The effects of chlorcyclizine-induced alterations of glycosaminoglycans on mouse palatal shelf elevation in vivo and in vitro

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SUMMARY

To define whether glycosaminoglycans play a role in palatal shelf movement, we studied the morphology and elevation behaviour of chlorcyclizine-treated mouse palatal shelves. Chlorcyclizine treatment was used because this agent enhances degradation of the palatal glycosaminoglycans, hyaluronate and chondroitin sulphates, with little or no effect on their synthesis. Use of in vitro and in vivo experiments enabled us to control the complicating effects of other factors on elevation.

Drug-administration resulted in a reduction in shelf size, as measured by cross-sectional surface area, in the posterior two-thirds of the palatal shelf. In vivo shelf reorientation was also inhibited. When elevation behaviour was observed in vitro, pronounced regional variation was noted. The anterior third of the shelf was able to reorient, the posterior two-thirds was not. This region also showed distinct histological changes as compared to controls. Mesenchymal cells were rounded with prominent nuclei and nucleoli and were more densely packed than in controls.

These results suggest that for at least the posterior two-thirds of the palatal shelf, the intrinsic reorientation ability may in large part be linked to the acquisition of a specific temporal and spatial distribution of hyaluronate and possibly other matrix components.

INTRODUCTION

The secondary palate is formed from two wedge-shaped masses of tissue, the palatal shelves, that initially grow down vertically from the maxillary processes to occupy positions on either side of the tongue. Subsequently the shelves undergo a reorientation or elevation to a horizontal position above the tongue where they meet in the midline, adhere and fuse to one another to form the secondary palate. The palatal shelves are not passively displaced, but are generally acknowledged to play a direct and active role in their reorientation (Walker & Fraser, 1956). The internal 'shelf force' underlying their movement has been the subject of considerable speculation and observation, but little direct experimentation.

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We have confirmed the idea that a reorienting force clearly resides within the shelf itself by demonstrating that palatal shelves in severed heads with the brain and tongue removed are able to elevate (Brinkley, Basehoar, Branch & Avery, 1975). Further, parts of shelves that have been lesioned transversely are able to reorient (Brinkley & Vickerman, 1979). Thus it seems that the shelves themselves are in some way responsible for generating the internal force that results in a form change.

Examination of the shelves' composition may help to generate hypotheses about the factors necessary for reorientation. The palatal shelves are composed of a core of mesenchymal cells surrounded by extracellular matrix and covered by an epithelium. The extracellular matrix of shelves just prior to reorientation has been shown to contain collagen, fibronectin and two glycosaminoglycans (GAGs): hyaluronate (HA) and chondroitin sulphates. HA is the predominant molecule present at this time, comprising about 60% of the extracellular GAGs (Pratt, Goggins, Wilk & King, 1973).

The presence, molecular state and interactions of HA with other extracellular molecules have been correlated with morphogenetic changes in a variety of embryonic systems (Toole, Underhill, Mikuni-Takagaki & Orkin, 1980). A number of unique physicochemical attributes of this macromolecule could provide the extracellular matrix with morphogenetically important properties. HA molecules occupy large domains and can enmesh 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). Glycosaminoglycans, particularly HA, are thought to play a role in palatal shelf movement, but little direct evidence for this suggestion exists (Lazzaro, 1940; Walker, 1961; Wilk, King & Pratt, 1978; Ferguson, 1978; Brinkley, 1980).

Study of the effects of disruptive agents can often clarify basic mechanisms. One such example is the potential effect of chlorcyclizine (CHLR) on palatal shelf morphology and behaviour. CHLR is an antihistaminic benzhydrylpiperazine compound which has been shown to enhance the degradation of HA and chondroitin sulphates with little effect on their synthesis. Both chlorcyclizine administered by gavage in vivo and exposure of isolated palatal shelves to its metabolite norchlorcyclizine (NORCHLR) in vitro causes the molecules to be degraded into smaller-molecular-weight pieces with lower charge densities (Wilk, et al. 1978). In vivo treatment of pregnant mice with this compound results in both cleft palate and mandibular reductions in their offspring (King, 1963; King, Weaver & Narrod; 1965; Wilk et al. 1978). Cleft palate could be a secondary result of the mandibular reduction, since a reduced mandible could cause the tongue to become wedged between the palatal shelves, thus providing a physical barrier to shelf elevation (Diewert & Pratt, 1979). Another possibility is that CHLR treatment is a direct cause of clefting through its effect on the GAGs within the shelves themselves (Wilk et al. 1978).
Chlorcyclizine and palate elevation in vivo and in vitro

To distinguish between these effects both in vivo and in vitro techniques must be used. We have previously shown that normal palatal shelves are able to elevate, adhere and fuse in our in vitro system (Brinkley et al. 1975; Lewis, Thibault, Pratt & Brinkley, 1980). We can use this culture system to determine directly the effects of CHLR treatment on the shelves without the complicating effects of the tongue. Comparison of in vivo and in vitro behaviour of the palatal shelves of CHLR-treated embryos should contribute to a better understanding of the possible role glycosaminoglycans play in shelf elevation.

MATERIALS AND METHODS

Animals. A random-bred CD-1 mouse strain was used for all experiments. Animals were maintained in quarters with controlled light cycles and fed Purina mouse chow and water ad libitum. Fertilization was assumed to occur between midnight and 2 a.m. of the morning the vaginal plug was found.

Chlorcyclizine administration. Sequential doses (250 mg/kg) of chlorcyclizine hydrochloride (CHLR) (Burroughs-Wellcome) were administered to pregnant mice via intragastric tube on gestational days 10.5, 11.5 and 12.5, as these three days were found to be the sensitive period for CHLR induction of cleft palate. Each dose was delivered in a total volume of 0.5 ml sterile water. Controls were given intragastric feedings of equal volumes of sterile water.
Animals were sacrificed on days 13-5, 14-25, 14-5 or 15-25, times which were approximately 1, 2 and 3 days after the final dose of CHLR. Palate closure in these mice normally occurs by day 14-5.

The drug treatment resulted in an 85% survival rate of the treated mice. Half of these mice had litters with 100% survival, a quarter had litters with a 10% resorption incidence and a quarter had litters with about 50% resorption. In all litters, the drug dosage given resulted in 100% cleft palate in offspring of treated mothers despite the fact that the treatment ended two days prior to palate closure.

Preparation of explants and culture technique. Control and CHLR-treated pregnant females were killed by cervical dislocation at day 13-5 or 14-25. Foetuses were removed under sterile conditions, measured for crown–rump length and assigned a morphological rating of the developmental state of fore and hindlimbs, ears, eyelids and hair follicles using the system of Walker & Crain (1960). Inter- and intralitter variability was minimized by excluding any individuals that differed from the group in either of these two parameters.

The brain and tongue were removed and then a small vent was cut in the floor of the posterior oral cavity to allow for better circulation of culture medium. The mandible was left in place, as described previously (Brinkley et al. 1975). Several of the heads were then fixed in phosphate-buffered formalin to serve as time zero, unincubated controls. The remainder were hung in a gassed, circulating culture chamber that we developed (Lewis et al. 1980).

Specimens with unelevated palatal shelves were incubated in either standard medium or standard medium containing norchlorcyclizine (NORCHLR), the metabolite of CHLR. In vivo CHLR was administered by gavage, as the animal converts the compound to its metabolite NORCHLR. For in vitro studies NORCHLR itself was used. This procedure provided for comparison of the elevation behaviour of the palatal shelves under recovery conditions provided by standard medium, or in the continued presence of the active drug.

Standard medium consisted of BGJb medium (Gibco) with 10% foetal bovine serum (KC Biologicals), supplemented with 8 mM glutamine and containing 50 μg/ml gentamicin (Schering Corp.). When norchlorcyclizine (NORCHLR) was added to the medium, a final concentration of 10 μg/ml was used. Cultures containing NORCHLR were always kept in the dark because of the compound’s light-sensitivity.

In all cultures the medium was constantly circulated, gassed with 95% O₂, 5% CO₂ using silicone copolymer hollow fibre devices, and maintained at 34 °C. Specific details of the procedure are described in Brinkley et al. (1975) and Lewis et al. (1980). Specimens were incubated 18 h, a time span previously shown to permit elevation and adhesion in day-13 palates (Brinkley, Basehoar & Avery, 1978).

Histological technique. Specimens to be examined histologically were fixed for 24 h in phosphate-buffered formalin, dehydrated through an alcohol series and
**Table 1. Chlorcyclizine effects on growth and morphology**

<table>
<thead>
<tr>
<th>Gestational age and treatment</th>
<th>Crown-rump length (mm)*</th>
<th>Morphological rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 13.5</td>
<td>Control (n = 12)</td>
<td>10.4±0.2</td>
</tr>
<tr>
<td></td>
<td>CHLR (n = 37)</td>
<td>9.8±0.3†</td>
</tr>
<tr>
<td>Day 14.25</td>
<td>Control (n = 24)</td>
<td>12.4±0.5</td>
</tr>
<tr>
<td></td>
<td>CHLR (n = 20)</td>
<td>10.6±0.5†</td>
</tr>
<tr>
<td>Day 14.5</td>
<td>Control (n = 59)</td>
<td>12.7±0.6</td>
</tr>
<tr>
<td></td>
<td>CHLR (n = 26)</td>
<td>11.1±0.1†</td>
</tr>
<tr>
<td>Day 15.25</td>
<td>Control (n = 27)</td>
<td>14.6±0.4</td>
</tr>
<tr>
<td></td>
<td>CHLR (n = 11)</td>
<td>14.2±0.4</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation.
† Different from age-matched control values, P < 0.005.

embedded in glycol methacrylate. Sections, 3 μm thick, were cut and stained with toluidine blue.

**Evaluation techniques**

**Palatal Shelf Index (PSI).** The degree of elevation of palatal shelves was assessed using the palatal shelf index (PSI). The PSI is a five-level scale to assess deviation of the palatal shelves from the horizontal (Wee, Wolfson & Zimmerman, 1976). A grid with five lines from vertical (1) to horizontal (5) was placed in the ocular of a dissecting microscope. Then, with the grid in place, freehand razor-blade sections were made transversely through the palate of each fixed specimen at four levels along the rostral-caudal axis. The slice was exposed to a drop of toluidine blue to enhance contrast. Then the shelf position was assessed by placing the horizontal line in register with the inferior surface of the nasal septum or cranial base and recording which of the five angular lines most closely corresponded to the position of the nasal surface of each shelf. This grid was also used to assess stained, mounted sections of palates.

**Cross-Sectional Area (CSA) measurements.** Sections of anterior, mid and posterior presumptive hard palate and presumptive soft palate were selected from each specimen. With the aid of a microscope equipped with a drawing tube, both shelves were traced. Anatomical landmarks visible in each region which were used to assign a given section to a palate region were those of Diewert (1978). The landmarks were: (1) anterior hard palate: nasal septum, vomeronasal organs, nasal cavity and palatal shelves; (2) mid hard palate: nasal septum posterior to vomeronasal organ, nasal cavity and palatal shelves; (3) posterior hard palate: posterior aspects of nasal cavity or immediately posterior to it, maxillary and mandibular toothbuds; (4) soft palate: cranial structures, the cranial base and palatal shelves. The shelf area measured for each region described is shown in Fig. 1.
Table 2. *Elevation behaviour of control and CHLR-treated palatal shelves* in vitro

<table>
<thead>
<tr>
<th>Gestational age and treatment</th>
<th>Palatal shelf index of shelf regions (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td>Day 13·5</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Time zero (n = 14)</td>
</tr>
<tr>
<td>CHLR</td>
<td>Cultured, standard medium (n = 20)</td>
</tr>
<tr>
<td></td>
<td>Time zero (n = 12)</td>
</tr>
<tr>
<td></td>
<td>Cultured, standard medium (n = 16)</td>
</tr>
<tr>
<td></td>
<td>Cultured, NORCHLR medium (n = 10)</td>
</tr>
<tr>
<td>Day 14·25</td>
<td>Time zero (n = 8)</td>
</tr>
<tr>
<td>Controls</td>
<td>Time zero (n = 14)</td>
</tr>
<tr>
<td>CHLR</td>
<td>Cultured, standard medium (n = 14)</td>
</tr>
<tr>
<td></td>
<td>Cultured, NORCHLR medium (n = 8)</td>
</tr>
</tbody>
</table>

* Different from age-matched, time zero, controls, \( P < 0·01 \).
† Different from age-matched, time zero, CHLRs, \( P < 0·01 \).
‡ Different from day-13·5 time zero, CHLRs, \( P < 0·05 \).
Table 3. Changes in shelf cross-sectional area over the time of in vivo palate closure

<table>
<thead>
<tr>
<th>Gestational age and treatment</th>
<th>Cross-sectional area of shelf regions (μm² × 10³) (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td>Day 13:5</td>
<td>Controls (n = 8)</td>
</tr>
<tr>
<td></td>
<td>CHLR (n = 12)</td>
</tr>
<tr>
<td>Day 14:25</td>
<td>Controls (n = 8)</td>
</tr>
<tr>
<td></td>
<td>CHLR (n = 14)</td>
</tr>
<tr>
<td>Day 14:5</td>
<td>Controls (n = 10)</td>
</tr>
<tr>
<td>Day 15:25</td>
<td>CHLR (n = 12)</td>
</tr>
</tbody>
</table>

* Different from age-matched controls, P < 0.05.
† Different from day-13:5 treatment-matched groups, P < 0.01.
‡ Different from day-13:5 and day 14:25 treatment-matched groups, P < 0.004.
§ Different from day-14:5 controls, P < 0.005.
The anterior and mid regions each represent about 20% of overall shelf length, while the posterior and soft regions comprise about 30% each (Brinkley & Vickerman, 1979).

**Sampling and statistical analysis.** Because of possible individual variability in all evaluation parameters samples were taken by randomly selecting two or more foetuses from each of 4–12 litters. Crown–rump length, PSI and CSA data were analysed by a univariate analysis of variance. Only differences found to be significant with \( P < 0.05 \) were accepted.

**RESULTS**

**In vivo**

**Growth and morphology.** Beginning on day 13-5, and continuing to day 14-5, the day of usual palate closure, CHLR-treated individuals were smaller as judged by crown–rump length and were less well developed as evidenced by lower morphological ratings (Table 1). By day 15-25, almost three days after the last drug treatment, CHLR-treated foetuses had apparently recovered and attained the same crown–rump length and morphological rating as control individuals. However, at all ages examined, CHLR-treated foetuses had both cleft palate and reduced mandibles.

**Palatal shelf elevation.** On day 13-5 no difference in shelf position was found between control and CHLR palates (Table 2). By day 14-25 palates of control foetuses had closed whereas only the anterior, and to a lesser degree the posterior of the CHLR-treated foetuses showed changes in shelf positions. The anterior had essentially reoriented, but despite some change in the position of the posterior shelves, it was insufficient to achieve elevation of this portion.

**Changes in shelf CSA.** Cross-sectional area of control and CHLR-treated shelves was measured at several times over the span of expected palate closure (Table 3). On day 13-5, one day prior to palate closure, a comparison of CSA values of control and CHLR shelves shows that for this parameter the posterior and soft palate regions are significantly affected by CHLR treatment. These two regions were reduced to 74% and 71% of control values, respectively.

After palate elevation, day 14-25, controls showed significant CSA increases in anterior (80%), mid (98%) and soft (58%) palate regions over that seen at day 13-5. But no significant change in the posterior region was seen. By the time adhesion had taken place (day 14-5), the anterior and mid shelf showed an additional increase in CSA.

During the same period, day 13-5 to day 14-25, only the anterior region of CHLR-treated palates had elevated (Table 2). The CSA values of control and CHLR shelves were similar in the anterior, but the CHLR values were significantly lower than those of controls in the remainder of the palate. CHLR-treated mid palate achieved only 66% of control surface area, whereas posterior
Table 4. In vitro changes in cross-sectional area of day-13.5 palatal shelves

<table>
<thead>
<tr>
<th>In vivo treatment and culture conditions</th>
<th>Cross-sectional area of shelf regions ($\mu$m$^2 \times 10^3$) (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>Time zero ($n = 8$)</td>
<td>61.7 ± 10.9</td>
</tr>
<tr>
<td>Cultured, standard medium ($n = 12$)</td>
<td>84.9 ± 16.3</td>
</tr>
<tr>
<td>CHLR</td>
<td></td>
</tr>
<tr>
<td>Time zero ($n = 12$)</td>
<td>59.4 ± 22.7</td>
</tr>
<tr>
<td>Cultured, standard medium ($n = 22$)</td>
<td>75.0 ± 22.5</td>
</tr>
<tr>
<td>Cultured, NORCHLR medium ($n = 20$)</td>
<td>66.1 ± 20.8</td>
</tr>
</tbody>
</table>

* Different from the treatment-matched time zero group, $P < 0.02$.
† Different from controls, time zero, $P < 0.02$.
‡ Different from controls, cultured in standard medium, $P < 0.03$.
§ Different from CHLR, cultured in standard medium, $P < 0.01$. 
Table 5. In vitro changes in cross-sectional area of CHLR-treated day-14-25 palatal shelves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cross-sectional area of shelf regions (µm²×10⁵) (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td>Time zero (n = 14)</td>
<td>97.7±35.4</td>
</tr>
<tr>
<td>Cultured, standard medium (n = 14)</td>
<td>119.3±30.6</td>
</tr>
</tbody>
</table>

* Different from time zero, P < 0.003.

and soft palate had CSAs which were 81% and 70% of control values, respectively.

Since no in vivo elevation of CHLR shelves had taken place by day 14-25, an additional day of in vivo development was allowed to determine what changes if any, would occur in shelf position and area. By day 15-25, three days after the last in vivo CHLR treatment, 5 of 12 individuals (42%) had shelves which were elevated, but all had large gaps between them. No statistically significant difference in CSA was found between elevated and une elevated day-15-25 shelves. Thus, for purposes of comparison with controls, the mean of all values was used.

No significant increase in CSA over that seen at day 14-25 was observed for the anterior, but the remaining shelf regions had increased. The cross-sectional areas achieved by the anterior, mid and soft palate regions of day-15-25 CHLR shelves were approximately those of day-14-25 controls, that had all elevated. However, the posterior region of day-15-25 CHLR shelves attained areas greater than those seen in either day-14-25 or -14-5 controls.

In vitro

Palatal shelf elevation. Palates of day-13-5 control specimens were able to elevate along the entire length of the shelf, and adhere in the posterior two-thirds of the shelf in 67% of the cases. When day-13-5 CHLR individuals were incubated in standard medium, elevation was achieved in the anterior and to some degree the mid regions, but no change in shelf position was observed in either the posterior or soft region. CHLR specimens cultured in medium containing NORCHLR exhibited the same regional pattern of shelf-elevation behaviour (Table 2).

Day-14-25 CHLR-treated palates were cultured to determine whether the posterior and soft regions of the shelves would now be able to elevate in vitro. By this age these regions had achieved CSAs similar to day-13-5 controls. CHLR-treated palates were indeed able to elevate in both regions whether cultured in standard medium or NORCHLR medium (Table 2).
Table 6. Summary of changes in shelf Palatal Shelf Index and cross-sectional area during palate closure

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shelf region (Palatal Shelf Index/cross-sectional area)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>+/+/+ +/+ +</td>
</tr>
<tr>
<td>CHLR</td>
<td>+/+/+ +/+</td>
</tr>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>+/+ + +/0</td>
</tr>
<tr>
<td>CHLR, standard medium</td>
<td>+/+ +/0</td>
</tr>
<tr>
<td>CHLR, NORCHLOR medium</td>
<td>+/+ +/0</td>
</tr>
</tbody>
</table>

PSI: No change = 0; significant increase to final PSI of: 2.25-3.0 = +; 3.0-3.75 = ++; 3.75-4.25 = +++; 4.25-5.0 = +++++.

CSA: No change = 0; significant increase of: 0-25 % = +; 25-50 % = ++; 50-75 % = +++; 75-100 % = +++++.
Fig. 2. Day-13-5 control (A and B) and CHLR-treated (C, D and E) shelves before and after incubation. (A) Time-zero controls, prior to incubation. (B) After culture, shelves have elevated and adhered. Areas of lowered cell density are obvious (→). (C) Unincubated, time-zero CHLR-treated shelves. (D) Incubated in standard medium. Note enlargement of vascular spaces (*). (E) Incubated in NORCHLR medium. Vascular spaces are not enlarged.

Changes in shelf CSA. Only the mid region of control day-13-5 palates increased significantly in surface area during the in vitro culture period (Table 4). CHLR specimens cultured in standard medium showed significant CSA increases in the mid, posterior and soft palate regions. Despite this increase, neither the mid nor soft palate of those specimens achieved CSA values attained by the same regions of cultured controls. The CSA increase found in the posterior brought the CHLR shelves to a size similar to that of uncultured control shelves that showed no CSA change during their culture period.

CHLR specimens cultured in NORCHLR-containing medium increased in
Chlorcyclizine and palate elevation in vivo and in vitro

Fig. 3. Comparison of mesenchymal cells of day-13.5 control (A and B), and CHLR-treated (C, D and E) shelves. (A) Mesenchymal cells of time zero, unincubated shelves. (B) After in vitro elevation and adhesion. (C) Mesenchymal cells of time zero, unincubated CHLR shelves. (D) After incubation in standard medium. (E) Cells of shelves incubated in NORCHLR medium. Although densely packed, several mitotic figures were observed in each field (→).

surface area only in the posterior, achieving a size similar to controls, but smaller than that of CHLR shelves cultured in standard medium.

When day-14-25 CHLR-treated palates were cultured in standard medium, the CSA of these shelves increased significantly in the posterior regions (Table 5), and achieved a CSA similar to the posterior region of day-14-25 controls by the end of the culture period (Table 3).

Summary of PSI and CSA changes in vivo and in vitro. Table 6 contains a tabulation of the significant changes observed in both elevation behaviour and shelf surface area in vivo and in vitro. In vivo, shelf elevation and expansion in cross-sectional area of controls occurred concurrently in the anterior, mid and
Fig. 4. Comparison of day-14-25 control (A) and CHLR-treated (B and C) shelves. (A) Control shelves. (B) Unincubated CHLR shelves. (C) CHLR shelves elevated in vitro. o, Areas of osteogenesis; t, tooth germ.

soft shelf regions, but elevation without expansion was seen in the posterior shelf. In vitro, only mid shelf of control specimens expanded during elevation. Thus, in normal palates, elevation of the anterior, posterior and soft palate regions does not appear to be linked to overall shelf expansion as measured by changes in CSA.

CHLR treatment effectively blocked shelf elevation in vivo in the mid and soft palate and retarded it to a large degree in the posterior region. However, increases in CSA of these regions was observed. In vitro behaviour of CHLR shelves reinforced the findings from controls that changes in overall shelf CSA are not necessarily linked to elevation in the anterior, posterior and soft palate regions. This may not, however, be the case for the mid-shelf region. It showed a pronounced divergence of behaviour in vitro as compared to in vivo. In vitro mid region was able both to elevate and to expand to some degree in standard medium, whereas in vivo only CSA expansion is observed.

The presence of NORCHLR in the culture medium permitted the same degree of shelf reorientation seen in standard medium, but effectively blocked or severely reduced CSA expansion in all shelf regions.

It is clear from both in vivo and in vitro studies that CHLR treatment rendered the caudal two-thirds of the palate, the posterior and soft palatal regions, unable to elevate. These were also the regions whose day-13-5 CSA was significantly reduced as compared to control shelves (Table 3).
Histological effects of chlorcyclizine

Day 13-5. The mid, posterior and soft palate regions of the shelf exhibited histological changes after CHLR treatment. Examined at low magnification, control shelves (Fig. 2A) had less densely packed mesenchymal cells than do CHLR-treated specimens of the same age (Fig. 2C). Local differences in cell packing were also apparent. An area of lower cell density medial to the tooth germ was observed in control shelves, which was less extensive in the CHLR-treated specimen. After in vitro elevation this area of lowered cell density was pronounced in control specimen (Fig. 2B), less so in CHLR shelves cultured in standard medium (Fig. 2D), and NORCHLR-cultured specimens showed little
change in this area (Fig. 2E). CHLR-standard-medium-cultured specimens also showed an area of decreased cell density on the nasal side of the shelf, which was not seen in controls or NORCHLR-cultured individuals. It was also consistently noted that the lumens of the blood vessels of CHLR-standard-cultured shelves were considerably expanded when compared to control animals or to NORCHLR-cultured specimen.

A qualitative comparison of cell density, size and morphology at higher magnifications revealed substantial differences between uncultured control tissue (Fig. 3A) and that of uncultured CHLR-treated individuals (Fig. 3C). Mesenchymal cells of control shelves appeared less densely packed, smaller, more elongate or stellate, and with relatively indistinct nuclei. In contrast, cells of uncultured CHLR-treated tissue (Fig. 3C) were more densely packed, larger, more rounded and exhibited distinct nuclei with prominent nucleoli. After culture (Fig. 3B), cells of control tissue appeared even more elongate and in fact both cells and nuclei exhibited wavy perimeters. Culture of CHLR-treated specimens in standard medium (Fig. 3D) resulted in shelves with mesenchymal cells which appear to be smaller than those of uncultured CHLR individuals, but which had a similar rounded shape with prominent nuclei and nucleoli. However, they remained larger and more densely packed as compared to incubated controls (Fig. 3B). Those cultured in NORCHLR medium (Fig. 3E) exhibited extremely dense mesenchymal cell packing, and cells which were about the same size as those seen in uncultured CHLR specimens. However, the nuclei were lighter staining with several prominent nucleoli; little cytoplasm was visible around them. Although densely packed with cells, several mitotic figures were observed in each field.

**Day 14-25.** All control specimens were elevated and adhered (Fig. 4A) with visible epithelial breakdown and areas of osteogenesis. CHLR individuals (Fig. 4B), however, showed no elevation, retarded tooth-germ development and less-developed areas of osteogenesis. If such treated individuals were cultured in standard medium (Fig. 4C), 70% elevated and adhered and ossification proceeded.

High-power views of control shelves (Fig. 5A, B) revealed mesenchymal cell morphologies similar to those seen in day-13-5 controls. Whereas mesenchymal cells of CHLR shelves which had elevated in vitro (Fig. 5C, D), remained more densely packed than controls and still exhibited the prominent nuclei and nucleoli observed in day-13-5 CHLR specimen. Cells of day-14-25 CHLR shelves (Fig. 5D) also appeared smaller than those of controls (Fig. 5B).

**DISCUSSION**

**Nature and distribution of CHLR effect**

**Direct effect on shelves.** Our experiments demonstrate that CHLR administration results in changes in shelf size and loss of the inherent ability to elevate.
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Other investigators have demonstrated direct effects of CHLR on GAGs in the palatal shelves, but have also shown an effect on mandibular growth (Wilk et al. 1978; King, 1963). Therefore, in our studies either GAG effects or mandibular reduction could be key elements in the observed clefting. The latter alternative can be eliminated. We have shown that the elevation ability of the palatal shelves is directly affected by CHLR treatment using in vitro techniques, wherein the tongue is removed and the behaviour of the shelves monitored directly.

Distribution of effects. CHLR effects on shelves were also observed to be regional, with the severity of response to drug treatment graded in an anterior-posterior manner. A similar gradation in in vitro elevation behaviour has been previously observed (Brinkley & Vickerman, 1979). The anterior shelf appeared to be unaffected by CHLR treatment. The loss of elevation ability and reduction of shelf CSA were evident in the mid to soft regions. The greatest effects were found in the posterior and soft areas.

There are two possible explanations for a lack of effect in the anterior. Either formation and elevation of the anterior palate are not dependent on production of an extensive extracellular matrix, and thus are unaffected by CHLR treatment, or the anterior recovers more rapidly and to a much greater extent than other regions. The former explanation seems most likely, not only because of the consistent lack of any drug effect on the CSA and elevation changes in this area, but also because the anterior is the most highly cellular region of the palate, with little extracellular space visible (Brinkley, 1980). Likewise, the pronounced effects observed in the posterior portion of the shelves suggest that these areas may be richer in the two CHLR-affected GAGs: HA and the chondroitin sulphates.

Shelf expansion and elevation ability

One of the oldest proposed mechanisms for the motive force underlying palatal shelf movement is a rapid increase in shelf volume due to increased intercellular volume in the connective tissue (Lazzaro, 1940; Walker, 1961). In vivo, shelf expansion appears to occur over the course of rat palate closure when observed in frozen sections (Diewert & Tait, 1979). Our in vivo findings of shelf cross-sectional area expansion are in agreement with those of Diewert & Tait for all shelf regions except the posterior, where we observed no significant shelf expansion. It is interesting that both the frozen tissue used by them and the fixed tissue used in the present study reveal a fairly similar pattern of in vivo expansion despite the tissue shrinkage associated with fixation.

Comparison of both in vivo and in vitro studies permits us to determine whether shelf expansion is actually necessary for shelf elevation. Our studies demonstrate that overt shelf expansion, as measured by change in shelf cross-sectional surface area, is not required for shelf reorientation in the anterior, posterior and soft palate, given the degree of maturity of a day-13.5 palatal shelf. In the mid palate though, elevation and expansion appear to be linked to some
degree. Present results also suggest that the in vivo condition is somehow related to shelf expansion. Given the physical properties of an HA-rich extracellular network it seems possible that in vivo shelf expansion is a response to the external compression of the shelves caused by their wedged position between the tongue and mandible. This expansion may be necessary for initiating the movement of the shelves around the tongue or for stimulating the tongue to move out of the way of the elevating shelves.

**GAGs and shelf elevation**

HA is the predominant GAG species present in the shelves around the time of elevation (Pratt et al. 1973). Thus CHLR effects on shelf morphology and elevation behaviour must in large part be attributable to degradation of this molecule into smaller pieces, resultant changes in its ionic responsiveness and/or altered interactions with other extracellular molecules. To understand how these changes might affect shelf elevation it is necessary to examine some of the properties of HA.

**HA in extracellular matrices.** The hyaluronate content of the extracellular matrix is known to be important in the morphogenesis of that tissue (Toole et al. 1977; Toole et al. 1980). HA is a very large, highly charged polyanion with a large molecular domain that tends to sequester water (Laurent, 1970). Its molecular weight, length and the presence of other molecules including proteins or cations can influence the viscosity of a hyaluronate solution (Balazs & Gibbs, 1970; Comper & Laurent, 1978; Morriss, Rees & Welsh, 1980). Hyaluronate often enmeshes with sulphated proteoglycans and collagen to form a gel-fibre network characteristic of extracellular matrices (Bernfield, 1981). Cellular behaviour is known to be directly influenced by its association with the extracellular matrix. For instance, anchorage of cells to a substratum determines cell shape and mobility and can thus alter cell metabolism (Bernfield, 1981). Indirectly, the gel-fibre matrix could simply contribute to a complex framework whose shape is the primary determinant of tissue form (Nakamura & Manasek, 1978).

These networks not only have specific physical configurations due to their molecular components, but also have unusual physiological properties. Such networks are capable of generating a 'swelling pressure' that is composed of an osmotic contribution from the hyaluronate portion's tendency to sequester water and an elastic contribution from the contractility of the fibres. If the equilibrium between osmotic and elastic components is disturbed the network will expand or shrink, depending on which components are affected (Comper & Laurent, 1978). Morphogenetic changes could then be brought about in tissues as a result of these physicochemical properties. Physical disturbance such as external compression of a tissue containing the network, or chemical changes such as local or systemic variations in the ionic environment, could alter the configuration of the network and in turn that of surrounding tissue. Such
remodelling could passively displace or translocate cells to areas of least resistance (Solursh, Fisher, Meier & Singley, 1979; Brinkley, 1980), or provide spaces for actual cell migration (Pratt, Larsen & Johnston, 1975; Tosney, 1978; Erickson, Tosney & Weston, 1980).

Consequences of altered HA. If one major component, for example HA, is abnormal, it is reasonable to expect an alteration in extracellular matrix organization. Such a disturbance could affect cell attachment to the matrix as well as the physicochemical properties of the matrix itself. If the cells were unable to attach properly a rounded rather than stellate appearance could result, and a change in cellular metabolism might occur which could preclude active cell migration. An altered gel-fibre network in the extracellular matrix might also be unable to undergo conformational changes necessary to displace cells passively and create local expansions and compressions.

Both the elevation behaviour of CHLR-treated shelves and the histological appearance of their mesenchymal compartment provide strong support for the idea that HA plays a central role in shelf elevation through its spatial distribution and macromolecular interactions in an extracellular network. The regional nature of CHLR effects on elevation suggests that there is an underlying spatial or temporal sequence of either HA and chondroitin sulphate production in the shelves or of differential recovery from the effects of CHLR. Histologically the mesenchymal cells of CHLR shelves appear large and rounded with prominent nuclei and nucleoli which may reflect direct metabolic alterations due to the CHLR. Their morphology might also be the result of an inability to adhere properly to the surrounding abnormal extracellular matrix. Another histological finding that may be related to the degradation of hyaluronate is that, following in vitro culture in standard medium, the vascular spaces of CHLR-treated shelves are enlarged as compared to controls. This result is comparable to that of Singley & Solursh (1981), who found similar vascular inflation after degradation of hyaluronate by local injection of Streptomyces hyaluronidase in chick wing buds.

Culture in NORCHLR medium, however, did not elicit this response, but did result in a noticeable increase in the number of dividing cells. It seems possible that further exposure to the drug may have resulted in sufficient additional alterations in the gel portion of the matrix both to change its osmotic properties, precluding vascular enlargement, and to alter the relationship of cells to matrix which may have resulted in mitotic stimulation.

Possible role of HA in shelf elevation. Our studies do not rule out the possibility that some subtle regional shelf expansion may indeed play a role in shelf elevation, but do preclude overall shelf swelling as the motive force. Alteration of the shape of a structure such as a shelf, with no change in surface area, could be accomplished by local expansions compensated for by other local compressions. Both the present results and changes previously observed in cell patterning over the course of shelf elevation (Brinkley, 1980), suggest that the internal compo-
ments must be rearranged but not expanded. Given the wide range of properties of gel-fibre matrices a highly patterned distribution of an HA-rich extracellular matrix with specific local variations in the nature of the matrix could be the underlying physical basis for such local tissue expansions and contractions. The development of ‘internal shelf force’, i.e. the intrinsic ability of shelves to elevate, may in large part be the acquisition of a specific temporal and spatial distribution of HA and other matrix components.

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