Effect of epidermal growth factor on secondary palatal epithelium in vitro: tissue isolation and recombination studies

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SUMMARY

Previous studies have shown that epidermal growth factor (EGF), a peptide of M.w. 6045, can specifically inhibit in organ culture the cessation of DNA synthesis and programmed cell death that normally occur in the presumptive fusion zone (PFZ) of the secondary palatal epithelium. The aim of this study was to determine if EGF acts directly on the epithelium to exert its effect and if there is a requirement for the underlying mesenchyme. Palatal processes from 13- and 14-day Swiss Webster embryonic mice were enzymatically separated into epithelium and mesenchyme which were then cultured alone or in transfilter recombination for up to 72 h. Tissues were examined by transmission- and scanning-electron microscopy and DNA synthesis was monitored autoradiographically using [³H]thymidine incorporation. In isolated epithelium cultured in control medium, cell death occurred in the PFZ and DNA synthesis did not occur in the oral and nasal epithelial regions. EGF (20–50 ng/ml) did not prevent cell death in the PFZ and failed to stimulate DNA synthesis in the isolated epithelium; EGF, however, did have an effect on epithelial cell morphology. In the presence of mesenchyme and EGF, there was extensive proliferation in the entire epithelium and cell death within the PFZ was not evident. The results indicate that the stimulation of DNA synthesis in the palatal epithelium by EGF requires the presence of the underlying mesenchyme and that EGF alone is not sufficient to inhibit programmed cell death within the PFZ of the isolated palatal epithelium.

INTRODUCTION

Palatal morphogenesis, in the embryonic mouse, entails the fusion of two palatal processes which initially grow vertically into the oral–nasal cavity along side the tongue, and subsequently, between the 14th and 15th days of gestation, reorientate to the horizontal position above the tongue. The palatal processes become apposed along their medial surfaces, and fusion between the processes effects the separation of the oral and nasal cavities. The dorsal epithelium of the palate, which constitutes the floor of the nasal cavity, differentiates into a

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pseudostratified ciliated columnar epithelium; and the ventral epithelium of the palate, which constitutes the roof of the oral cavity, differentiates into a keratinizing stratified squamous epithelium. The medial epithelial lamina which is formed between apposing processes undergoes a programmed cell death allowing the mesenchymal tissues of the two processes to become confluent. The first sign of programmed cell death within the medial epithelium is a cessation of DNA synthesis 24 h prior to fusion (Hudson & Shapiro, 1973; Pratt & Martin, 1975).

Organ culture studies have demonstrated that cell death within the medial palatal epithelium does not depend upon contact with an apposing palatal process (Smiley & Koch, 1972; Tyler & Koch, 1975), nor does it depend upon the presence of an underlying mesenchyme for at least 3 days prior to fusion (Tyler & Koch, 1977). Cell death occurs within the medial region of cultured palatal epithelium that has been isolated from its mesenchyme, and the timing of this cell death is according to the in vivo schedule. Other in vitro studies on intact palatal processes have shown that the cessation of DNA synthesis and cell death within the medial palatal epithelium can be inhibited in organ culture by the addition of epidermal growth factor (EGF) to the culture medium (Hassell, 1975; Pratt, Figueroa, Nexo & Hollenberg, 1978). If administered after the cessation of DNA synthesis within the medial epithelium, EGF does not reinitiate DNA synthesis, but does inhibit cell death within this region (Hassell & Pratt, 1977). It has been determined that palatal epithelium has receptors for EGF (at 13 days of gestation) (Nexo, Hollenberg, Figueroa & Pratt, 1979), indicating that EGF in culture may be acting directly on the epithelium. The aim of the present study was to investigate this possibility and to determine whether the effect of EGF on the epithelium is dependent upon the presence of the underlying mesenchyme. A portion of this study has been presented as an abstract (Tyler & Pratt, 1979).

MATERIALS AND METHODS

Tissue preparation

The palatal processes used in this study were taken from 13- and 14-day embryonic mice (NIH, Swiss Webster). The adult animals were mated overnight; the following morning, the presence of spermatozoa in vaginal smears was evidence of mating and this day was designated as day zero of gestation. Pregnant females were killed by cervical dislocation on the day that tissues were dissected; fetuses were aseptically removed from the uterus, and organ dissection was carried out immediately in a mixture of phosphate-buffered saline (PBS), pH 7.4, and fetal calf serum (1:1, v/v).

Separation of the epithelial and mesenchymal tissues of palatal processes was achieved by treatment with a 3% trypsin–pancreatin solution (3:1; w/w, in calcium- and magnesium-free PBS) at 4 °C for 30–45 min. After enzymic
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treatment, the organ rudiments were transferred to the PBS-serum mixture, and agitation with a small-bore pipette was used to separate the loosened epithelium from the mesenchyme.

**Culturing procedures**

Tissues were cultured on Millipore filter discs (0.45 μm porosity and 25 ± 5 μm thick, from the Millipore Filter Corp., Bedford, Mass.) supported by plexiglass rings (Grobstein, 1956). The filter was in contact with 1 ml of complex culture medium (modified Ham’s F-12 medium supplemented with 10% fetal calf serum, 1% glutamine, 50 μg/ml ascorbic acid, and 50 μg/ml gentamycin) Epidermal growth factor (EGF; Collaborative Research, Waltham, Mass.) (20 or 50 ng/ml) was added to the culture medium of experimental cultures. Cultures prepared for autoradiographic analysis of DNA synthesis were labelled with [3H]thymidine (20 μCi/ml) for 3–6 h prior to fixation.

Isolated palatal epithelium was cultured with its basal surface against the upper surface of the Millipore filter at the gas–fluid interphase. Notches were made in the Millipore filter marking the region of the medial epithelium at the time of explantation. In transfilter recombinations, epithelium was placed on the upper surface of the filter; mesenchyme was placed on the lower surface of the filter and held in place with a plasma clot. Recombining the tissues in a trans-filter arrangement provided a mechanism for maintaining the epithelium as a flattened cell-sheet. This facilitated scanning-electron microscopic observations of epithelial differentiation.

Cultures were maintained at 37-5 °C in a humidified incubator gassed with 5% CO₂ in air. The culture medium was replaced every 48 h, and cultures were maintained for up to 72 h. The living cultures were photographed at the onset and the termination of the culture period.

**Histological procedures**

Cultures were fixed on their Millipore filters in 2.5% glutaraldehyde (Ladd) in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Fixed cultures were then rinsed with 0.1 M cacodylate buffer (pH 7.4) and post-fixed in 1% OsO₄ in veronal acetate buffer (pH 7.4) for 2 h at 4 °C. Cultures were subsequently processed either for scanning electron microscopy or for autoradiographic analysis.

**Scanning electron microscopy**

Cultures prepared for scanning electron microscopy were dehydrated in a graded ethanol series and dried by the critical-point method using liquid CO₂ in a Toussimis Samdri pvt-3 model critical-point drier. Dried specimens were mounted on aluminum stubs with silver conductive paint and coated with 20 nm of gold–palladium (60:40) in a Denton DV-502 vacuum coating unit.
Coated specimens were examined on an AMR 1000 A scanning electron microscope at 20 kV.

**Transmission electron microscopy**

Following observations by scanning electron microscopy, coated specimens were prepared for transmission electron microscopy. Specimens were removed from the aluminum stubs, were placed in propylene oxide, infiltrated with Epon-Araldite and propylene oxide (1:1) and subsequently embedded in Epon-Araldite. Ultra-thin sections cut with a diamond knife on an LKB ultramicrotome were mounted on slot grids with a composite carbon/formvar film for support. Sections were stained with uranyl acetate and lead citrate and examined on a Philips EM 201 electron microscope.

**Light microscopy and autoradiography**

Following fixation, specimens that had been labelled with [3H]thymidine during the culture period were dehydrated in a graded series of ethanol and embedded in Spurr. Sections 1 μm thick, were mounted on albumin-coated slides and stained with 0.1 % toluidine blue. Unstained labelled sections were dipped in NTB-2 nuclear track emulsion (Kodak), exposed at 4 °C for 7 days, developed, and stained with 0.1 % toluidine blue.

**RESULTS**

**Isolated palatal epithelium**

Palatal epithelium isolated from its mesenchyme and cultured in contact with control medium adhered to the substrate and underwent limited differentiation in both the nasal and oral regions. Cell death occurred within the medial

**Figures 1-5**

Fig. 1. Scanning electron micrograph of 14-day palatal epithelium grown in isolation for 48 h. Medial epithelial cell death has occurred creating a disruption in the epithelial cell sheet. Cellular debris remains within the medial region (arrow). (OE and NE designate the oral and nasal epithelial regions, respectively.) × 180.

Figs. 2–3. Higher magnification scanning electron micrographs of the oral (Fig. 2) and nasal (Fig. 3) regions shown in Fig. 1. The oral epithelium has become layers of flattened cells some of which have short microvillar extensions. The nasal epithelium has differentiated as flattened-to-rounded cells many of which are ciliated. × 1800

Figs. 4–5. Transmission electron micrographs of the oral (Fig. 4) and nasal (Fig. 5) regions of 13-day palatal epithelium grown in isolation for 72 h. Differentiation is similar to that of isolated 14-day palatal epithelium. The oral epithelium consists of squamous cells, two to three cell layers thick. The nasal epithelium is ciliated and is one to two cell layers thick. (f, m, and mf designate fibrillar components, mitochondria, and the supporting Millipore filter, respectively.) × 3000.
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epithelial region at a time which corresponded to the same event in vivo (Fig. 1). These results confirm those of a previous study (Tyler & Koch, 1977). The nasal epithelium was 1–2 cell layers thick and included numerous ciliated cells that had characteristic rounded apical surfaces; sparsely ciliated or unciliated cells of the nasal region had flattened apical surfaces (Fig. 3). Cell nuclei were basally positioned and mitochondria were concentrated in the apical region of the cells (Fig. 5). The oral epithelium consisted of two to three layers of squamous cells. The cell nuclei were characteristically flattened and fibrillar components were concentrated in the apical region of the surface cells (Fig. 4). The surface layer of cells was arranged as an irregular pavement of squamous cells which had short microvillar extensions (Fig. 2).

Autoradiographic studies indicated that incorporation of [3H]thymidine does not occur within the isolated epithelium during the labelling period (6 h) prior to fixation.

**EGF-treated cultures**

Palatal epithelium isolated from its mesenchyme and cultured in the presence of EGF underwent more extensive differentiation than did isolated epithelium in contact with control medium. Exposure to EGF, however, did not alter the pattern of cell death within the medial epithelial region. Medial epithelial cell death occurred as it did in control cultures of isolated epithelium (Fig. 6).

Cells of the nasal epithelium were more heavily ciliated than those of control cultures, and a greater proportion of the nasal cells were ciliated as compared with control cultures (Figs. 8 and 10). The surface cells of the oral epithelium were arranged in a more regular pavement-pattern and had more numerous microvilli than the oral epithelium of control cultures (Fig. 7). In addition, the oral epithelium was thicker than that of control cultures consisting of five to six

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**Figures 6–10**

Fig. 6. Scanning electron micrograph of isolated 14-day palatal epithelium grown for 48 h in contact with culture medium containing EGF (20 ng ml). Medial epithelial cell death has occurred, and cellular debris remains within the medial region (arrow). (OE and NE designate the oral and nasal epithelial regions, respectively.) × 180.

Figs. 7–8. Higher magnification scanning electron micrographs of the oral (Fig. 7) and nasal (Fig. 8) regions shown in Fig. 6. The oral epithelium exhibits a more organized array of flattened cells with more numerous microvillar extensions than in control cultures (cf. with Fig. 2), and the nasal epithelium is more heavily ciliated than in control cultures (cf. with Fig. 3). × 990 and × 1800, respectively.

Figs. 9–10. Transmission electron micrographs of the culture shown in Figs 6–8. The oral epithelium (Fig. 9) is thicker and contains more numerous microfilament bundles (f) than in control cultures of isolated epithelium (cf. with Fig. 4). The nasal epithelium is ciliated but is not as thick as in control cultures (cf. with Fig. 5). × 3000.
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cell layers, and more numerous bundles of microfilaments than were seen in control cultures were found in the outer two to three cell layers (Fig. 9).

Autoradiographic studies showed that EGF did not stimulate DNA synthesis in the isolated epithelium (Fig. 16).

**Palatal epithelium recombined transfilter to palatal mesenchyme**

**Control cultures**

Results from transfilter recombinations in control medium confirmed those of earlier reports (Tyler & Koch, 1974, 1977) that culturing palatal epithelium transfilter to palatal mesenchyme allows normal histodifferentiation of the epithelium. Cell death occurs within the medial epithelial region, and differentiation within the oral and nasal regions is similar to that *in vivo* (see Tyler & Koch, 1977, fig. 3). In addition, the timing of these events *in vitro* is similar to that *in vivo*.

Unlike palatal epithelium cultured in isolation, palatal epithelium cultured transfilter to mesenchyme does not maintain the discontinuity within the medial region caused by cell death (Fig. 11). Instead, the differentiating oral and nasal epithelial regions spread into the medial region and often spread to overlap one another (see Tyler & Koch, 1977, fig. 3). Cellular debris resulting from medial epithelial cell death remains within the medial region, and scanning electron microscopic observations showed filamentous material within the medial

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**Figures 11-15**

Fig. 11. Scanning electron micrograph of 13-day palatal epithelium grown transfilter to palatal mesenchyme for 48 h. The nasal epithelium (NE) and oral epithelium (OE) have spread into the region of the medial epithelium where cellular debris from dying medial epithelium remains (arrow). × 380.

Fig. 12. Higher-magnification scanning electron micrograph of the medial region of the culture shown in Fig. 11. Cilia are present on cells of the nasal epithelium (NE), and the outer layer of oral epithelial cells (OE) are flattened. Cellular debris (arrow) from the dying medial epithelium is present between the nasal and oral epithelial regions. × 1800.

Fig. 13. Scanning electron micrograph of 13-day palatal epithelium that has been grown transfilter to palatal mesenchyme in the presence of EGF (20 ng ml) for 48 h. The nasal epithelium (NE) is ciliated, and the flattened cells of the oral epithelium (OE) have microvilli. Cellular debris is not apparent within the medial epithelial region (ME). × 950.

Figs 14-15. Transmission electron micrographs of a culture similar to that shown in Fig. 13. The nasal epithelium (Fig. 14) is thicker than that of isolated epithelium treated with EGF (cf. with Fig. 10). The oral epithelium (Fig. 15) is five to seven cell layers thick with outer layers of sloughing keratinizing cells not found in EGF-treated isolated epithelium (cf. with Fig. 9). The left margin of a region of epithelial cells (probably of medial origin) which extends below the oral epithelium and is separated from it by a layer of cellular debris (arrow) is shown in the lower left corner of the micrograph. × 4260 and × 2130, respectively.
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Fig. 16. Autoradiograph of a culture of isolated 14-day palatal epithelium grown for 48 h in contact with culture medium containing EGF (20 ng/ml) and pulse labelled with [3H]thymidine for 4 h prior to fixation. There are a few (<4 %) labelled nuclei within the nasal (NE) and oral (OE) epithelial regions. No cells remain within the medial region (ME) where cell death has occurred. (mf designates the supporting Millipore filter.) × 330.

Fig. 17. Autoradiograph of a culture of 13-day palatal epithelium grown transfilter to palatal mesenchyme for 48 h and pulse labelled with [3H]thymidine for 4 h prior to fixation. Many labelled nuclei are present within the mesenchyme (mes) which lies below the Millipore filter (mf), but few (<1 %) nuclei are labelled within the epithelium (ep). × 330.

Fig. 18. Autoradiograph of a culture of 13-day palatal epithelium grown transfilter to palatal mesenchyme in the presence of EGF (20 ng/ml) for 48 h and pulse labelled with [3H]thymidine for 4 h prior to fixation. Labelled nuclei are numerous within both the mesenchyme (mes) and the epithelium (ep). The number (>50 %) of nuclei labelled within the epithelium is significantly greater than that of control cultures (cf. with Fig. 17). (The mesenchymal tissue pulled away from the Millipore filter (mf) during the histological processing.) × 330.

region that is characteristic of the fusion zone during epithelial cell death in vivo (Fig. 12; cf. Waterman, Ross & Meller, 1973, fig. 17).

Autoradiographic studies showed that DNA synthesis was negligible within the epithelium but did occur within the mesenchyme during the label period prior to fixation (Fig. 17).
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EGF-treated cultures

Palatal epithelium cultured transfilter to palatal mesenchyme in the presence of EGF was altered in its differentiation in comparison with control cultures. The oral and nasal epithelial regions appeared continuous with one another as in control transfilter cultures, however, cellular debris and the filamentous material characteristic of the medial region of control cultures were not present within the medial epithelial region of EGF-treated recombinants (Fig. 13). Ultrastructural studies of sections through the medial region showed a layer of epithelial cells without distinctive differentiative features lying below the oral epithelium and separated from it by a layer of cellular debris (Fig. 15).

Both the oral and nasal epithelia consisted of more numerous cell layers than that of control cultures and EGF-treated isolated epithelium (three to four cell layers in the nasal epithelium; five to seven cell layers in the oral epithelium) (Figs. 14 and 15). The nasal epithelium was not more heavily ciliated than that of control cultures or of EGF-treated isolated epithelium. The oral epithelium included layers of sloughing keratinizing cells not found in cultures of EGF-treated isolated epithelium but which were found, though in fewer numbers, in control transfilter cultures.

Autoradiographic studies revealed numerous labelled nuclei in both the mesenchyme and the epithelium of EGF-treated recombinants, indicating that DNA synthesis had been stimulated within the epithelium in the presence of both mesenchyme and EGF (Fig. 18).

DISCUSSION

Epidermal growth factor (EGF) is a small polypeptide (MW 6045) that is found in large amounts in the submandibular gland of the adult male mouse (Cohen, 1962). EGF is also found in various tissues of the human, and appears to be identical to human urogastrone, a hormone which inhibits gastric acid secretion (for review, see Hollenberg, 1979). In the newborn rodent, EGF injection results in precocious eruption of the incisor teeth and eyelid opening (Cohen, 1962). A number of cell types can respond to EGF by increased keratinization (Cohen & Elliot, 1963), and for several cell types, EGF has been shown to stimulate cell proliferation and protein synthesis (e.g. for human fibroblasts, Hollenberg & Cuatrecasas, 1973; embryonic chick epidermis, Hoobner & Cohen, 1967).

Previous studies with embryonic rat palatal processes in organ culture have shown that EGF can prevent medial epithelial cell death and result in altered protein synthesis and epithelial hypertrophy (Hassell, 1975; Hassell & Pratt, 1977). Results from the present study indicate that limited cell death may occur in the presence of EGF as evidenced by the presence of cellular debris between the medial and oral regions of EGF-treated palatal epithelium cultured transfilter to mesenchyme. Previous studies have shown that EGF does not reinitiate
DNA synthesis within the medial epithelium if added to the cultures after cessation of DNA synthesis within the medial epithelium (Hassell, 1975; Hassell & Pratt, 1977). In more recent studies (Pratt et al, 1978), however, it was found that when palatal processes were treated with EGF at an early stage of development prior to cessation of DNA synthesis (mouse, 12 days of gestation), the medial epithelial cells were maintained in a proliferative, nondegenerative state; similar effects were not produced by other peptides tested (insulin, fibroblast growth factor, and growth hormone).

In the present study, results confirmed previous reports (Tyler & Koch, 1974, 1977) that palatal epithelium separated from its mesenchyme (at 13 and 14 days of gestation in the mouse) and grown in isolation undergoes limited differentiation within the oral and nasal regions and that cell death occurs within the medial region. DNA synthesis does not occur within the isolated epithelium under these conditions. It was also found that EGF does not stimulate DNA synthesis or prevent medial epithelial cell death in the cultured palatal epithelium isolated at 13 or 14 days of gestation. EGF, however, did affect the morphology of the isolated epithelium; the nasal epithelial cells were more heavily ciliated and there were increased numbers of microfilament bundles within cells of the oral epithelium than were found in control cultures. These results suggest that EGF does affect the cells of the isolated palatal epithelium and that these effects are independent of the effect of EGF on cell proliferation in the palatal epithelium.

The results showed further that EGF does stimulate DNA synthesis in palatal epithelium cultured transfilter to palatal mesenchyme, indicating that the palatal epithelium requires the presence of the mesenchyme in order to respond to the mitogenic effects of EGF. The data do not rule out the possibility that EGF may exert its effect on the epithelium indirectly by acting upon the mesenchyme. Results from other studies, however, indicate that it is more likely that EGF is acting directly upon the epithelium and that the mesenchyme provides a substrate for the epithelial cells that allows the epithelium to respond to the mitogenic effect of EGF. It has been shown in a variety of instances that the substrate of an epithelium is important in determining the epithelial sensitivity to EGF. EGF has been shown to have a mitogenic effect on chick epidermis (Cohen, 1964), rat mammary gland epithelium (Turkington, 1969), and corneal epithelium (Savage & Cohen, 1973) when these epithelia are grown in combination with their mesenchyme. Further investigations of corneal epithelium have shown that corneal epithelial cells respond to EGF when grown on a feeder layer of fibroblasts (Rheinwald & Green, 1977) or on a collagen substrate (Gospodarowicz, Greenburg & Birdwell, 1978; Liu & Karaseck, 1978) but do not respond to EGF when grown in isolation on a plastic substrate (Gospodarowicz et al. 1978).

The mechanism by which EGF interferes with the programmed cell death of the medial palatal epithelium has not been determined, though our studies
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indicate that this effect of EGF is dependent upon the presence of mesenchyme. It has been shown that dibutyryl cyclic AMP (db-cAMP) and theophylline block the effect of EGF on medial palatal epithelium (Hassell & Pratt, 1977). Since it is presumed that the effect of db-cAMP and theophylline on the palate is to increase intracellular levels of cAMP, it was suggested that EGF may act by lowering the levels of epithelial cAMP allowing the medial cells to escape their terminal differentiation (Hassell & Pratt, 1977). It has been shown that db-cAMP can cause precocious degradation of the medial palatal epithelium of intact palates in vitro (Pratt & Martin, 1975).

Recent findings that EGF receptors appear in the embryonic mouse on the 11th–12th days of gestation and can be detected in the palatal epithelium on the 13th day of gestation by autoradiographic localization (Nexo et al. 1979) suggests that EGF may be involved in embryonic epithelial growth and differentiation. It has also been shown that an embryonic EGF, distinct from the maternal mouse EGF, appears during organogenesis (11 to 12 days of gestation) (Nexo et al. 1979), which further suggests that EGF plays a role during embryogenesis in epithelial growth and differentiation.

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REFERENCES


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