Autoradiographic study of macromolecular synthesis in the fusion epithelium of the developing rat primary palate in vitro

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SUMMARY
The facial processes involved in primary palate formation undergo epithelial fusion in a manner morphologically analogous to that observed during secondary palate formation. We have used whole embryo culture to analyze the synthesis of macromolecules (DNA, protein, glycoprotein) in the primary palate, based on the incorporation of various labeled precursors. The results of this study demonstrate that changes in the synthesis of macromolecules occur during the fusion of the facial processes, which resemble those previously reported to occur during secondary palate development. These changes include cessation of DNA synthesis in cells in a restricted zone of the epithelium, concomitant with maintenance of glycoprotein synthesis. These findings indicate that the molecular events underlying the development of the primary and secondary palate may be similar.

INTRODUCTION
Formation of the rat primary palate occurs from day 11 through 13 of gestation. This process begins with the formation of the nasal placodes, ectodermal thickenings on both sides of the developing embryonic face. Subsequent invagination of the nasal placode epithelium (Smuts, 1977; Wilson & Hendrickx, 1977) and proliferation of the surrounding mesenchymal cells gives rise to the maxillary and nasal processes (medial and lateral), which enclose the nasal pit. The medial-nasal and maxillary processes merge first at the most caudal portion of the nasal pit, followed later by contact and fusion of the lateral and medial-nasal process. Epithelial contact between the medial-nasal process and the lateral-nasal process produces a temporary epithelial structure, the nasal fin. Autolytic and phagocytic activities in the most posterior portion of the nasal...
fin result in its breakdown (Gaare & Langman, 1977a, b), in this way connecting the nasal pit with the primitive oral cavity, thus establishing the primary palate. Excellent morphological descriptions of these different stages of primary palate development are available for human and rodent embryos (Warbrick, 1960; Patten, 1961; Trasler, 1968; Trasler & Fraser, 1977; Pourtois, 1972; Waterman & Meller, 1973); however the biochemical mechanisms involved in the formation of this structure are unknown.

Analogous events can be observed during formation of the secondary palate where contact and fusion of opposing palatal shelves occurs, early on day 16 of gestation in the rat (Greene & Pratt, 1976). It has been suggested that epithelial cells of the presumptive fusion zone (medial edge) of the secondary palate are 'programmed' to begin terminal cell differentiation prior to epithelial adhesion (Shapiro & Sweney, 1969; Hudson & Shapiro, 1973; Pratt & Martin, 1975).

These studies of secondary palate development have been facilitated by the application of organ culture techniques (Pourtois, 1966; Tyler & Koch, 1975). Currently such techniques are not possible with the primary palate since the morphology is complex and it is difficult to maintain the proper orientation of the tissues in vitro. However, in vitro development of the primary palate is possible using whole embryo culture (New & Stein, 1964), where the time of explantation and culture of the embryos, coincides with the period when the primary palate develops. Whole embryo culture has been simplified to the extent that the system can now be used in developmental and teratological studies (Cockroft, 1973, 1976; New, Coppola & Terry, 1973; Kochhar, 1975). This technique has been applied recently in the study of normal and abnormal primary palate development (Eto & Horigan, 1977; Eto, Figueroa, Tamura & Pratt, 1978; Figueroa & Pratt, 1978).

Here we report on the biosynthetic activities in the developing primary palate, using autoradiographic techniques to assess the distribution of incorporated radiolabeled thymidine, leucine and fucose.

**MATERIALS AND METHODS**

**Source of embryos and culture**

Mature virgin Sprague–Dawley female rats, weighing approximately 250 g were placed overnight with males of known fertility. The morning vaginal smears were found to be sperm positive was considered day 0 of gestation.

On day 12 of pregnancy, the rats were killed by exposure to carbon dioxide gas, the uterus was removed and placed in phosphate-buffered saline (PBS) at 4 °C in a sterile Petri dish. Embryos containing four to six tail somites were explanted using techniques previously described (New & Stein, 1964; New, 1967; Cockroft, 1973; New, Coppola & Terry, 1973; New, Coppola & Cockroft, 1976; Cockroft, 1976). The yolk sac and amnion were incised exposing the embryos, the embryos were transferred to 15 ml screw cap vials containing 2 ml
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of Waymouth's 752/1 media and 50 % heat-inactivated rat serum (New et al. 1976) plus streptomycin at a final concentration of 50 µg/ml. Two embryos were placed in each vial and the vials were gassed at the beginning of the culture period with 95 % O₂ and 5 % CO₂, placed on a rotator in an incubator at 37 °C, and turned at 10 rev./min.

Radioactive precursors and autoradiography

After 18 or 23 h in culture, embryos with a visible heart beat and visible blood circulation were exposed to media containing 10 µCi/ml of either [³H]-thymidine ([methyl-³H]-50 Ci/mmol, New England Nuclear), [³H]leucine (L-[4,5-³H(N)]-50 Ci/mmol, New England Nuclear) or 50 µCi/ml of [³H]fucose (L-6-³H]-12-06 Ci/mmol, New England Nuclear) for 1–6 h.

Afterwards the embryos were washed twice in PBS and immediately fixed in 2-5 % glutaraldehyde in 0-1 m cacodylate buffer (pH 7-4) at 4 °C for 1–7 days. The presence of 5 mM precursor was included in the fixative and washes to ensure minimal presence of unincorporated labeled precursors in the autoradiograms. The nasal pit areas were then carefully dissected, ethanol dehydrated and embedded in epoxy resin (Spurr, 1969). One micron sections of the tissue were placed on albumin coated slides, dipped in nuclear track emulsion NTB-2 (Kodak). The slides were exposed at 4 °C for 2–3 weeks, developed, fixed and stained with 0-1 % toluidine blue.

RESULTS

The facial processes that form the primary palate in the rat make contact and undergo fusion during days 12–13 of gestation. This study concentrated on the cellular events in the fusion process, both prior to as well as during contact of the medial and lateral nasal processes. Tissue at different stages of the fusion process can be observed in the same nasal pit, allowing a more reliable correlation of metabolic activities and cellular events with development than can be obtained by comparing tissue from different embryos. As can be seen in Figs 1, 2A, B, two different planes of section (A and B) in the same nasal pit enable us to determine the cellular and biochemical changes that take place in the presumptive fusion epithelium during these stages of development.

Whole rat embryos were exposed to [³H]thymidine for 1 or 6 h to determine which cells in the presumptive fusion epithelium were synthesizing DNA. The autoradiograms show that the presumptive fusion epithelium of the lateral (Fig. 3A) and medial (Fig. 3B) nasal processes have far fewer cells with labeled nuclei than either the underlying or adjacent nasal epithelium (Fig. 3D). This same phenomenon also occurs during contact of the nasal process (Fig. 3C), although a few labeled nuclei can however still be seen. Autoradiograms of the presumptive fusion epithelium of embryos exposed to [³H]thymidine approximately 12 h prior to contact of the nasal processes, showed similar labeling to that seen in Fig. 3D.
Fig. 1. Head from a rat embryo explanted at day 11½ of gestation and cultured for 24 h. Line A indicates the section plane for Figs. 2A, 3A, B, D, 4A-C; B indicates section plane for Figs. 2B and 3C.

Fig. 2. (A) (B) Frontal sections illustrating the nasal pit during fusion. LNP, lateral-nasal process; MNP, medial-nasal process; PFE, presumptive fusion epithelium; FZ, fusion zone. × 130.
Fig. 3. Autoradiograms of nasal pits from rat embryos which were cultured for 24 h, and exposed the last hour of culture to 10 μCi/ml of [3H]thymidine. In (A) and (B) significantly less labeling is observed in the presumptive fusion epithelium of the lateral and medial-nasal processes, when compared to adjacent nasal epithelium (D) and underlying mesenchyme. Few cells in the fusion zone (C) are labeled when compared to underlying mesenchyme. LNP, lateral-nasal process; MNP, medial-nasal process; PFE, presumptive fusion epithelium; NE, nasal epithelium; MES, mesenchyme; NP, nasal pit; FZ, fusion zone. × 860.
Fig. 4. Autoradiograms of nasal pits from rat embryos exposed for 1 h and 6 h to \([\text{³H}]\)leucine and \([\text{³H}]\)fucose respectively. \([\text{³H}]\)Leucine incorporation is similar in the presumptive fusion epithelium (A), adjacent nasal epithelium (B) and underlying mesenchyme (C). The presumptive fusion epithelium of the nasal processes incorporated higher amounts of \([\text{³H}]\)fucose than the underlying mesenchyme. Note the distribution of \([\text{³H}]\)fucose grains in the surface epithelium and basal lamina (arrows). LNP, lateral-nasal process; MNP, medial-nasal process; PFE, presumptive fusion epithelium; MES, mesenchyme; NP, nasal pit. × 860.
The synthetic capability of these post-mitotic cells of the presumptive fusion epithelium of the nasal processes were examined for protein and glycoprotein synthesis, by following the incorporation of $[^3H]$leucine or $[^3H]fucose$. The incorporation of $[^3H]leucine$ into protein was observed in the cells of the presumptive fusion epithelium of the nasal processes (Fig. 4A), but in contrast to $[^3H]$thymidine incorporation it did not differ from either the underlying mesenchyme or adjacent cells of the nasal epithelium (Fig. 4B). Fucose, which is incorporated specifically into glycoproteins and glycolipids (Bocci & Winzler, 1963), was incorporated into the cells of the presumptive fusion zone and nasal epithelium of the nasal processes (Fig. 4C), and appears to be there in slightly greater amounts than in the underlying mesenchyme. A higher incorporation of $[^3H]fucose$ in the basal lamina and superficial cells of the epithelium (arrows) was observed.

**DISCUSSION**

Evidence obtained from avian and rodent embryos (Johnston & Pratt, 1974; Johnston, Morris, Kushner & Bingle, 1977) indicates that the early phase of primary palate development involves migration of cranial neural crest cells into the frontonasal processes. Differential proliferation of mesenchymal- and neural crest-derived cells (Minkoff & Kuntz, 1977) and increased matrix formation gives rise to the facial processes that eventually fuse to form the primary palate.

Relatively little is known of the biochemical changes that occur during primary palate development. However this is not the case for the secondary palate, a structure that undergoes similar developmental steps regarding processes of contact and fusion of opposing epithelial surfaces. It is thought that the midline epithelium (medial-edge) of the secondary palatal shelf is ‘programmed’ to undergo certain biochemical changes that prepare the presumptive fusion epithelium for cell death and fusion with the opposing shelf. DNA synthesis ceases in the medial-edge epithelium approximately 24 h prior to contact (Pratt & Martin, 1975) and the synthesis of complex glycoconjugates by the presumptive fusion epithelium increases (Pratt & Hassell, 1975). These surface-associated macromolecules appear to be necessary for adhesion of the opposing epithelia *in vitro* (Greene & Kochhar, 1974; Pratt, Greene, Hassell & Greenberg, 1975; Greene & Pratt, 1977; Meller & Barton, 1978).

To determine whether similar biochemical changes occurred in the primary palate epithelium, we made use of autoradiographic techniques. Labeling of the primary palate tissues was carried out in whole embryo culture, since this method (New & Stein, 1964; New et al. 1976) provides a reliable and consistent means of exposing the embryo to defined periods of labeling.

In the present study we have demonstrated that the presumptive fusion zone of the rat primary palate epithelium undergoes a selective decrease of DNA synthesis well before apposition of the processes. The exact time of the decrease
is not precisely known, but appears to be as early as 6 h prior to contact. This decrease is limited to the presumptive fusion epithelium and fusion zone (nasal fin), and does not occur in either the adjacent epithelium or underlying mesenchyme. In the present study we are not able to explain the presence of few labeled nuclei in the presumptive fusion epithelium and nasal fin, phenomenon also observed in the developing primary palate of the Rhesus monkey in vivo by Wilson & Hendrickx (1977).

While cell division was stopped in the presumptive fusion epithelium, other activities were maintained by these post-mitotic cells, for example, the incorporation of labeled leucine and fucose. Fucose is a specific precursor for glycoproteins and glycolipids (Bocci & Winzler, 1963) which suggests that there is an accumulation of such glycoconjugates at the presumptive fusion epithelium as was described in the secondary palate (Pratt & Hassell, 1975; Pratt et al. 1975).

These results correlate well with the accumulation of ruthenium red positive material (Gaare & Langman, 1977a, b) and [3H]Con A binding (Smuts, 1977) on the surface of the presumptive fusion epithelium of the primary palate, suggesting that secretion of cell surface glycoconjugates may be involved in the initial steps of primary palate adhesion.

The present study has provided evidence that similar changes in macromolecular synthesis precede fusion of both the primary and secondary palate. Whether these changes occur by similar mechanisms or are sensitive to alteration by the same teratogens is currently under study.

REFERENCES


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(Received 24 July 1978, revised 30 October 1978)