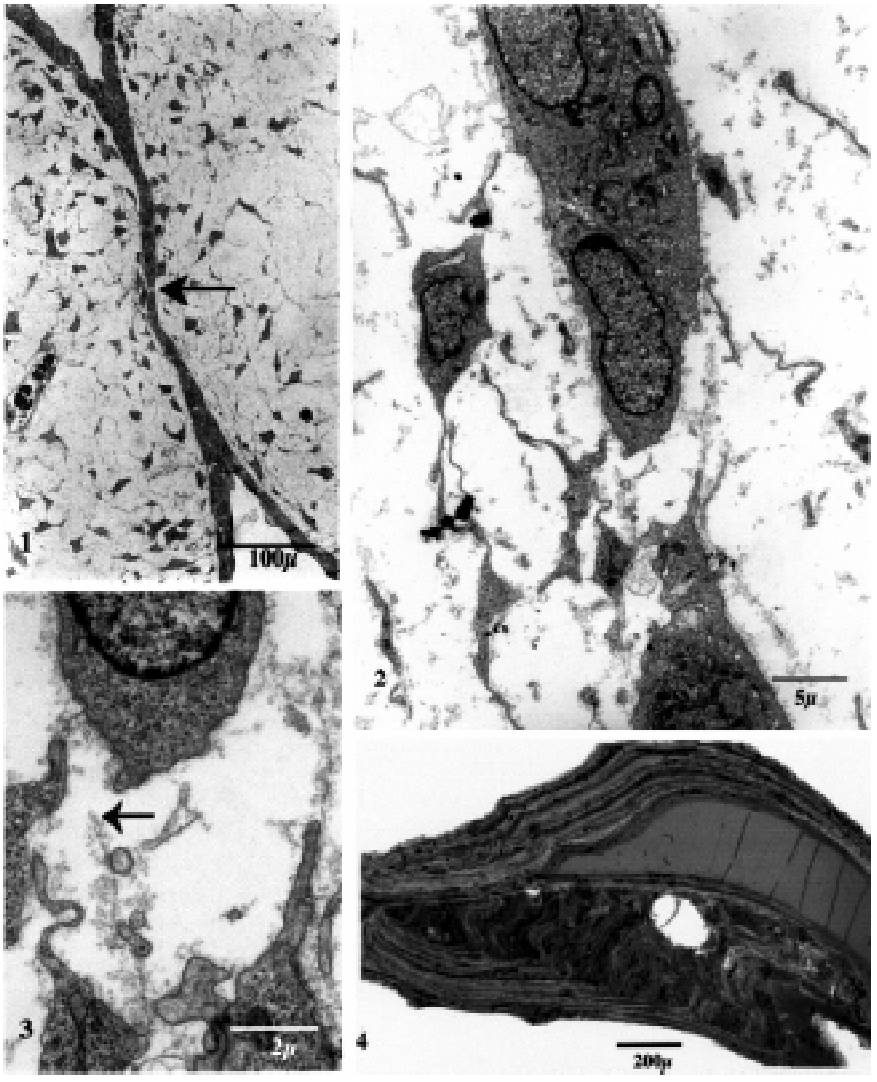

Fig. 1. A point of failure in medial epithelial seam (MES). *The medial edge epithelia (MEE) on each palatal shelf have merged to form the MES and become attenuated to a single layer of cells near the mid-region. The MES has become discontinuous in the attenuated region (arrow). Light micrograph of a Toluidine Blue stained 1 μ resin cross-section of a palate from a 16.5 dpc rat embryo.* **Fig. 2. Absence of intercellular attachment at a point of failure in the MES.** *Separated cells in the attenuated epithelial seam are not connected by cytoplasmic processes below the resolution of the light microscope. There is no morphological evidence of apoptosis or necrosis in the region of cell separation. Uranyl and lead stained cross-section of a palate from a 16.5 dpc rat embryo.* **Fig. 3. Loss and fragmentation of basal lamina at a point of failure in the MES.** *The plasma membranes of the epithelial cells on opposite sides of the break are intact and devoid of basal lamina. Fragments of electron dense material interpreted as remnants of the basal lamina (arrow) lie adjacent to region of cell separation. Higher magnification view of the region in Figure 2.* **Fig. 4. Focussed region of early breakdown of the MES in a 3-dimensional reconstruction of a palate from a 16.5 dpc rat embryo.** *A focussed defect interpreted as the initial region of mesenchymal bridging in this fusing palate. The region of breakdown of the MES has a rounded outline and is located nearer to the nasal than the oral margin of the MES. Three-dimensional reconstruction from 1 μ sections in the sagittal plane of a palate from a 16.5 dpc rat embryo. The direction of the anterior end of the palate is indicated by the arrow and the oral surface by the label on the lower edge of the reconstructed image.*

is restricted to defined micro-regions of the seam. In the early development of the cardiac outflow tract, the majority of endothelial cells lining the atrioventricular (AV) canal become activated, but only a small subset undergo an EMT and contribute to the mesenchyme (Wunsch *et al.*, 1994). Unlike the majority of the endothelium of the AV canal, which is retained in subsequent development, the entire epithelium of the MES is removed rapidly, and in rodents completely, to allow palatal fusion and normal development of the maxilla. This rapid and total disappearance of the MES complicates efforts to identify regional differences in the mechanisms responsible for the breakdown of the seam. Any proposition that mechanisms for breakdown of the medial seam may be regionally restricted presupposes accurate information on the detailed structure of the seam and the pattern of breakdown of the seam during the fusion process. The majority of previous studies with a structural emphasis have, with some notable exceptions (Ferguson, 1978, 1981; Knudsen *et al.*, 1985), employed coronal sectioning techniques almost exclusively. Knowledge of the structure of the medial epithelial seam during the process of disintegration is necessary to determine if breakdown of the epithelial sheet occurs uniformly or exhibits a pattern of discrete regions indicative of defined sites where cells could be targeted for distinct fates.

This paper reports the three-dimensional structure of the medial edge seam as revealed in reconstructions with volume rendering software from 1 μ serial epoxy resin sections in the sagittal plane of rat palates fixed *in situ* during the process of fusion. The information obtained from the reconstructions and the results of a study initiated in the light of this information indicates that the epithelial cells of the palatal seam may be capable of generating tension during the fusion event.

Results

Loss of the majority of the cells of the periderm-like layer, melding of the remaining layer of epithelium on each shelf into a bilayered seam and attenuation of the seam as the palatal shelves increase in volume as described in other studies of palatal fusion (Ferguson, 1988; Fitchett and Hay, 1989; Shuler *et al.*, 1991; Shuler, 1995), were observed in palates in this study. Breakdown was not observed before a medial seam had formed along the anterior two thirds of the secondary palate.

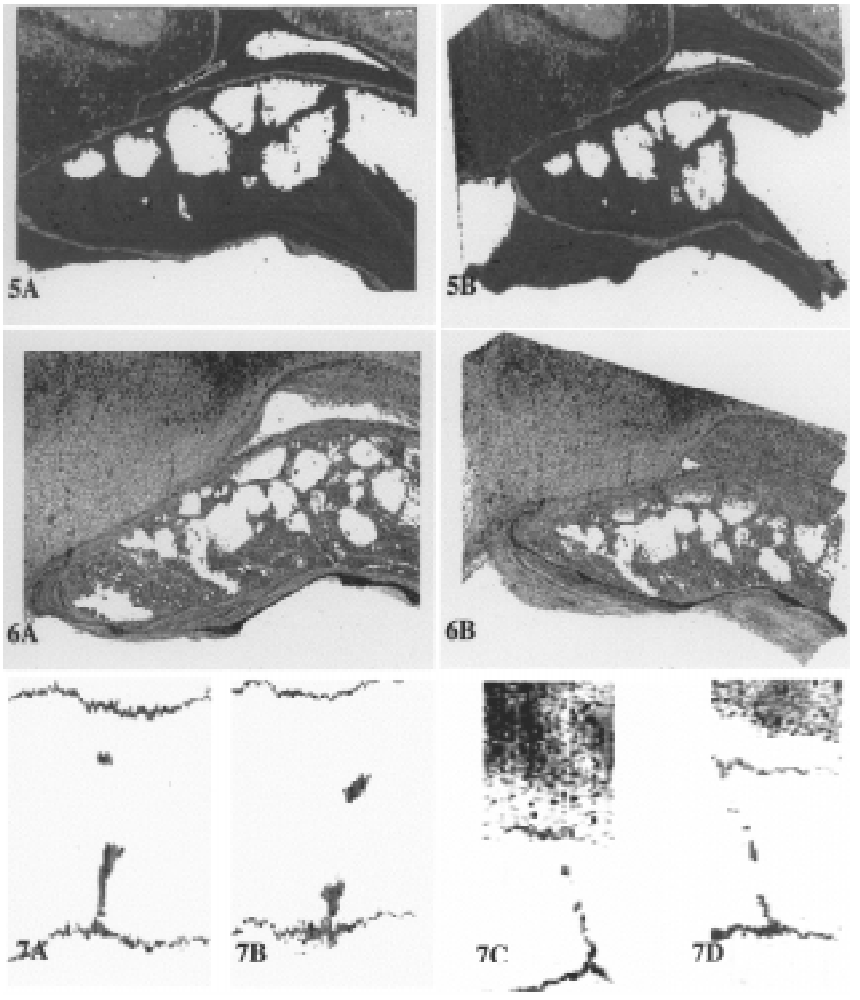

Fig. 5. Multiple discrete regions of breakdown of the MES in a 3-dimensional reconstruction of a palate from a 16.5-17 dpc rat embryo. (A) *Multiple discrete regions of breakdown with rounded outlines lie along the nasal third of the MES. These regions of breakdown increase in size from the anterior end of the palate to the posterior margin included in the specimen and are separated by columns of residual epithelial cells. The more posterior columns have detached from the epithelium forming the inferior lining of the nasal cavity while the MES is still intact along the oral margin and maintained in continuity with epithelium lining the developing oral cavity.* **(B)** *The same reconstruction tilted to show the depth of the reconstruction and the separation of the most posterior column of epithelium from the nasal surface. Three-dimensional reconstruction from 1 μ sections in the sagittal plane of a palate from a 16.5-17 dpc rat embryo. The direction of the anterior end of the palate is indicated by the arrow and the oral surface by the label on the lower edge of the reconstructed image.* **Fig. 6. Preferential breakdown of the nasal margin of the MES by multiple discrete regions. (A)** *Reconstruction showing a similar pattern of breakdown by multiple discrete regions with rounded outlines preferentially located towards the nasal margin of the MES. In this palate, some of the discrete regions of breakdown have merged and extended towards the oral margin in the anterior part of the seam.* **(B)** *The depth of reconstruction and the preferential breakdown along the palatal margin evident in a tilted view of the same reconstructed image as in A. Three-dimensional reconstruction from 1 μ sections in the sagittal plane of a palate from a 16.5-17 dpc rat embryo. The direction of the anterior end of the palate is indicated by the arrow and the oral surface by the label on the lower edge of the reconstructed image.* **Fig. 7. Pseudo-islands of epithelial cells are a consequence of the pattern of breakdown in multiple discrete regions.** *Volume slicing in the coronal plane of the images reconstructed from sagittal sections shows apparent islands are artifacts due to the pattern of breakdown which produces multiple discrete regions separated by columns of epithelial cells.* **(A** *and* **B)** *Coronal slices from the reconstruction in Figure 5.* **(C** *and* **D)** *Coronal slices from the reconstruction in Figure 6. Isolated islands of cells are not evident in the reconstructed images viewed en face. Virtual slices in the coronal plane of volume rendered 3-dimensional reconstructions in Figure 5 and Figure 6 of palates from 16.5-17 dpc rat embryos. The oral surface is indicated by the label on the lower edge of the reconstructed image.*

Initial break in epithelium due to isolated cell separation

The earliest signs of breakdown of the seam were discrete microscopic defects in attenuated regions of the seam (Fig. 1, arrow) without evidence of interconnecting fine intercellular processes detectable in electron micrographs over a sequence of several sections (Fig. 2). The surfaces of the MES cells immediately adjacent to the defects were devoid of basal lamina but profiles interpreted as fragments of basal lamina could be observed outlining defects (Fig. 3, arrow).

Epithelial breakdown occurs preferentially along the nasal margin

The earliest breakdown revealed in 3D reconstructions was a single, focussed, essentially circular defect, located towards the nasal margin of a substantially intact MES (Fig. 4). Consistent with a later stage of breakdown, multiple individual defects were located along the length of the seam closer to the nasal margin with the oral margin of the seam still essentially intact (Figs. 5A,B). Individual defects had a rounded profile and were separated by columns of remaining epithelial cells. The number and extent of the individual defects varied, but the same pattern of breakdown of isolated regions near the nasal edge of the seam with rounded defects separated by epithelial columns was observed in other reconstructions. Defects with irregular outlines were evident where partial breakdown of epithelial columns had occurred (Figs. 6A,B). This pattern of breakdown produced interconnected strands of cells that maintained continuity with the epithelium on the oral and nasal surfaces of the fusing palate until a substantial breakdown of the seam had occurred. Preferential breakdown of the seam along the palatal margin resulted in a more extensive and more protracted connection with the oral epithelium during the fusion process.

Pattern of early breakdown of the seam can produce apparent islands of epithelial cells

Apparent 'islands' of epithelial cells in the mesenchyme would be seen in cross-sections of the fusing palate as a consequence of the

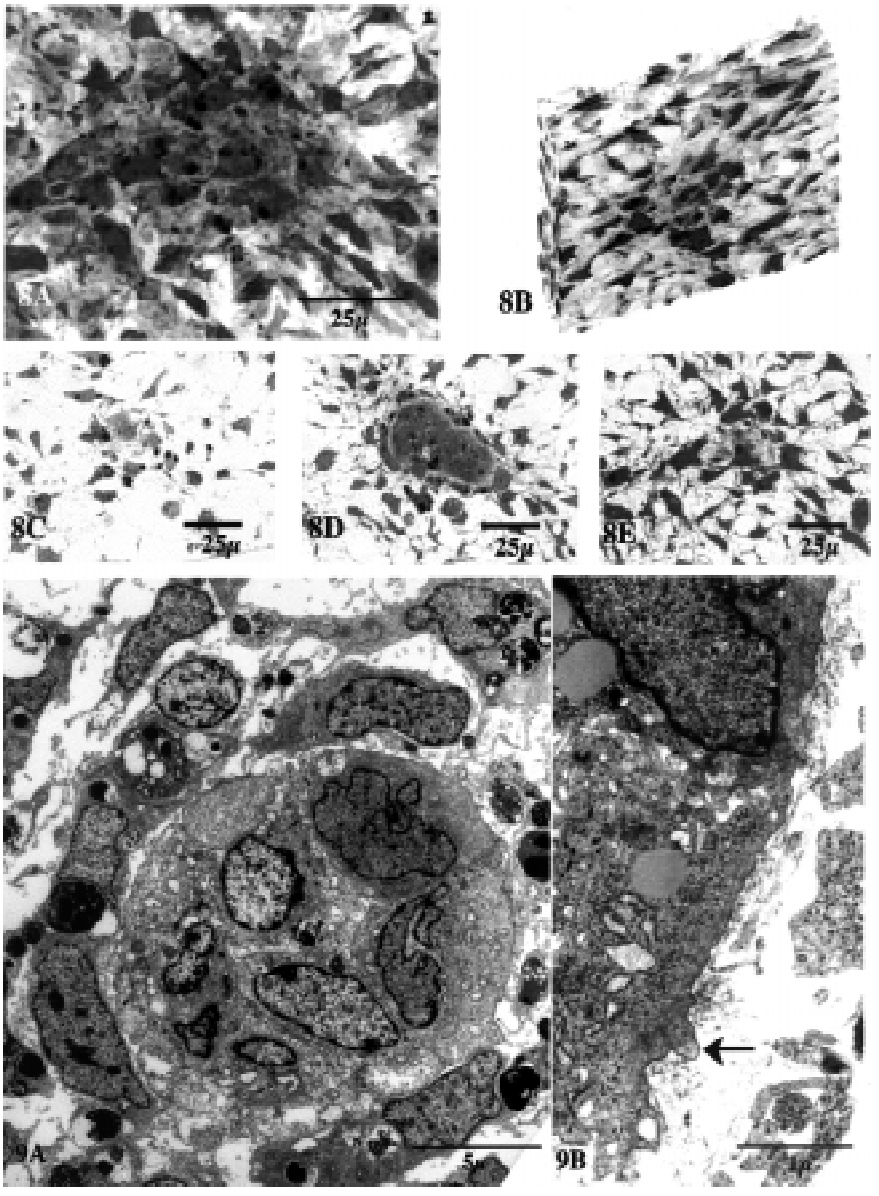

Fig. 8. True islands of epithelial cells formed late in the breakdown of the MES. *Segmentation within the columns of epithelial cells produces isolated islands of epithelial cells unconnected with the oral or nasal surfaces and surrounded by mesenchymal cells. The epithelial cells of the islands in the center are occluded by mesenchymal cells when viewed either en face* **(A)** *or obliquely* **(B)** *as the mesenchymal cells were not erased from the higher power optical images used in this reconstruction. Individual sections from the beginning* **(C)**, *middle* **(D)** *and end* **(E)** *of the series used to generate the reconstruction in A and B show the epithelial structure and the isolation of the island. The epithelial island is surrounded by a halo of cells containing densely stained granules. Three-dimensional reconstructions generated from images of Toluidine blue stained 1 μ resin sections in the sagittal plane without erasure of the mesenchymal cells of a palate from a 17 dpc rat embryo.*

Fig. 9. Detailed structure of true islands of epithelial cells formed late in the breakdown of the MES. (A) *The epithelial islands have a rounded, almost spherical, outline and are surrounded by a halo of cells containing phagocytosed apoptotic debris.* **(B)** *The epithelial cells in the islands retain desmosomes but are devoid of an intact basal lamina and well formed hemi-desmosomes. The cells also exhibit cytoplasmic extensions into the adjacent matrix (arrow). Uranyl and lead stained cross-section of a palate from a 16.5-17 dpc rat embryo.*

breakdown of the sheet of cells forming the MES via multiple discrete defects that generated interconnected strands of cells running obliquely in the plane of the seam. The volume rendering software was used to generate cross sections of the fusing palates as two-dimensional slices of the reconstructed images in the plane at right angles to the plane of the original sections and revealed apparently isolated islands in fusing palates when no discrete islands were evident in the same reconstructions viewed en face. Figures 7A and B show two cross-sectional slices of the reconstructed palate in Figure 5 and Figures 7C and D, two slices of the palate in Figure 6.

True transient islands of epithelial cells generated late in breakdown of the seam

True islands of epithelial cells were found in reconstructed palates when the majority of the seam had broken down. True epithelial islands encased in mesenchymal cells and unconnected with other cells in the seam could be demonstrated when reconstructions were viewed *en face* (Fig. 8A) or tilted (Fig. 8B). Mesenchymal cells surrounding the islands obscured the epithelial cells in the reconstructions but individual sections from either extreme (Figs. 8C and E) and the middle (Fig. 8D) of the set used to reconstruct the island show that the group of epithelial cells in the center is surrounded by mesenchyme and completely separated from other epithelial cells. The true islands of residual MES had rounded profiles and were surrounded by a cuff of closely applied cells containing densely stained granules identified as apoptotic debris in electron micrographs of similar islands of epithelial cells (Fig. 9A). Cells in the isolated islands had well-defined interconnecting desmosomes establishing them as epithelial but had reduced hemi-desmosomes, lacked a defined basal lamina, except for a few residual remnants (Fig. 9B), and showed irregular extensions of cytoplasm into the surrounding matrix (Fig. 9B, arrow).

Retention of continuity with oral epithelium until late in fusion

A consistent feature of the breakdown of the MES was the preferential breakdown along the nasal margin and retention of the

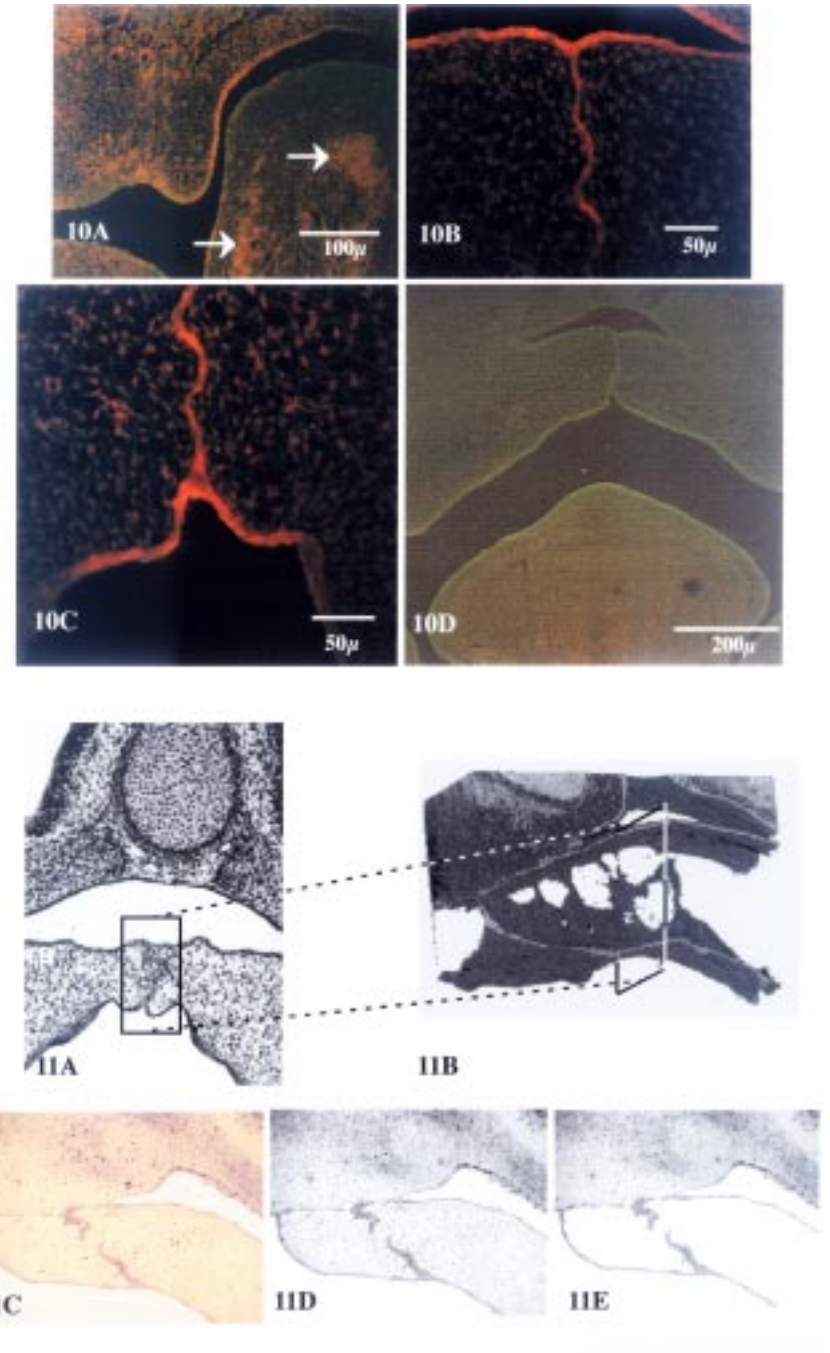

Fig. 10. Immunofluorescent detection of α-smooth muscle actin expression in sections of fusing palates. **(A)** *α-smooth muscle actin expression in the epithelial cells of the palatal shelf in the developing oral cavity (o) prior to elevation of the shelves in a 15.5 dpc rat embryo. The immediately adjacent epithelium on the lateral border of the tongue shows a weak signal while the epithelium on the dorsum of the tongue is negative. Expression of this isotype of actin is also evident in the striated muscle developing in the tongue at this stage (arrows).* **(B)** *The epithelium forming the nasal surface of the developing nasal cavity (n) and the palatal region of the MES following contact of the palatal shelves in the midline in a palate from a16.5 dpc rat embryo shows a positive signal for α-smooth muscle actin expression.* **(C)** *The epithelium on the oral end of the MES and the contiguous epithelium on the oral surface (o) show strong expression for α-smooth muscle actin.* **(D)** *A negative control specimen reacted with isotype-matched irrelevant monoclonal antibody shows no red fluorescent signal in the epithelium in the oral (o) or the nasal (n) regions of the fusing palate. Confocal laser microscopy of paraffin sections of paraformaldehyde fixed palates from rat embryos.* **Fig. 11. Relative orientations of conventional cross-sections and the 3-dimensional reconstructions of the 1 μ sagittal sections of fusing palates (A,B) and the preparation of section images of the 1 μ sagittal sections for reconstruction (C,D,E). (A)** *A conventional cross section through a palate at the stage of formation of the MES. The region outlined connected by dotted lines with the approximately corresponding region in the reconstruction.* **(B)** *An oblique view of a reconstruction from 1 μ sagittal sections showing the approximate location and orientation of the region connected by dotted lines to the cross section in A. The anterior end of the palate is on the left side of the reconstructed image.* **(C)** *A digital image captured from a single 1 μ sagittal section. Only a portion of the seam is contained in each section.* **(D)** *The same captured image converted to grey scale rendering with 256 greys.* **(E)** *The same image processed by erasing the mesenchymal tissue that would occlude the epithelium in the reconstruction. The final processed image was assembled with other final images in a set to generate the reconstructed image with the volume rendering software. The oral surface of the developing palate is indicated and the arrows show the direction of the anterior end of the palate.*

connection with the oral epithelium until late in the fusion process (evident in Figs. 5,6 and 7). Fingers of residual seam remained when complete breakdown of the nasal edge of the seam had occurred. Palates at later stages of fusion (greater than 17.5 dpc) were not sectioned in the sagittal plane and reconstructed, but cross-sections of palates indicated that the oral edge of the seam also disappeared as fusion progressed.

Expression of smooth muscle actin in palatal epithelium before and during palatal fusion

Alpha-smooth muscle actin was strongly expressed in the walls of the developing arteries in the mesenchyme of the palates. When the palatal shelves were lateral to the tongue (at approximately 15 dpc), epithelial cells of the presumptive MEE also showed expression of this marker (Fig. 10A). Immediately adjacent epithelium on the lateral surface of the tongue was unreactive for expressed protein but the skeletal muscle fibers beginning development in the body of the tongue at this stage (Fig. 10, arrows) showed expression of this smooth muscle protein. After the medial seam had formed, α-smooth muscle actin was also expressed in the MES in the midline and in the adjacent epithelium continuous with the MES on the nasal and oral surfaces of the fusing palate (Fig. 10B,C). Control sections reacted with an isotype-matched antibody to an irrelevant antigen were negative (Fig. 10D).

Relationship of the 3D reconstructions to conventional cross sections of the head

The relationship of a conventional cross section of a fusing palate (Fig. 11A) to a volume-rendered reconstruction (Fig. 11B) is illustrated to assist in the interpretation of the reconstructed and rotated images. The regions of mesenchymal confluence appear as holes in the reconstructed images as the mesenchyme must be erased in the individual sections prior to reconstruction to prevent masking of the internal detail in the volume rendered image. The process of preparing the sections for reconstruction is also illustrated in Figure 11 with an original color image of a 1 μ section (Fig. 11C), the same image converted to 256 shades of grey (Fig. 11D) and a final image used for reconstruction following erasure of the mesenchyme (Fig. 11E).

Discussion

Breakdown of the medial seam due to confluence of initially independent foci

The epithelium of the palatal shelves has been shown to undergo changes in gene expression in anticipation of fusion that indicate this epithelium is responding to some form of 'priming' signal (Yano *et al.*, 1996; Gibbins *et al.*, 1999). These changes include downregulation of E-cadherin and syndecan-1 (Sun *et al.*, 1998a) upregulation of cytokeratin K5 and vimentin expression (Gibbins *et al.*, 1999) and, as shown here, expression of α-smooth muscle actin. The changes in gene expression appear to involve a majority of the cells in the MEE and the MES, which suggests that the cells in the MES are similarly primed and therefore equivalent in regard to subsequent processes required for breakdown of the seam. It would be expected, therefore, that dispersion of the MES would occur uniformly and not exhibit any pattern, but this is clearly not the case. Reconstruction of sections of rat palates fixed when the medial epithelial seam is breaking down during palatal fusion revealed an unexpected focal pattern of disappearance of epithelium. Neither a uniform disappearance nor an evenly spread 'peppered shot' pattern of breakdown was observed in any of the reconstructions. Rather, a single defect and then, multiple defects, with rounded outlines offset towards the nasal edge of the seam were seen that could be interpreted as localized initial breaks followed by radial expansion of each defect to intersect and merge with adjacent regions undergoing a similar process. When the observations from the 3D reconstructions and the electron microscopic studies were combined, the pattern of breakdown was more consistent with regional breakdown in the MES rather than with a process affecting the whole MES evenly.

Pattern of breakdown consistent with failure of the medial seam under tension

The pattern of breakdown revealed in this study is consistent with a pattern of failure expected in an elastic sheet under sustained overall tension but with a few discrete points of relative weakness. An obvious mechanism to propose for generating points of relative weakness in a continuous sheet of cells would be individual cell death. Earlier evaluations of palatal fusion considered cell death identified by morphological criteria to be entirely responsible for the breakdown of the seam (Shapiro and Sweney, 1969) but more recent studies using detection of nicked DNA to demonstrate apoptotic cells failed to show evidence of programmed cell death in the MEE prior to fusion or in the intact seam at the time of fusion (Mori *et al.*, 1994; Yano *et al.*, 1996) despite success in locating apoptotic cells later in the remains of the seam in oral and nasal triangles (Mori *et al.*, 1994). A mechanism that would best fit the available evidence is to postulate a reduction in intercellular adhesion in a sheet of epithelial cells under uniform tension generated by contraction of the elements of the sheet. In support of this interpretation, the intercellular adhesion molecule E-cadherin is strongly expressed in the epithelium of the oral cavity (Luning *et al.*, 1994) and this calcium ion-dependent epithelial cell specific adhesion molecule, together with the transmembrane heparan sulfate proteoglycan, syndecan-1, has been shown to be downregulated in the cells of the MES during palatal fusion (Sun *et al.*, 1998). In a strongly interconnected epithelial sheet under uniform tension with intercellular adhesion decreasing progressively due to downregulation of intercellular adhesion molecules, cell separation would occur in regions that reached the threshold level for the maintenance of intercellular attachment. The cells contiguous with these regions, still exerting tension, would retract, producing rounded, essentially circular, defects. In the columns of cells between the larger defects, isolated groups of cells would result from the same mechanism of sustained tension and differentially reduced intercellular adhesion. Continued contraction in the cells of these isolated islands of epithelial cells would then force the islands to adopt a spherical shape. All of these features were observed in specimens fixed during breakdown of the MES.

Expression of smooth muscle actin in epithelial cells of the medial seam

An absolute requirement for a mechanism of failure under active tension as proposed here would be a system to generate tensile force within the sheet of epithelial cells of the medial seam while intercellular adhesion was being reduced. Earlier studies with a polyclonal human autoantibody against smooth muscle obtained from patients with chronic aggressive hepatitis had shown reactivity in oral epithelium in mouse heads at the time of formation of the palate (Krawczyk and Gillon, 1976). The muscle actins, despite being highly conserved at the amino acid level, are expressed as isoforms in distinct tissue-dependent expression patterns that correlate with functional requirements (Vandekerckhove and Weber, 1979). Expression of the α smooth muscle isoform of actin is normally associated with sustained tonic contraction as occurs in vascular smooth muscle cells, while the enteric γ smooth muscle isoform is associated with pulsatile contraction, as in the intestine. Binding of a monoclonal antibody to the α SMA isoform in the epithelial cells in the fusing palate at the precise location and time of seam breakdown is consistent with generation of sustained contractile tension within this epithelium.

Expression of smooth muscle actin in epithelial cells is not a feature of adult tissues, except in the myoepithelial cells in exocrine glands where it is co-expressed with cytokeratin pairs K5/K14 and K7/K17 (Schon *et al.*, 1999). It is interesting to note that the MEE, a simple epithelium expressing cytokeratin K8, has been shown to express cytokeratin K5/6 at the same time as the α-smooth muscle actin expression shown here (Gibbins *et al.*, 1999). Adult cardiac microvascular endothelial cells, unlike aortic endothelial cells, have been shown express α-smooth muscle actin *in situ* in the myocardium and in culture (Ando *et al.*, 1999). While epithelial and endothelial cells do not normally express α-smooth muscle actin, transforming growth factor β can induce expression *in vitro* in lens epithelial cells

(Gordon-Thomson *et al.*, 1998), in retinal pigment epithelium (Kurosaka *et al.*, 1996) and in endothelial cells that differentiate into mesenchymal cells to form the primordia of the cardiac valves and septa (Nakajima *et al.*, 1997). Both TGFβ-3 and antibody to the receptor TGF βII induce upregulation of expression of α-smooth muscle actin in endothelial cells of the embryonic heart (Boyer *et al.*, 1999) that undergo an EMT during cardiac morphogenesis (Nakajima *et al.*, 1994, 1998). TGFβ–3 has been shown to be a specific component of the signal for dispersion of the palatal seam and fusion of the secondary palate (Kaartinen *et al.*, 1995, 1997; Proetzel *et al.*, 1995), but not for fusion of the mandibular processes (Chai *et al.*, 1997). A correspondence between the requirement for TGFβ-3 in fusion of the secondary palate and ability of this growth factor to upregulate α-smooth muscle actin in epithelial cells (Hales *et al.*, 1994; Gordon-Thomson *et al.*, 1998) and in endothelial cells undergoing the changes of an EMT (Nakajima *et al.*, 1994, 1998) suggests that expression of α-SMA may also be a component of the process of EMT in the cells of the MES.

Disruption of the medial seam under tension reconciles apparently incompatible data

The data presented in this paper indicating a contractile role for the epithelial cells of the MES reconciles apparently conflicting results supporting at least three independent mechanisms for the breakdown of the MES, and accommodates many other observations from earlier studies. In rodents, fusion of the palatal processes is rapid with the formation and breakdown of the MES occurring within approximately twelve hours. This is insufficient time for a complete cell cycle. The cells forming the seam, therefore, must be competent to complete the process of breakdown at the time of formation of the seam. Elevation of the palatal shelves prior to fusion has been known for some time to be dependent on the integrity of the epithelium (Bulleit and Zimmerman, 1985), and neither elevation nor fusion requires cell replication (Abbott *et al.*, 1993). This study shows that the cells in the MEE have begun expression of a component of a potentially contractile apparatus prior to contact of the palatal shelves and that expression continues in the MES at the time of breakdown of the seam. Hydration of hyaluronic acid in the mesenchyme has been proposed as responsible for the rapid swelling of the palatal shelves observed at the time of fusion and this swelling would be expected to exert an intrinsic pressure in the palate (Knudsen *et al.*, 1985). The MEE and the MES are structurally integrated by desmosomes and have been shown to express specific cytokeratins not usually found in simple epithelia (Gibbins *et al.*, 1999). A differentially contractile epithelium encasing a swelling palatal shelf could contain and direct an intrinsic pressure and could cause a sudden separation of the epithelium at points where intercellular adhesion has been reduced to a threshold level. Fusion of the palatal shelves also occurs in organ cultured explants of palates of mouse (Carette and Ferguson, 1992) and rat (Fitchett and Hay, 1989) and in chick palates if the developing beak is removed and TGFβ3 added (Sun *et al.*, 1998b), indicating that the generation of tension in the epithelium as proposed must be localized and independent of other supporting structure. We have used organ cultures of rat palates to study other aspects of the fusion process (Gibbins *et al.*, 1999) and α-smooth muscle actin is expressed in the MES in these organ cultures (Tazawa, unpublished observation) so that an experimental analysis of a proposed role for this protein in palatal fusion should be possible.

Epithelial retraction as proposed also accommodates the apparent migration of cells of the MES into the oral epithelium described during fusion (Carette and Ferguson, 1992), where the cells in the MEE fluorescently labeled prior to fusion could remain within the epithelial sheet of the MES as it withdraws preferentially towards the oral surface. Subsequent cell division would dilute the labeled cells to produce a picture of labeled cells in a background of unlabelled epithelium remote from region where the seam had formed.

The formation of true islands relatively late in the fusion process also means that mechanisms in addition to contraction-retraction must occur in the breakdown of the MES. These epithelial islands rapidly disappear in rodents, although some remain in humans to form microcysts in the midline palatal mucosa in newborn infants (Friend *et al.*, 1990). For these islands of epithelia unconnected to either the oral or the nasal epithelia to disappear they must be either destroyed by apoptosis, as suggested by the halos of cells containing phagocytosed apoptotic debris surrounding the islands, or undergo EMT and disperse into the surrounding mesenchyme (Fitchett and Hay, 1989; Shuler *et al.*, 1992).

A more detailed knowledge of the role of changes in gene expression in these cells and the factors controlling these changes could help to elucidate this and other midline fusion events that are so crucial for normal development and are major contributors to a variety of congenital defects when a fusion process fails during embryogenesis.

Materials and Methods

Animals

Animals from an out-bred line of Sprague-Dawley rats bred at the Institute of Dental Research (IDR), Surry Hills, Sydney, Australia were housed in cages containing wood-chip litter and allowed water and commercial pellets *ad libitum*. Timed pregnancies were obtained in animals between 70 and 120 days of age by adding males to cages of females at 5 pm and removing the males at 8 am the following morning with the gestational age of the embryos taken as 0.5 days. Embryos were harvested by surgical delivery from the pregnant dams under Fluothane anaesthesia at intervals of 15, 15.5, 16.0, 16.5, 17.0 and 17.5 days post coitum (dpc), dissected immediately from the embryonic membranes and dispatched by decapitation. A minimum of 5 animals was harvested for each time point.

Dissection and processing

The maxilla containing the region of the palatal seam was dissected from the heads, placed immediately in Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde) in PBS and stored in ice overnight then trimmed, post-fixed in osmium tetroxide, dehydrated and embedded in Spurr's Resin in an orientation for sectioning in the sagittal plane.

Electron microscopy

The block face was trimmed under a dissecting microscope to the region of the medial epithelial seam and ultrathin sections of the seam stained with lead citrate were examined and recorded in a Philips CD10 electron microscope.

3D reconstruction of 1 μ sagittal sections of the fusing palate

Sectioning

Excised maxillae were inspected under a dissecting microscope and the specimens showing contact of the palatal shelves were trimmed and blocked. The block containing the trimmed palate was mounted for

sectioning in the sagittal plane and 1 μ sections cut with sample sections examined every 5 μ for the first appearance of medial edge epithelial cells, then all sections were collected without loss until sectioning had passed completely through the epithelial seam. Slides numbered serially and containing no more than three sections were stained with 1% Toluidine Blue in 1% sodium borate. Contact of the palatal shelves obscured the region of breakdown of the MES both prior to fixation and following processing so that direct observation of the stage of breakdown of the MES was not possible. The combination of an obscured MES, the brief time for formation and breakdown of the MES (16.5-17 dpc), the error inherent in the timing of fertilization (12 h) and the slight but distinct variations in the stage of development of individual embryos within any one litter meant that it was not possible to predict the extent of breakdown of the seam prior to completion of sectioning and image reconstruction. Series of sections from 6 blocks were suitable for reconstruction and three series are illustrated in this paper.

Image digitization and processing

Images were recorded using a 4x objective on a Zeiss Photomicroscope fitted with a rotating stage and a Sony 300D color video camera (Model DXC 93 OP) connected to a Power Macintosh 7100/80 computer. The island illustrated in Figure 8 was recorded with a 25x objective lens. Images were aligned using anatomical features as reference points, captured with ImageGrabber 2.1 software (Fig. 11C) and converted from color to grey scale images with 256 shades of grey (Fig. 11D). The mesenchymal tissue, clearly distinguishable from the epithelium in the individual images, was then erased from each image by hand (with the exception of the island in Fig. 8), so that only epithelial cells were retained in the images used for reconstruction at a final size of 375x215 pixels (Fig. 11E). The image set for the island in Figure 8 was reconstructed at 750x430 pixels without erasure of the mesenchymal cells prior to reconstruction. Image sets were aligned on the middle image of each set with Adobe Photoshop 4.0 (Adobe Systems, Inc) using the transparency and free transfer utilities. The image sets were then reconstructed and manipulated using Voxblast 3.0 (Vaytek, Inc) software on an Optima Pentium computer and the final images printed at 1470 dpi resolution. The 3D reconstructions of the palates have been arranged in an order interpreted as a progressive disintegration of the medial seam.

Immunodetection of smooth muscle actin

Paraffin sections

Heads removed from rat embryos as above were fixed immediately in ice-cold 4% paraformaldehyde in phosphate buffered saline (PBS) at pH 7.4 for 24 h, dehydrated, embedded in paraffin wax, sectioned at 7 μ then sections de-waxed, re-hydrated and reacted for 1 h at room temperature (RT) with a 1:250 dilution of monoclonal anti-α smooth muscle antibody (Sigma A-2547) in 10% fetal calf serum (FCS) in 0.1M Tris pH 7.4 after blocking for 2 h at RT in 20% v/v horse serum in the same buffer. After three washes in buffer the slides were reacted for 1 h at RT with alkaline phosphatase-conjugated rabbit anti-mouse antibody (Dako D0314) at 1:200 in 10% FCS, then Vector Red (Vector Labs SK-5100) at pH 8.2 for 30 min at RT after washing three times in Tris buffer at pH 8.2 to ensure the higher pH for reaction, dehydrated and mounted in non-aqueous mounting medium. Controls were processed identically except that an isotype-matched irrelevant primary antibody (mouse IgG-2a DAKO Z014) replaced the anti-smooth muscle actin antibody.

Laser confocal microscopy

Sections and whole-mount preparations reacted with Vector Red as chromogen were examined and recorded without a counterstain with an Optiscan Laser confocal F900e scanning system (Optiscan, Victoria, Australia) mounted on an Olympus BHX microscope using an argon ion laser with 488 nm excitation filter in 3 channel mode detecting the Vector Red signal with a 590 nm barrier filter in channel 2. As no counterstain was used, the gain of the green channel was set to display the mesenchymal tissue.

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