Differential Cell Proliferation of Embryonic Rat Palatal Processes as Determined by Incorporation of Tritiated Thymidine

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Introduction

During normal secondary palate morphogenesis, three distinct sequences of events take place, namely: a) transposition of vertically directed palatal processes into a horizontal position; b) growth of the horizontal processes until their approximation in the midline; and c) ultimate breakdown of epithelial wall between the two processes and fusion.

Literature is abundant on the study of the above phases in normal human and laboratory animal fetuses, and these major steps of palatal closure are agreed upon (*6*). However, the mechanism by which each individual sequence occurs is not well understood. The past investigations have explained the development of the secondary palate as being a result of changes within the palatal processes or from changes in the surrounding craniofacial structures, or combination of both.

Regarding the changes within the palatal processes, Schaeffer (1920) reported that horizontalization is accomplished by differential growth. Similarly, other factors attributed for horizontalization and closure of palatal processes have been the presence of an "intrinsic force" (*21*), cellular growth (*11, 15*), buildup of acid mucopolysaccharides (*10, 20*), and vascular development within the processes (*1*).

Several studies have reported that changes in spatial relationship of the tongue to the palatal processes plays a minor role in the horizontalization (*11, 16, 19*). Other craniofacial changes reported are: reduction in the cranial flexure angle (*2, 18*), mandibular movements (*19*), and orofacial reflexes (*5*).

In several of the above mentioned studies authors have alluded to differential growth of the palatal processes as a vital factor in the development of the secondary palate. However, a majority of the conclusions are based upon histological observations and inadequate methodology. In general very little attention has been paid by investigators to the normal pattern of cell dynamics during the development of the palatal processes. Few investigators have made an

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attempt to study the DNA synthesis of the cells of the palatal processes prior to and during fusion (*9, 12, 13*). The emphasis in these investigations was to study the effect of hypervitaminosis A (*9, 14*) and cortisone (*12*), on the ability of the cells of the palatal processes to proliferate rather than sequential normal cell dynamics and palatal closure. Their results as shown by labeling index indicate that the drugs significantly reduce the number of tritiated thymidine labeled cells in unfused processes of drug induced fetuses. This finding as well as lack of studies on the role of cell proliferation in the development of normal secondary palate, emphasizes the need of an organized investigation on this subject.

The present study was undertaken to define the role of cellular activity, as determined by tritiated thymidine uptake, within the palatal processes during the different days of palatal development in normal rat fetuses.

Materials and methods

Sixteen female Wistar albino rats were utilized in the present study. All rats were housed in a constant temperature room with a controlled light cycle (12 hours of light and 12 hours of dark), with mating occurring during the dark period. Female rats were placed with male rats at 5:00 p.m. and were removed at 8:00 a.m. Vaginal smears were obtained. If this test was positive it was taken as day 0 of gestation.

The pregnant rats were divided into four equal groups. Group 1 received one intraperitoneal injection of 250 μCi/Kg of rat body weight of tritiated thymidine (sp. act. 20Ci/m mole) on the 14th day of gestation. Similarly, groups 2, 3, and 4 received the the same dosage of isotope on days 15, 16, and 17 of gestation respectively. All rats were killed one hour after the tritiated thymidine (^{3}HTdr) administration. Four fetuses from each rat were recovered and placed in Bouin's solution for 24–48 hours. The fetus heads were embedded in paraffin and 5 μm thick serial sections were cut. The sections were coated with Kodak NTB-2 emulsion and exposed in the dark for 3 weeks. The sections were developed and stained with hematoxylin-eosin.

The autoradiograms were examined under Zeiss photomicroscope. The palatal processes in the frontal plane of the fetus head were studied in three regions. The total number of sections was counted from the first appearance of the secondary palatal processes or palate in the serial frontal sections to the last section, where the palate was no longer visible. The total number of sections was divided into three equal parts. The sections belonging to the anterior part or near primary palate were grouped as the anterior region and the other two as the middle and posterior regions.

The palatal processes were divided into three arbitrarily defined areas, namely, the tip, middle, and lateral, in each of the previously described regions (Figure 1). The labeling index of the cells of the palatal processes was calculated by recording the percentage of labeled cells in a sample of total population after an exposure of 1 hour to ^{3}HTdr. A total number of 1000 mesenchymal cells was counted in each area using a counting grid placed in the eye piece of the microscope. The epithelial cells of the palatal processes were counted separately in each of the following three areas: cells at the presumptive zone of fusion, cells facing the nasal septum, and cells facing the tongue. The epithelial cells of the

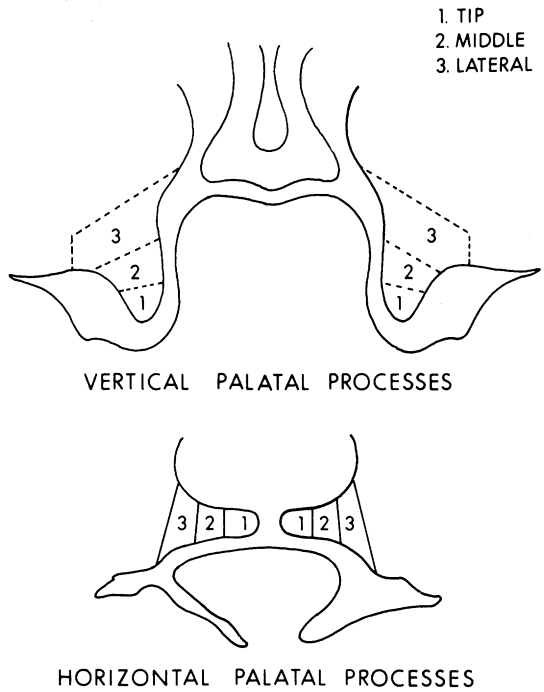

FIGURE 1. Diagrammatic frontal section of a rat fetus showing the arbitrary division of vertical and horizontal palatal processes into three different geographic areas

tongue towards the palatal surface were also counted as controls. A total of 250 epithelial cells were counted in each of the above described areas.

The labeling indices obtained for the different mesenchymal areas and regions as well as epithelial areas were statistically analyzed utilizing the standard analysis of variance and Duncan's multivariant analysis. These tests were utilized to find any significant differences in DNA activity which may exist between different days of gestation, as well as any differences in activity between areas and regions within the same days.

Results

On days 14 and 15 the palatal processes were vertically directed with the tongue lying between them. On day 16 the processes were horizontal and superior to the tongue. On day 17 various stages of fusion were observed. Some fetuses showed total mesenchymal fusion, and in others epithelial fusion or an epithelial seam in the midline was present.

Table 1 and Figures 2 and 3 show the mean percentage of labeled cells in different regions and areas of the palatal processes on different days of gestation. On day 14, the tip area showed significantly greater numbers of labeled mesenchymal cells ($p < 0.01$) in all three regions as compared to the respective middle and lateral areas.

In addition, on day 14 the cumulative labeling index of the different areas in the anterior region of the palate was significantly higher than in the middle and posterior regions.

TABLE 1. Mean labeling index in per cent for different regions and areas by days of gestation.

day 14					*day 15*				
region	*area*				*region*	*area*			
	tip	*middle*	*lateral*	*avg. of regions*		*tip*	*middle*	*lateral*	*avg. of regions*
Anterior	28.2	18.0	14.8	20.4	Anterior	22.9	18.4	14.0	18.5*
Middle	22.4	16.3	13.0	17.2	Middle	25.8	17.5	12.9	18.7*
Posterior	18.7	15.2	11.3	15.1	Posterior	18.6	12.4	10.2	13.7
Average of areas	23.1	16.6	13.0	52.7	Average of areas	22.5	16.2	12.3	50.9
day 16					*day 17*				
region	*area*				*region*	*area*			
	tip	*middle*	*lateral*	*avg. of regions*		*tip*	*middle*	*lateral*	*Avg. of regions*
Anterior	19.0	17.2	9.92	16.9*	Anterior	6.98	9.93	10.1	9.01*
Middle	11.3	14.8	10.3	13.0	Middle	7.20	10.4	8.35	8.64*
Posterior	14.4	17.4	10.6	15.3*	Posterior	6.18	7.18	7.25	6.86
Average of areas	17.1*	17.9*	10.2	45.2	Average of areas	6.78	8.35*	8.58*	24.5

* These values are not statistically significant. All other values are significant at $P < .05$ level as determined by Duncan's multivariant analysis.

The pattern of labeled cells in different areas and different regions on day 15 was essentially the same as was observed for day 14 (Table 1).

On day 16 (Table 1) there was a significant decrease in the number of labeled cells at the tip and lateral areas as compared to the activity in the same areas on days 14 and 15. Over all, a slight increase in the number of labeled cells was observed in the middle area as compared to the previous ages. Within day 16, the middle area of the palatal process showed significantly higher labeling activity ($p < 0.01$) in all regions except in the anterior region, as compared to the tip and lateral areas.

On day 17 (Table 1) an overall decrease in the labeling index was seen in all areas, except that the decrease was least in the lateral area. Over the 4-day period, the lateral area of the palatal processes showed the most consistent level of labeling. The tip area on day 17, which is fused at this time, showed least number of labeled mesenchymal cells as compared to the other two areas.

The mean percentage of labeled epithelial cells showed a constant decrease with advanced age (Table 2). However, a significant decrease in the number of labeled epithelial cells in the presumptive zone of fusion was observed between days 15 and 16 and also between days 16 and 17. The epithelial cells facing the nasal septum showed greatest decrease after 16 days.

Discussion

The mechanism of palatal closure is presently not well understood. The investigations in the past have hypothesized many factors for this biological

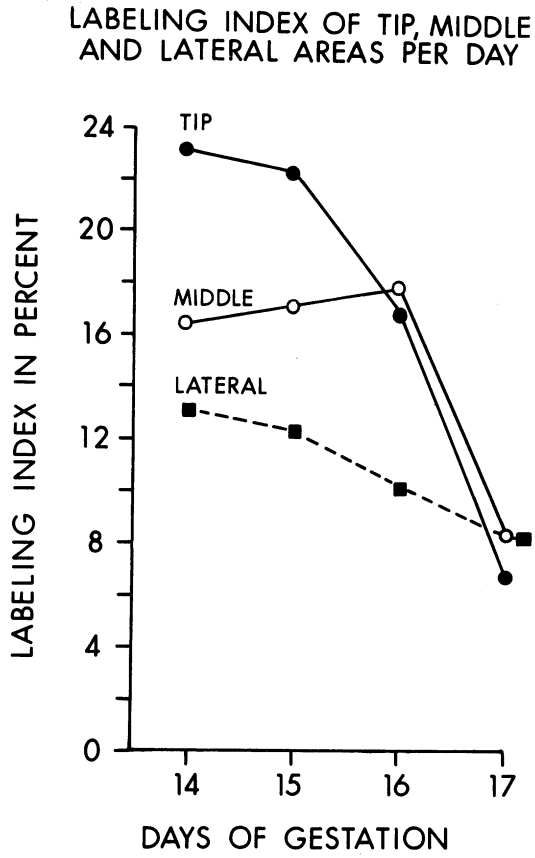

FIGURE 2. Mean labeling index of mesenchymal cells of the palatal processes on different days of gestation in different areas of the processes

phenomena ranging from the role of the tongue and associated craniofacial structures to "intrinsic" factors within the palatal processes. Although differential cell proliferation (*11, 15*) has been alluded to in the previous studies as a primary factor in the horizontalization and fusion of the palatal processes, to date only a few investigations have made an attempt to study this variable.

A recent study of Jelinek and Dostal (*7*), who utilized colchicine in mice to study cell proliferation in the palatal processes has suggested that highest cell proliferation activity was at least 24 hours prior to horizontalization and was also greatest in the apical parts of the processes. The findings of the present study support their results. Furthermore, the results of the present study reveal a differential trend in the DNA synthesis of the mesenchymal cells of the palatal processes on different days of gestation and also in different areas and regions of the processes.

Our data suggests that the vertical growth of the palatal processes on days 14 and 15 of gestation is associated with the peak labeling index observed on these days as compared to days 16 and 17. Furthermore, a high labeling index observed at the tip and middle areas may suggest a possible relationship of cell

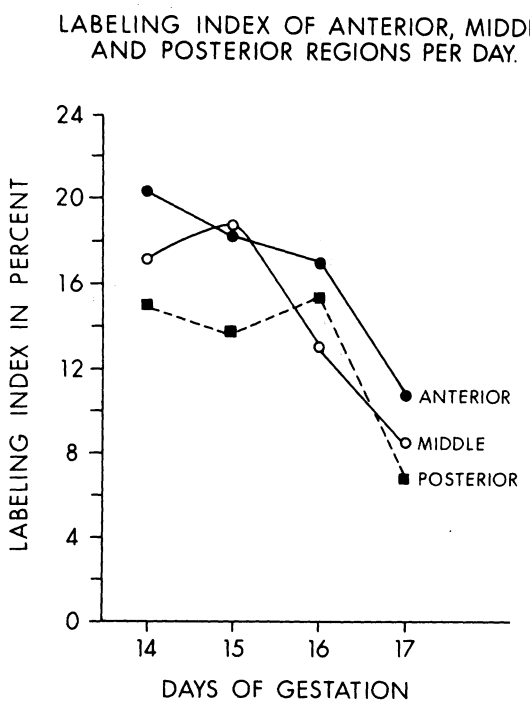

FIGURE 3. Mean labeling index of mesenchymal cells of the palatal processes on different days of gestation in different regions of the palate

proliferation in horizontalization (*15, 17*). However, cell proliferation can not be called the primary factor as the tongue, mandibular movements (*19*), and changes in the cranial base flexure (*2*) have been shown to be associated with horizontalization of the palatal processes. Interestingly, Jelinek and Dostal (*8*) recently reported that they found horizontalization and fusion in three fetuses with aglossia. This finding somehow rules out that the tongue is a primary factor in the mechanism of palatal closure.

The present results also showed an increase in the DNA synthesis of the mesenchymal cells in the middle area and a fairly constant activity in the lateral area of the 16 day old fetuses. This shows that during horizontal growth of the

TABLE 2. Mean labeling index in per cent for epithelial cells

area of epithelium	*days*			
	14	*15*	*16*	*17*
Facing** Nasal Septum	30.2	32.1	28.7	7.2
Facing** Oral Surface	32.1	27.5	21.6	19.4
Presumptive** Zone of Fusion	36.3	27.2	18.9	4.2
Tongue (Control)	40.5	33.8	27.8	22.4

** Cumulative results for the 4 days showed these areas significantly different ($P < .01$) utilizing the standard analysis of variance.

palatal processes towards each other differential cellular proliferation takes place in different areas.

The epithelial cells showed a decrease in the ^{3}HTdr uptake with advanced age and the cells at the presumptive zone of fusion showed the greatest decrease after day 16 of fusion. This finding supports the results of Hudson and Shapiro (*4*), who also concluded that decline in mitotic activity of palatal tip epithelium (presumptive zone of fusion) supports the concept of programmed cell death (*3*) for this tissue.

One factor which may have influenced results might be our arbitrary division of palatal processes in different areas and regions. However, with the lack of reproducible landmarks in the fetus, this was the only mechanism available to study different areas and regions. Utmost care was taken to imbed fetus heads in paraffin to control the plane of sections. Since significant differences were observed in the labeling index in different areas and regions, we believe a small difference in arbitrary division of regions and areas may not have made a significant difference in the final results.

Our investigation primarily attempted to define variations in cellular proliferation, as indicated by tritiated thymidine uptake by the cells of the palatal processes which may be responsible for various stages of palatal development. Thymidine uptake in different areas and regions of the palatal processes is highly suggestive of the levels of cellular proliferation, but it is not a rigorous proof that all labeled cells will undergo replication. At the present state of knowledge, it is probably safe to assume that more than one mechanism is involved in the various stages leading to palatal fusion. However, to understand the role of these variables, it is necessary to establish their ultimate contributions to palatal formation.

Summary

The differential cellular proliferation of the palatal processes in the Wistar albino rat was studied utilizing tritiated thymidine labeling. ^{3}HTdr was given to pregnant rats at 14, 15, 16, and 17 days of gestation. The animals were killed one hour after injection. The fetuses were removed and the heads were embedded, sectioned, and an autoradiographic technique utilized. The autoradiograms were examined microscopically and labeling indices for various areas and regions of the palatal processes were calculated. The findings revealed that the pattern of cellular activity of mesenchymal and epithelial cells changes in different areas and regions of the palatal processes with different stages of secondary palate development.

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