Cell distribution during mouse secondary palate closure
II. Mesenchymal cells

LINDA L. BRINKLEY*

Department of Anatomy and Cell Biology, The University of Michigan Medical School, 4722 Medical Science II Building, Ann Arbor, Michigan 48109, USA

AND FRED L. BOOKSTEIN

Center for Human Growth and Development, The University of Michigan, Ann Arbor, Michigan 48109, USA

SUMMARY

The patterns of distribution of both total mesenchymal cells and the ratios of [3H]thymidine-labelled to total cells were mapped during secondary palatal shelf reorientation in vivo and in vitro. Smoothed spatial averaging, a computer-assisted method which takes into account the positions of all cells across an entire histological section of the shelf, was employed. Changes in shelf cross-sectional area and cell size were also measured. Three shelf regions, anterior and posterior presumptive hard and presumptive soft palate, were studied at developmental stages which were 30, 24 and 18 h prior to expected in vivo elevation, after in vivo reorientation and during the course of in vitro reorientation. Region-specific patterns of cell distribution change with shelf reorientation. These changes were observable within 6 h. Increases in cell number by cell division may enhance some high local cell densities, but cannot account for decreases in cell density. Increase in cell size is not a factor in decreasing cell density, nor is cell death. Displacement of cells by expansion of the extracellular matrix may be involved.

INTRODUCTION

During morphogenesis of the secondary palate, the palatal shelves reorient from a vertical position on either side of the tongue to a horizontal position above it. This remodelling derives from the nature and activities of the basic structural components: mesenchymal cells, extracellular matrix, and surrounding epithelium. Monitoring the activities of these components during shelf movement can provide information that is basic to determining their roles in the reorientation process. We have previously shown that pronounced, often transient, local increases in epithelial cell density, not attributable to cell division or perimeter changes, accompany shelf reorientation in vivo and in vitro (Brinkley, 1984). Mesenchymal cells have been observed to disperse just prior to shelf reorientation (Kochhar & Johnson, 1965; Sweeney & Shapiro, 1970; Ferguson, 1977; Brinkley,

* Author to whom reprint requests should be addressed.

Key words: palate closure, mesenchymal cells, cell patterning, mouse, secondary palate.
1980), yet there have been no systematic quantitative studies of their patterns of distribution during the course of palate development.

For studying patterns of cell distribution across an entire cross section of a shelf, it is not sufficient to compare selected sites in a section by ordinary statistical means. A method for identifying patterns must take into account the positions of all cells. The present study exploits a method of smoothed spatial averaging to visualize general trends and to highlight systematic variation of cell density and labelling ratios across the section.

We studied the patterns of distribution of both total mesenchymal cells and the ratios of $[^3H]$thymidine-labelled to total cells at several times during shelf reorientation; this enabled us to separate the roles of cell division and cell displacement in generating the observed patterns. Because cell displacements may be transient, it is an ineffective design to observe palatal shelves at experimentally fixed times before and after in vivo reorientation. A more complete picture of the timing and magnitude of cell displacement associated with shelf movements derives from a combination of in vivo and in vitro observation.

We have developed an in vitro system that supports palatal shelf reorientation with good tissue vitality. This system allows us to follow events associated with shelf movement beginning up to 36h prior to expected in vivo palate closure (Brinkley, Basehoar, Branch & Avery, 1975; Lewis, Thibault, Pratt & Brinkley, 1980; Brinkley & Vickerman, 1982). Analysing both in vivo and in vitro data by smoothed spatial averaging, we find that shelf reorientation is accompanied by distinctive mesenchymal cell displacements, displacements that cannot be accounted for by either increase in cell size or overall shelf expansion. In some shelf regions, but not in others, thymidine-labelling patterns are similar to the patterns of cell distribution. Thus, while increase in cell number by cell division may intensify the pattern of mesenchymal cell distribution, it cannot be totally responsible for generating that pattern.

METHODS

Animals

Random-bred CD-1 mice were housed with a dark cycle from 8.00 p.m. to 6.30 a.m., and fed Purina Mouse chow and water ad libitum. Fertilization was assumed to occur between midnight and 2.00 a.m. of the morning a vaginal plug was found. Timed pregnant dams were sacrificed by cervical dislocation at four different developmental times that span the course of secondary palate formation. Shelf reorientation and initial adhesion normally occurs by gestational day 14-5 in these mice. The foetuses were removed under sterile conditions. They were then assigned to one of four developmental stages based on their crown–rump length (CRL) and a morphological rating (MR) based on the developmental state of fore- and hindlimbs, ears, eyelids, and hair follicles (Walker & Crain, 1960). Four groups were defined as follows: (1) CRL 9.0–9.5 mm and MR 3–4; (2) CRL 10.0–10.5 mm and MR 5–6; (3) CRL 11.0–11.5 mm and MR 7–8; (4) CRL 12.5–13.0 mm and MR 12–14. We will refer to these groups by their MR scores. These groups were aged approximately 30, 24, 18 and 0h prior to the time of expected in vivo palate closure.

Immediately after the foetuses were obtained, they were partially dissected as described in Brinkley et al. (1975), to remove the tongue and brain while leaving the maxillary–mandibular
relationship intact. Some heads were fixed immediately in phosphate-buffered formalin to serve as Time 0 (T0) unincubated controls; the rest were hung in a gassed, circulating culture system as previously described in Lewis et al. (1980). Presumptive anterior and posterior hard and soft palatal shelf regions were identified using the anatomical landmarks of Diewert (1978) that have been previously described and illustrated (Brinkley, 1984).

**Culture, labelling and histological techniques**

The culture medium and conditions and the [3H]thymidine-labelling method were described in detail elsewhere (Brinkley, 1984) and will only be briefly reviewed here. Specimens were incubated for 6, 12 or 18 h in the culture system containing BGJb (GIBCO) medium with 10% foetal bovine serum (KC Biologicals) supplemented to a final concentration of 8 mM-glutamine and containing 50 µg garamycin (Schering Corporation). The medium was kept at 34°C, constantly circulated and gassed with 95% oxygen, 5% CO2 using silicon copolymer hollow-fibre devices to maintain a pO2 of 600±30 Torr. Methyl [3H]thymidine (specific activity 40–60 µCi mmole⁻¹, New England Nuclear) was present in the culture medium to a final concentration of 20 µCi ml⁻¹ for the entire 6 h incubation period and was added in the same concentration for the last 6 h of both 12 and 18 h incubation periods.

Following incubation, specimens were rinsed in three changes of medium with no isotope, fixed in phosphate-buffered formalin, dehydrated through an ethanol series and embedded in glycol methacrylate. Serial cross sections (3 µm) were taken through the anterior, posterior and soft palatal shelf region. Contiguous pairs of sections from each of the regions were separated and placed on two different gelatin-coated slides. The sections of one set were used for autoradiography. These were dipped in NTB-2 photographic emulsion (Kodak), placed in light-tight boxes, and exposed for two weeks at 0°C. The slides were then developed using D-19 developer (Kodak) and stained with toluidine blue. All labelled nuclei were densely covered by blackened silver grains. Background labelling was approximately 5 grains cm⁻². Sections of the other set were stained with toluidine blue, with Feulgen's (1 h in 5N-HCl, followed by 2 h in Schiff's reagent), or with both. These sections yielded the measurements of nuclear and cell size described below.

**Data collection**

**Nuclear and cell size**

Using measurements of nuclear cross-sectional area and total cross-sectional area of nucleated cells, we investigated whether changes in cell distribution were related to changes in cell size along the longitudinal (rostral-caudal) axis of the shelves, that is between anterior, posterior and soft palatal shelf regions. Measurements were made by either a Quantimet Image Analyzer (Cambridge–Imanco) or a Zeiss IBAS. For each shelf region, oral, medial and nasal areas of the mesenchymal compartment were selected together with others taken arbitrarily. We measured between 5 and 20 cells or nuclei per sampling site from the shelves of four to six individual specimens.

**Cross-sectional area**

The boundary of each stained section was traced with the aid of a microscope and drawing tube (the superior-nasal and lateral-oral delimiters of the shelf were described in Brinkley, 1984). The shelves' mesenchymal compartment was considered to be bordered by the basal lamina. The contours of these tracings were digitized using a Summagraphics digitizing tablet interfaced with a Tektronix 4054 computer running a simple micrograph measurement program. Cross-sectional area was calculated in µm² and will be referred to as ‘area’ elsewhere in this paper.

**Smoothed spatial averages**

For each combination of stage, shelf region and incubation time, a minimum of four and a maximum of eight shelves were studied, each from a different individual and the individuals
taken from at least three different litters. Using a Leitz Orthoplan microscope, photomicrographs were taken of the developed autoradiographs of complete frontal sections of each shelf. A montage of photographic prints was produced at ×900. All labelled and unlabelled nuclei were located and digitized together with a string of points representing the shelf's mesenchymal perimeter.

These data were transmitted to the University of Michigan's Amdahl 5860 computer for analysis by FORTRAN programs coded by one of us (FLB), and used previously by Brinkley (1980) and Connelly & Bookstein (1983). The analytic method is reviewed briefly here and described in detail in the Appendix. Morphometric processing begins by placing a grid of square smoothing boxes over the image of each section. Unlabelled and labelled cells are counted within each grid box. During the development of the method several sections were selected at random and the total number of cells as well as cells per grid box were calculated manually. These counts were in complete agreement with those done by the computer.

Counts of unlabelled and labelled cells are averaged over adjacent squares in a manner which takes account of the location of the mesenchymal perimeter (see Appendix). From these smoothed cell counts are computed labelling ratios (labelled cells divided by total cells) and densities (labelled, unlabelled and total cells) per 600 \( \mu \text{m}^2 \) inside the section. These values, the cell densities and the labelling ratio, are linearly interpolated between adjacent smoothing box centres and converted to contour maps at convenient spacing over the entire interior of the section outline. Fig. 1 presents an example of a photomontage of a palatal shelf section and its contour map. Both the general pattern and individual variations over specimens are easily discernible in these plots.

RESULTS

Cell and nuclear size

After areas of Feulgen-stained nuclei and toluidine-blue-stained nucleated cells were measured, we calculated the percentage of the cells' cross-sectional area occupied by nucleus. Because mesenchymal cell nuclei tend to be oval or round, a cross-sectional area may be used as a reasonable measure of size. As the mesenchymal cell bodies are more irregular in shape, their cross-sectional areas in any given plane of section are more variable.

The cross-sectional areas of nuclei and cell bodies from all three mesenchymal sampling sites of the three palatal shelf regions were measured for MR 5 specimens at 0 and 18h incubation and for MR7 specimens after 18h incubation. Nuclear areas tended to be about 90% of measurable cell area, with no statistically significant dependency upon age or incubation time. Furthermore, nuclear area itself showed no differences across sampling sites within a shelf region. Hence, the three nuclear sampling sites of each set were grouped for comparisons across stage, incubation time and shelf region. The results are shown in Table 1.

Nuclear size is similar in all three shelf regions at MR 5 and 7. The nuclei of MR 3 shelves are larger in the posterior than in the anterior or soft regions. This is

---

Fig. 1. A photomontage of a histological section (A), and the contour map (B) of its pattern of cell distribution. Numbers are cells per 600 \( \mu \text{m}^2 \). The smoothed spatial averaging algorithm used to draw the contour map is described in the Appendix. Single lines delineate the boundaries between integers, a double line indicates integer multiples of five times the basic spacing. Local interior points which are above or below the value of the surrounding field are represented by small diamond-shaped contours, e.g. 4-6, 7-1. \( mx \), maxillary; \( n \), nasal; \( m \), medial; \( o \), oral.
### Table 1. Changes in nuclear cross-sectional area in vivo and in vitro

<table>
<thead>
<tr>
<th>Incubation Stage</th>
<th>Time (h)</th>
<th>Anterior (μm²×10⁶)</th>
<th>Posterior (μm²×10⁶)</th>
<th>Soft (μm²×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR 3</td>
<td>0</td>
<td>19.3 ± 8.2</td>
<td>24.1 ± 12.7*</td>
<td>21.2 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>(n = 238)</td>
<td>(n = 93)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>26.3 ± 10.1*</td>
<td>16.5 ± 7.5</td>
<td>17.3 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>(n = 108)</td>
<td>(n = 132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR 5</td>
<td>0</td>
<td>20.4 ± 7.3</td>
<td>21.7 ± 10.0</td>
<td>22.2 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>(n = 120)</td>
<td>(n = 207)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>29.7 ± 9.1*</td>
<td>32.4 ± 10.7</td>
<td>32.3 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>(n = 170)</td>
<td>(n = 180)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR 7</td>
<td>0</td>
<td>25.2 ± 12.7</td>
<td>25.7 ± 8.4</td>
<td>26.9 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>(n = 36 )</td>
<td>(n = 165)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>41.9 ± 18.0*</td>
<td>36.8 ± 17.7</td>
<td>38.2 ± 14.2</td>
</tr>
<tr>
<td></td>
<td>(n = 128)</td>
<td>(n = 191)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR 12</td>
<td>0</td>
<td>36.7 ± 11.7</td>
<td>40.7 ± 9.2</td>
<td>28.1 ± 10.9*</td>
</tr>
<tr>
<td></td>
<td>(n = 121)</td>
<td>(n = 60)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Changes in size seen after 18 h incubation in all three shelf regions are significant, \( P \leq 0.05 \).

* Different from other two regions of the same stage and incubation time, \( P \leq 0.05 \) by analysis of variance.

also the case at MR 12. During the 30 h \textit{in vivo}, from MR 3 to 12, nuclear area doubles in the anterior and posterior, but not in the soft shelf region (Table 1). Most of the size increase occurs during the 18 h between MR 7 and 12. Regional variation is also seen after 18 h incubation. In all three pre-elevation developmental stages nuclei of the anterior shelf increase in size with incubation. In the posterior and soft regions, MR 5 and 7 nuclei increase in size with incubation, while those of MR 3 decrease in size.

\section*{Shelf area}

Over the course of \textit{in vivo} shelf elevation, MR 3 to 12 (Table 2), all three shelf regions increase their area by about 70%. There are regional differences in shelf expansion during \textit{in vitro} reorientation. In the anterior, only MR 3 shelves expanded concomitant with elevation. MR 5 and 7 anterior shelves reoriented without change in surface area. By contrast, in all three pre-elevation stages the posterior shelves increased in area during \textit{in vitro} elevation. MR 3 shelves expanded by 60% and those of MR 5 and 7 by about 18%. Some expansion of the soft shelf region was also observed, but only that seen in MR 3 specimens after 6 h incubation was statistically significant. The increases noted in MR 5 shelves after 6 h and in MR 7 shelves after 12 and 18 h were not statistically significant because of sample size, but are consistent with the other trends.
Spatial patterns of cell density

Examining the contour maps of the smoothed spatial averages of individual shelves allows us to correlate changes in patterns of spatial distribution of mesenchymal cells with changes in shelf morphology. Figs 2–4 display contour maps of actual anterior, posterior and soft palatal shelf regions typifying the patterns of each group. The representative maps we have chosen correspond to the shortest incubation times after which complete shelf reorientation occurred in any of the three stages. The patterns observed after other incubation times are discussed below, but are not illustrated.

Anterior (Fig. 2)

In vivo. All three pre-elevation stages present a similar spatial pattern of cell distribution: cell density is lowest in the superior-nasal region and increases in a medial–oral direction. Stages MR 3, 5, and 7 differed in these patterns principally in the compactness of populated areas and in the degree of cell density in those areas. Some areas of MR 3 shelves reached densities of about 9 cells per 600 μm², whereas those of MR 5 and 7 attained highs of 14 cells per 600 μm². From MR 3 to 7, the regions of highest cell density occurred increasingly toward the medial region. At MR 12 after in vivo elevation, cell density had dropped well below that seen at earlier stages. Only isolated medial and medial-oral concentrations of cells

Table 2. Changes in shelf cross-sectional area during palate closure in vivo and in vitro

<table>
<thead>
<tr>
<th>Stage</th>
<th>Incubation time (h)</th>
<th>(n)</th>
<th>Cross-sectional area of shelf regions (μm²×10³) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td>MR 3</td>
<td>0</td>
<td>(7)</td>
<td>70.1 ± 14.6*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(9)</td>
<td>89.7 ± 21.3(e)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>(7)</td>
<td>91.3 ± 7.6(e)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>(4)</td>
<td>87.1 ± 18.2(ea)</td>
</tr>
<tr>
<td>MR 5</td>
<td>0</td>
<td>(8)</td>
<td>79.3 ± 13.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(11)</td>
<td>84.4 ± 16.7(e)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>(5)</td>
<td>85.2 ± 8.7(ea)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>(11)</td>
<td>82.2 ± 15.1(ea)</td>
</tr>
<tr>
<td>MR 7</td>
<td>0</td>
<td>(9)</td>
<td>105.4 ± 36.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>(5)</td>
<td>102.4 ± 27.9(e)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>(8)</td>
<td>109.7 ± 21.3(ea)</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>(6)</td>
<td>106.3 ± 24.6(ea)</td>
</tr>
<tr>
<td>MR ≥12</td>
<td>0</td>
<td>(6)</td>
<td>116.7 ± 14.4(ea)</td>
</tr>
</tbody>
</table>

* Different from other incubation times of the same region and stage. P ≤ 0.05 by analysis of variance. pe, partially elevated (medial portion of shelf is less vertically oriented than prior to incubation, but is not yet horizontal); e, elevated (medial portion of shelf is horizontal); ea, elevated and adhered.
were still observed, and their densities were considerably less than those seen prior to shelf elevation.

**In vitro.** Anterior shelves of MR 3, 5, and 7 specimens all reoriented by 6 h *in vitro*. MR 3 shelves exhibited large medial cell accumulations ranging from 8 to 12 cells per 600 μm², with slightly lower cell populations in the area adjacent to the maxillary process. Small superior-nasal and lateral-oral concentrations were also apparent. In MR 5 shelves, cell distribution in the medial portion of the shelves was essentially unchanged from that seen prior to incubation. However, superior-nasal areas of accumulation, which were more extensive than in MR 3 shelves, were now seen. In the medial and nasal areas of MR 7 anterior shelves, only small peripheral concentrations of increased density (10 cells per 600 μm²) were found whereas pronounced lateral-oral concentrations often remained. A definite decrease in cell density was observed at the origin of the shelf from the maxillary process.

These patterns of cell distribution, characterizing each of the three stages after 6 h incubation, remained essentially unchanged after 12 or 18 h incubation. Although the locations of the most densely populated areas were essentially unchanged after 12 h incubation, their extent had decreased. By 18 h *in vitro* MR 3 and 7 shelves showed cell density similar to that seen after *in vivo* elevation: large central areas of 4 to 6 cells per 600 μm² with persisting medial and oral peripheral zones of 6 to 8 cells per 600 μm². MR 5 shelves showed similar spatial patterns of cell distribution but the cell densities of both the central and peripheral zones were twice those of their MR 3 and 7 counterparts.

**Posterior** (Fig. 3)

**In vivo.** Again the patterns of distribution were similar at all three pre-elevation stages. A large proportion of the MR 3 shelves was populated with a reasonably homogeneous density of 3 to 4 cells per 600 μm², which increased to 6 to 8 cells per 600 μm² at the two later stages. All three stages also showed oral concentrations of slightly greater density. No other consistent cell concentrations were observed. After *in vivo* elevation and initial adhesion at MR 12, a density of 4 cells per 600 μm² was again observed over most of the shelves. Pockets of 5 to 6 cells per 600 μm² were retained at the medial and oral peripheries and occasionally at the origin of the shelf from the maxillary process.
In vitro. After 6 h *in vitro*, most MR 3 and 5 shelves were only partially elevated, that is the medial portions of the shelves were not yet horizontal, while most MR 7 shelves were fully elevated. The only increase in cell density in the MR 3 shelves was at the oral periphery; MR 5 shelves displayed concentrations both orally and medially. Fully reoriented MR 7 shelves displayed similar patterns of distribution, but, in the medial and oral areas, cell densities were somewhat lower than those seen at MR 5. 12 h *in vitro* resulted in reorientation of MR 3 and 5 shelves and initial adhesion of MR 7 shelves to each other. Both MR 3 and 5 shelves displayed some reduction in the size of the oral and medial zones of cell density, but little change in numerical cell density of these regions. The MR 7 shelves that had achieved adhesion greatly resembled MR 12 *in vivo* elevated shelves with overall cell densities of 2 to 4 cells per 600 \( \mu m^2 \) and medial and oral pockets of twice that concentration.

Shelf adhesion occurred in MR 3 and 5 specimens after 18 h *in vitro*. The observed patterns of cell distribution were also similar to those seen after *in vivo* elevation and adhesion, except that the medial area of cell density remained at about 6 cells per 600 \( \mu m^2 \), instead of dropping to the 4 cells per 600 \( \mu m^2 \) seen *in vivo*. MR 7 shelves showed no additional changes in cell distribution or density after 18 h *in vitro*.

Soft (Fig. 4)

In vivo. MR 3 shelves had a relatively homogeneous cell density. The maxillary one-half to two-thirds of the shelf was populated by 5 to 6 cells per 600 \( \mu m^2 \); the medial portion had a slightly lower cell density. Shelves of MR 5 specimens had greater cell density: superior-nasal, medial, oral and maxillary concentrations ranged from 10 to 14 cells per 600 \( \mu m^2 \). By MR 7, the medial one-half to two-thirds of the shelf was again populated with the same cell density as seen 12 h earlier at MR 3. The remaining shelf, that closest to the maxillary process, had higher densities of 6 to 9 cells per 600 \( \mu m^2 \). MR 12 shelves, elevated *in vivo*, had patterns of distribution and numerical densities similar to those seen at MR 7.

In vitro. Shelves of all three ages attained only partial elevation after both 6 and 12 h *in vitro*. All three showed no change in pattern of cell distribution from that seen at the outset of the incubation period. Numerical cell densities also remained

---

Fig. 3. Representative contour maps of posterior shelves before and after reorientation *in vivo* and *in vitro*. Shelves of MR 7 and some of those of MR 5, as illustrated here, had reoriented so that the medial aspect of the shelf was horizontal after 6 h incubation. Whereas those of MR 3 and some of MR 5 required 12 h incubation to reorient fully. During the course of reorientation, cell density decreases in the central regions and areas of cell accumulation at the mid-oral periphery become apparent. Transient increases in cell density are also observed at the superior-nasal and medial peripheries. *mx*, maxillary; *n*, nasal; *o*, oral; *pe*, partially elevated; *e*, elevated; *ea*, elevated and adhered.
MR3

Oh

6h incubation

500 urn

Cells/600 urn\^2

1-2

3-4

5-6

7-8

9-10

≥11

MR7

MR5

MR3

MR2

mix

0 h

6 h incubation

500 \( \mu \text{m} \)
similar, with the MR 5 shelves still displaying higher cell densities and more pronounced areas of increased density than the other two stages.

By 18 h \textit{in vitro} both MR 5 and 7 shelves had elevated and sometimes adhered, while only a minority of MR 3 had done so. In the latter case, the shelves had a more even distribution of cell density with occasional medial and oral pockets of 7 to 9 cells per 600 \(\mu m^2\). Even after elevation, MR 5 shelves continued to show the higher cell densities observed prior to incubation, but displayed a distribution pattern similar to that seen in MR 7 shelves after shorter incubation times. The medial one-third to one-half of the shelf had lower cell density compared to the remainder. After 18 h of incubation, MR 7 shelves displayed a uniform cell density of 3 to 4 cells per 600 \(\mu m^2\) across most of the shelf with occasional pockets of 5 to 6 cells per 600 \(\mu m^2\).

\textit{Relationship of labelling ratio to cell distribution patterns}

\textit{Anterior}

For all three pre-elevation stages patterns of distributions of LRs after 6 h incubation were similar to those of cells. The central shelf region had 20 to 25 \% of its cells labelled; this ratio increased to 30 to 40 \% at the medial and oral peripheries. MR 5 shelves showed an additional area of increased LR at the superior nasal surface, the same place at which they often displayed increased cell density. No change in the pattern or level of labelling was observed in MR 3 or 7 shelves after either 12 or 18 h incubation. However, in MR 5 shelves labelling in the medial periphery increased to 40 to 65 \% after 12 h incubation, then dropped back to 30 to 35 \% by 18 h \textit{in vitro}.

\textit{Posterior}

LR patterns followed patterns of cell density for all ages and incubation periods. After 6 h incubation, 20 to 25 \% of the cells in the central portions of the shelves were labelled, with the medial and mid- to lateral-oral peripheral shelf regions displaying 35 to 45 \% labelling. Although the spatial patterns of labelling ratio remained similar after 12 h \textit{in vitro}, MR 3 shelves had greater proportions of cells labelled in one or both areas of highest cell density, ranging as high as 80 \% in some cases. No changes in LR patterns were observed in MR 5 and 7 shelves. By 18 h \textit{in vitro} shelves of all three ages also displayed similar patterns. LR in the

Fig. 4. Representative contour maps of soft shelf before and after reorientation \textit{in vivo} and \textit{in vitro}. After 18 h incubation all MR 5 and MR 7 shelves had reoriented, while only some of those of MR 3 had. All examples shown here are reoriented in order to facilitate comparisons of patterns. Especially at MR 3 and MR 7 the maxillary halves of the shelves tend to have higher cell densities than the medial halves. After reorientation a nasal-to-oral gradient of increasing cell density is often observed at MR 3, while MR 5 shelves usually display a decreasing distribution of cells from maxillary-to-medial aspects. Whereas those of MR 7 show a rather uniform drop in cell distribution following reorientation. \textit{mx}, maxillary; \textit{n}, nasal; \textit{m}, medial; \textit{o}, oral; \textit{pe}, partially elevated; \textit{e}, elevated; \textit{ea}, elevated and adhered.
central portion of the shelves now dropped to 15 to 20% with medial and oral cell concentrations remaining at 30 to 45% labelled cells.

**Soft**

MR3 cells showed pronounced LR patterns after 6h incubation. Labelling ratios increased steadily in waves emanating from the maxillary origin of the shelf and progressed medially. Labelling increased from 10% at the maxillary origin to 60% at the medial surface. This trend did not coincide with the cell distribution patterns observed in MR3 shelves after various incubation periods; it is, in fact, the exact inverse of the patterns of cell distribution observed later at MR7. Similar waves of increasing LR, ranging from 20 to 45%, were observed in MR5 and 7 shelves, but only the medial one-half of the shelves was involved. The maxillary portion of the shelves maintained reasonably consistent LRs of 10 to 15%.

Similar patterns of LRs were observed at all three ages after both 12 and 18h incubation. The ranges of labelling ratio declined in both MR5 and 7 shelves after 18h incubation; areas of greatest cell density reached an LR of only 30%. In contrast, MR3 shelves showed increased LRs after 12h incubation, ranging from 20% at the maxillary origin of the shelf to, in some cases, as high as 75% at the medial edge. After 18h in vitro the low had decreased to 10%, the high to 50%.

**DISCUSSION**

The present study provides data on the patterns of mesenchymal cell distribution and proliferation during the course of shelf reorientation which are basic to studies that seek to understand how the mesenchymal compartment may be influenced by genetic or environmental perturbations leading to cleft palate. The statistical method of smoothed spatial averaging permits objective identification of patterns of mesenchymal cell distribution and labelling ratio across the entire cross-sectional profile of a palatal shelf. At this stage in the development of the method statistical comparisons between groups of contour maps are not possible. Commonalities among groups of contour maps are to be discerned by the researcher. It is generally true of the patterns of cell distribution in all shelf regions that over time, *in vivo* and *in vitro*, there are discrete areas of initial increase in cell density. Subsequently, cell density decreases in the central or maxillary areas, but peripheral patches of higher cell density remain. The areas of change are the same areas that are responsible for the distinct regional patterns of cell distribution associated with shelf reorientation (Figs 2–4).

Our shortest time frame for observing pattern changes *in vivo* or *in vitro* was 6h; actual changes may have occurred more rapidly. Because of this time span, the degree to which some factors are operating in determining cell distribution may be limited.

Increase in cell density could be accomplished by increasing cell number by cell division or by movement of cells into an area. Whereas density could be decreased by an increase in cell size, cell death or displacement of cells away from a given
Mesenchymal cell patterns and palate closure

area. Present results suggest that localized cell division may play a role, but certainly not a major one, in generating the patterns of distribution observed to be associated with shelf reorientation. Increase in cell number may enhance some high local cell densities observed in the anterior and posterior, but do not appear to do so in soft regions. In the anterior and posterior, but not in the soft, the patterns of distribution of labelling ratios coincide to some degree with patterns of cell distribution. The two simplest means of decreasing density, increase in cell size and cell death, also do not appear to be contributing to pattern changes. No cell death was observed, and, as judged by nuclear size, cell size seems not to have increased a great deal in this time span. The greatest nuclear size increase observed in any region after an 18 h incubation period was 67%; most regions showed an increase of less than 50% (Table 1). If a gradual, even increase is assumed, this would mean nuclei might have enlarged by 22% or less in a 6 h period. Such an increase would result in only a small drop in net cell density.

Present results suggest the patterns of cell distribution are altered by cells moving from one place to another: decreasing cell density in one region while increasing it in another. This could be accomplished by active change or passive displacement. Active contractility and migration of mesenchymal cells (Zimmerman, Wee, Clark & Venkatasubramanian, 1980; Zimmerman & Wee, 1984) and expansion of the extracellular matrix, thereby passively displacing mesenchymal cells (Ferguson, 1978; Brinkley, 1980; Brinkley & Morris-Wiman, 1984) have both been suggested to play important roles in shelf reorientation. The present findings provide circumstantial support for the idea that extracellular matrix expansion is involved. Although it is possible that active mesenchymal cell movement may take place in specific local regions of the epithelial-mesenchymal interface, active cell migration does not seem likely to be a principal factor in accounting for the observed major cell displacements. Fisher & Solursh (1979) have shown that cells within a mesenchyme are normally not locomotory, but rather the mesenchyme expands in three dimensions as a unit. Hydration of the extracellular matrix is probably involved. Prior to reorientation palatal shelves are rich in hyaluronate (Pratt, Goggins, Wilk & King, 1973). These molecules occupy large domains and mesh with themselves and other extracellular molecules to form networks which can sequester water and are responsive to changes in ionic strength and tissue compression (Laurent, 1970; Comper & Laurent, 1978). In this way they serve to expand the extracellular matrix; for a review, see Toole (1982).

Expansion of a hyaluronate-rich matrix could result in: (1) overall or local shelf expansion and large cell displacements, thus remodelling the contours of the shelf; or (2) smaller or undetectable cell displacements and an increase in overall turgor of the shelf but with no significant expansion or change in shelf shape. The patterns of cell distribution seen during reorientation of posterior and soft shelf regions are consistent with the first suggestion. Both shelf expansion and remodelling of the contours were noted. In the case of the posterior region, cell displacements seem to be from the central region toward the nasal, medial and oral surfaces. Expansion of the soft region appears to emanate from a more medial area of the
shelf. Such expansion would encounter little resistance in the medial direction and thus might result in a medial ballooning of the shelf and decreased cell density in that portion. In contrast, matrix expansion in the maxillary direction would encounter the resistance of developing ossification centres, cartilaginous elements and the denser, more differentiated mesenchymal tissue surrounding them, causing cells to accumulate. Anterior shelf patterns of cell distribution are more consistent with the second suggestion. Both shelf expansion and cell displacements are limited over the 12 h from the MR 3 to MR 7 as well as during their \textit{in vitro} reorientation. The shape of the shelf also remains reasonably constant.

Other studies also support these suggestions. The pattern of hyaluronate distribution has been observed to be first condensed and then dispersed in the central areas of the posterior and soft shelf regions concomitant with shelf expansion and reorientation \textit{in vivo} (Brinkley & Morris-Wiman, 1984). Previous studies have also shown that perturbation of GAGs affects these two regions preferentially. Treatment with chlorcyclizine, which degrades GAGs, including hyaluronate, into smaller pieces with lower charge densities, reduces shelf surface area and blocks reorientation in the posterior and soft regions, but not in the anterior (Brinkley & Vickerman, 1982).

In addition to the tissue characteristics and possible patterning of hyaluronate distribution, other local factors within the posterior and soft shelf regions may affect cell displacements by imposing directionality on the expansion. Overlying the nasal and mid- to lateral-oral regions of peripheral mesenchymal cell accumulation, pronounced increases in epithelial cell packing occur concomitant with shelf reorientation (Brinkley, 1984). Local conditions at these regions of the epithelial-mesenchymal interface may encourage or permit cells to move or be drawn preferentially toward or along the basal lamina from relatively nearby. One function of these peripheral regions of the shelf's perimeter, which have increased mesenchymal and epithelial cell packing, may be to direct the shelf's remodelling by applying local compression. This would also serve to buttress against expansion of the extracellular matrix in those directions and so channel all remodelling in a nasal–medial direction. In contrast, the anterior is considerably more cellular in composition than the remainder, and has an additional rigidity derived from its intense cell packing. Mere peripheral epithelial constrictions may induce it to move. Some amount of extracellular matrix expansion emanating from the maxillary region may also be involved, but, judging from our data, it is clearly not the crucial factor.

This work was supported by NIH-NIDR grants DE02774 and 5K0400104 to LLB.

\textbf{APPENDIX}

\textit{Smoothed spatial averaging of cell distributions over palatal sections}

A palatal shelf section is presented to this algorithm as an outline form (the mesenchymal perimeter) together with two lists of cell locations (labelled and
unlabelled). The intention of the spatial averaging is to compromise between two different aspects of undesirable biometric error in cell-distribution data. The fewer the cells involved in a particular count, density estimate or ratio, the more variable are those counts and derived estimates from section to section. For instance, under conditions of Poisson noise, a count of about a hundred cells is $\sqrt{10}$ times as precise as a count of ten cells. There is also an opposite sort of error: with larger numbers of cells there is a larger area over which they must be counted; this can obliterate true differences of distribution.

The algorithm we used here attempts to balance these competing sources of error by adapting the notion of blurring (a familiar idea in medical image processing) to the discrete cell-location data. In effect, we assign each cell to many different locations: not only to the position at which it was in fact observed, but also to eight other positions bordering the observed location in a square, tic-tac-toe fashion. The counts attributed to all locations, both the observed and the artificial, now are fractional: we count $3/8$ cell at the location actually observed, $3/32$ cell at the neighbouring locations north, east, south and west, and $1/16$ cell at neighbouring locations northeast, northwest, southeast and southwest. These fractions total $32/32$, corresponding to the one cell observed, so that our net cell totals are not altered even though we have split each cell into nine different fragments. The patterns of these fractions can be set down in a matrix $W_{ij'}$ whose row and column indices run from $-1$ to $+1$, as follows:

$$
\begin{array}{ccc}
  i' & -1 & 0 & 1 \\
-1 & 1/16 & 3/32 & 1/16 \\
 0 & 3/32 & 3/8 & 3/32 \\
 1 & 1/16 & 3/32 & 1/16 \\
\end{array}
$$

(Its subscripts run from $-1$ to $+1$ because we use them as offsets of square box subscripts $i$ and $j$ in the formulae to come.) The centre of the matrix is given more value (weight), $3/8$, than the surrounding positions and thus the matrix may be termed a 'moderately centre-loaded' weighting field.

We do not wish to count cells where there is no tissue. Therefore, our procedure has to be adjusted whenever any of the 'remote locations' fall outside the boundary of the shelf. To manage this exclusion, the algorithm counts cells accurately in small squares, and then adjusts the totals for the squares in light of the geometric outline of the shelf. The fractional counts $3/8, 3/32, 1/16$ thereby become weights applied to the actual counts encountered in neighbouring accumulation boxes. The adjustment for area consists in multiplying each of the weights $W$ by a further factor $A$, the area fraction available for each neighbouring square. Thus if the upper left box falls wholly outside the mesenchymal boundary, its weight of $1/16$ is replaced by 0, so that the resulting weighted average is corrected by a division by $15/16$. By this means, once again, the total number of cells is preserved in the smoothing.
The computation is carried out at a fixed spatial scale. A 19-by-19 grid of square smoothing boxes is centred over the section. For each smoothing box, the fraction of its area inside the shelf outline, ranging from 0-0 to 1-0, is computed and the information stored in the appropriate entry of a 19-by-19 matrix $A$. Labelled and unlabelled cells are counted separately within each smoothing box and also stored in 19-by-19 matrices of counts of number labelled ($NL$) and number unlabelled ($NU$).

Smoothed data fields $TOTAL$ and $RATIO$ are computed, representing estimated cell density and labelling ratio (labelled cells divided by total cells) in the vicinity of each smoothing box. Let $W_{ij^*}$ be the moderately centre-loaded weighting field mentioned before. Correcting for boxes partially outside the section, the smoothed counts are:

\[
LABELLED_{ij} = \frac{\sum_{i'=-1}^{1} \sum_{j'=-1}^{1} W_{i'j'} NL_{i+i',j+j'}}{\sum_{i'=-1}^{1} \sum_{j'=-1}^{1} W_{i'j'} A_{i+i',j+j'}}
\]

and

\[
TOTAL_{ij} = \frac{\sum_{i'=-1}^{1} \sum_{j'=-1}^{1} W_{i'j'} [NL_{i+i',j+j'} + NU_{i+i',j+j'}]}{\sum_{i'=-1}^{1} \sum_{j'=-1}^{1} W_{i'j'} A_{i+i',j+j'}}
\]

In the denominator for these expressions you see the correction of net weight to account for neighbours outside the form. These boxes, of course, have $NL = NU = 0$, so that they do not contribute to the numerator of the estimate either.

The smoothed labelling ratio is then computed as:

\[
RATIO_{ij} = \frac{LABELLED_{ij}}{TOTAL_{ij}}.
\]

These formulae are applied not only to squares inside the mesenchymal shelf boundary but also to squares straddling the boundary and even to squares lying slightly outside the boundary (and thus having no cell counts of their own at all). Both of these special cases correspond in the formulae to factor $A$ multiplying the central weight $W_{00} = 3/8$ and lowering it partly or all the way to zero. These boxes are assigned cell counts that draw upon values for whichever of their neighbouring squares overlap the interior. The satisfactory extrapolation of contours is thereby made possible right out to the boundary of the section.
In this way we produced a field of cell counts corresponding to a specialized sort of blurring of the original locations so that fractional cell counts are never attributed to areas outside the original mesenchymal boundary.

It remains to display this pattern of smoothed counts and ratios as if it represents a continuous spatial field of values instead of as a square meshwork. To this end, the values of TOTAL and RATIO are linearly interpolated along all edges connecting adjacent smoothing box centres vertically and horizontally. The points where these interpolants cut integer multiples of 0.05 (for RATIO) or 1.0 (for TOTAL) are noted. For either quantity, TOTAL or RATIO, these marked points are converted to a contour map over the interior of the section outline. As a smoothing box is scanned, any particular integer multiple may be encountered zero, two, or four times in the circuit of the boundary of that box. Multiples encountered twice are connected with a straight segment (doubled in the drawing for multiples of five times the basic spacing). Whenever a particular integer multiple is encountered four times, a saddle point of the interpolated surface is indicated. The saddle is resolved arbitrarily by pairing the first encounter clockwise from the southwest box corner with the first encounter counterclockwise. The contours are truncated at the section boundary and the cut levels they represent printed there. Interior points at which the field is higher or lower than at all four of the adjacent box centres, and which thus are likely to be at the centre of small diamond-shaped contours, have the field value printed. The reader may locate all of these diagrammatic features here and there in Fig. 1.

REFERENCES


(Accepted 13 March 1986)