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Inhibition of Programmed Cell Death in Mouse Embryonic Palate *in Vitro* by Cortisol and Phenytoin: Receptor Involvement and Requirement of Protein Synthesis (41731)

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Abstract. In an *in vitro* model cortisol and phenytoin inhibit the precisely timed process of palatal development, the lysosomally mediated cell death of the medial edge palatal epithelium. This inhibition of programmed cell death of the palatal midline epithelium by each drug is virtually completely blocked by the antiglucocorticoid, cortexolone, whose blocking action results from competitive binding of the glucocorticoid receptor site. The inhibition produced by each of these drugs is prevented by the protein synthesis blocker, cycloheximide. Thus, blockade of programmed cell death by each of these drugs involves the glucocorticoid receptor site and requires protein synthesis.

In mammals after elevation of the embryonic palatal shelves from a vertical to a horizontal position over the tongue, fusion of the palatal processes takes place in the midline in two phases: (a) contact and adherence of the two edges with formation of an epithelial seam and (b) epithelial breakdown followed by mesenchymal confluence (1). These developmental changes have been experimentally reproduced in culture by explanting paired palatal shelves (2, 3). In addition, the process of epithelial breakdown has been demonstrated in single palatal shelves *in vitro* and appears in culture at a time corresponding to the same event *in vivo* (4–6). This programmed cell death of the palatal epithelium is mediated by the intracellular release of hydrolytic enzymes from lysosomes (7–9). Cortisol prevents medial edge breakdown and lysosomal activity in single shelves *in vitro* (10, 11). *In vivo,* cortisol also prevents medial edge breakdown and alters the synthesis and/or release of lysosomal enzymes in the medial edge epithelia of the palatal shelves of glucocorticoid-sensitive mice (11, 12).

It is generally believed that the mechanism of the anti-inflammatory action of glucocorticoid hormones includes as an initial step, the specific binding of the steroid to the receptor proteins in the cytosol fraction obtained from target tissues (13). The resulting steroid–receptor complex then undergoes a temperature-dependent translocation to the nucleus where it interacts with specific nuclear sites (14). The latter interaction triggers transcriptional changes resulting in the synthesis of a specific protein(s) which ultimately leads to the observed anti-inflammatory or teratogenic responses. This action can be blocked by glucocorticoid receptor antagonists, such as the antiglucocorticoid, cortexolone (15, 16), and by inhibitors of protein synthesis, such as cycloheximide (17, 18). The present report presents the results of *in vitro* studies with the embryonic mouse palate demonstrating that the blockade of programmed cell death by cortisol can be prevented by cortexolone and cycloheximide.

Several recent studies indicate that the production of cleft palate by 5,5-diphenylhydantoin (DPH) has a common pathway with that produced by glucocorticoids (19). When given *in vivo* with cortisone, DPH does not increase the frequency of cleft palate (20), and a probit analysis of cleft palate produced by DPH and cortisone indicates an identical mechanism (21). Both drugs delay shelf elevation from a vertical to a horizontal position (22). Susceptibility to cleft palate induced by both drugs is influenced by the *H-2* and *H-3* histocompatibility loci (23, 24). In our first study of the association between DPH teratogenicity and the glucocorticoid receptor, we demonstrated the blocking of [^{3}H]dexamethasone incorporation into human embryonic palatal cells by 0.5 μM DPH (25). Recently, we have

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presented evidence that DPH and glucocorticoids bind to a common receptor which is responsible for the inhibition of prostaglandin generation and the teratogenic effects of these drugs (19). In view of these findings, we demonstrate in this report that DPH, like cortisol, inhibits the programmed cell death in the palatal medial edge epithelium *in vitro* and that this inhibition is also reversed by cortexolone and cycloheximide.

Materials and Methods. CD-1 (Crl; CD-1(1CR)BR; Charles River Breeding Laboratory) male and female mice were maintained in a 12-hr light cycle with the dark phase extending from 5 AM to 5 PM. Females were mated with males of the same strain from 10 AM to 2 PM.

Single palatal shelves from 13-day, 0-hr (±2 hr) embryos were dissected in minimal essential medium with Earle's salts (MEM; Flow Laboratories). Left and right shelves from each embryo were routinely used to compare control and test responses. Each shelf was explanted oral side up on a Millipore filter (0.45 μm; Millipore Corp.). The filter was supported by a glass ring in a well of a depression slide. The culture medium in the well (approximately 0.75 ml) consisted of 10% fetal bovine serum (Flow Laboratories) and 2% penicillin–streptomycin mixture (5000 units-μg/ml; Flow Laboratories) in MEM. Cortisol (cortisol-21-phosphate; Sigma Chemical Co.) and cycloheximide (Sigma Chemical Co.) were each solubilized in MEM, sterilized by Millipore filtration, and included in the culture medium as 1% additions. DPH (Phenytoin, 50 mg/ml; Elkins-Sinn, Inc.) was diluted in MEM and included as a 1% addition. An appropriate aliquot of the DPH solvent (40% propylene glycol, 10% ethanol, and 50% H_2O, pH 12) was included in DPH controls. Cortexolone (Reichstein's Substance S; Sigma Chemical Co.) was solubilized in absolute ethanol (1 mg/ml) and included as a 0.5% addition. In a few cases, cortexolone was included as a 1% addition in absolute ethanol. Controls received a 0.5% (or 1%) addition of absolute ethanol. Unpublished results show that absolute ethanol in volumes as high as 1% of the culture medium has no effect on the normal breakdown of the medial edge epithelium in cultured shelves. All additions to the culture medium were made at the expense of appropriate volumes of MEM. The cultures were gassed with 5% CO_2–95% air mixture and incubated at 37°C in a humidified atmosphere.

All shelves were collected after 48–60 hr of culture and fixed in a 4% formaldehyde–1% $CaCl_2$ solution (pH 7.1) for 12–24 hr. Thereafter, the shelves were dehydrated and then infiltrated and embedded in glycol methacrylate. Sections (approximately 2 μm) were prepared using glass knives. The sections were then stained with Lee's methylene blue–basic fuchsin which gives good differential staining of epithelial and mesenchymal cells in palatal shelves. Sections were routinely cut through the middle third of both control and test shelves (11). Epithelial breakdown in the shelves was defined as the complete loss of epithelial lining at the medial edge. Previous studies (10, 11) have demonstrated that a cultured shelf with an epithelial-free edge is easily differentiated histologically from a shelf with an intact medial edge epithelium. The medial edge of each shelf was routinely scored as epithelial-free or epithelial-bound by at least two experienced observers working independently.

Statistical comparisons were made using the χ^2 test.

Results. The medial edge epithelium of all control shelves broke down (Tables I and II). Similarly, the medial edge epithelium of shelves cultured in either cortexolone or cycloheximide alone showed 100% breakdown

TABLE I. EFFECTS OF CORTEXOLONE AND CYCLOHEXIMIDE ON MEDIAL EDGE EPITHELIAL BREAKDOWN IN THE PRESENCE AND ABSENCE OF CORTISOL

Group	Concentration (μg/ml)	Breakdown
Control	—	72/72[a] (100)[b]
Cortisol	0.1	12/23 (52)
Cortexolone	5	16/16 (100)
Cortisol plus cortexolone	0.1 5	15/15 (100)
Cycloheximide	2	9/9 (100)
Cortisol plus cycloheximide	0.1 2	9/9 (100)

[a] Ratio represents number of shelves with epithelial breakdown per total number of shelves.
[b] Percentage of shelves with epithelial breakdown.

TABLE II. EFFECTS OF CORTEXOLONE AND CYCLOHEXIMIDE ON MEDIAL EDGE EPITHELIAL BREAKDOWN IN THE PRESENCE OF DPH

Group	Concentration (μg/ml)	Breakdown
Control	—	43/43[a] (100)[b]
DPH	1	13/23 (57)
DPH plus cortexolone	1 5	10/11 (91)
DPH plus cycloheximide	1 2	9/9 (100)

[a] Ratio represents number of shelves with epithelial breakdown per total number of shelves.
[b] Percentage of shelves with epithelial breakdown.

(Table I). In contrast, the epithelium of shelves cultured with cortisol broke down only 52% of the time ($P < 0.001$, Table I); while those cultured with DPH broke down only 57% of the time ($P < 0.001$, Table II). The medial edge epithelium of shelves cultured in cortisol plus cortexolone and cortisol plus cycloheximide broke down 100% of the time ($P < 0.001$, $P = 0.01$, respectively, Table I). Similarly, the medial edge epithelium of shelves cultured with DPH and cortexolone broke down 91% of the time ($P = 0.04$) while that in DPH plus cycloheximide broke down 100% of the time ($P = 0.02$, Table II).

Cortexolone-treated shelves were histologically indistinguishable from controls. On the other hand, cycloheximide (2 μg/ml) inhibited the normal development of the mesenchymal cell population in cultured shelves. Interestingly, cycloheximide did not prevent the programmed loss of the medial edge epithelium (Fig. 1 and Table I). The oral and nasal epithelia remained intact in the presence of cycloheximide indicating that the loss of the medial edge epithelium was a physiological event and not due to general tissue breakdown. The failure of cycloheximide to block breakdown of the palatal epithelium disagrees with observations made in an earlier study by Pratt and Greene (27). In that report, cycloheximide (1 and 10 μg/ml) prevented epithelial loss in cultured shelves from rat fetuses. These differences may reflect different sensitivities to cycloheximide in tissues from mice and rats. In any case, the results from the present study with fetal mice suggest that protein synthesis is not required for palatal epithelial cell death.

Discussion. The present results demonstrate that cortisol prevents breakdown of the medial edge epithelial cells. Cortisol is somewhat less effective as an inhibitor of breakdown than previously reported for shelves from CD-1 embryos (10, 11). However, this appears to correlate with the *in vivo* observation (unpublished data) that glucocorticoid-induced clefting percentages for the CD-1 strain are presently at 66% in contrast to the 90% reported in an earlier study (26). Despite these differences, cortexolone completely antagonizes the inhibition by cortisol thereby implicating involvement of the glucocorticoid receptor in this developmental event. Similarly, cycloheximide also completely prevents the inhibition by cortisol implicating a requirement of protein synthesis for this teratogenic action of cortisol *in vitro*. Similar conclusions were drawn from the observation that cycloheximide depressed the production of cleft palate by glucocorticoids when administered concurrently in mice *in vivo* (28).

It is particularly interesting that DPH also blocks breakdown of the medial edge epithelial cells *in vitro* and that this blockade also appears to be mediated by the glucocorticoid receptor in the embryonic palatal shelf and to require protein synthesis.

Susceptibility to cortisone- and DPH-induced cleft palate in the mouse is regulated in part by two genes acting by complementation within the H-2 histocompatibility complex on chromosome 17 (23, 24). The same genetic region also appears to influence the level of glucocorticoid receptors in mouse embryonic palatal cells (29, 30) and lungs and thymuses (31). Recently, we have found that susceptibility to cortisone- and DPH-induced cleft palate in mice is also influenced in part by a gene(s) within the *H-3* histocompatibility complex on chromosome 2 (23, 24).

The present report indicates that inhibition of programmed cell death of the medial edge epithelium is one common site of the action of both glucocorticoids and DPH. The present report also provides evidence that the glucocorticoid receptor is a biochemical site of the teratogenic action of each drug. It further suggests that each drug produces a protein(s) necessary for this action.

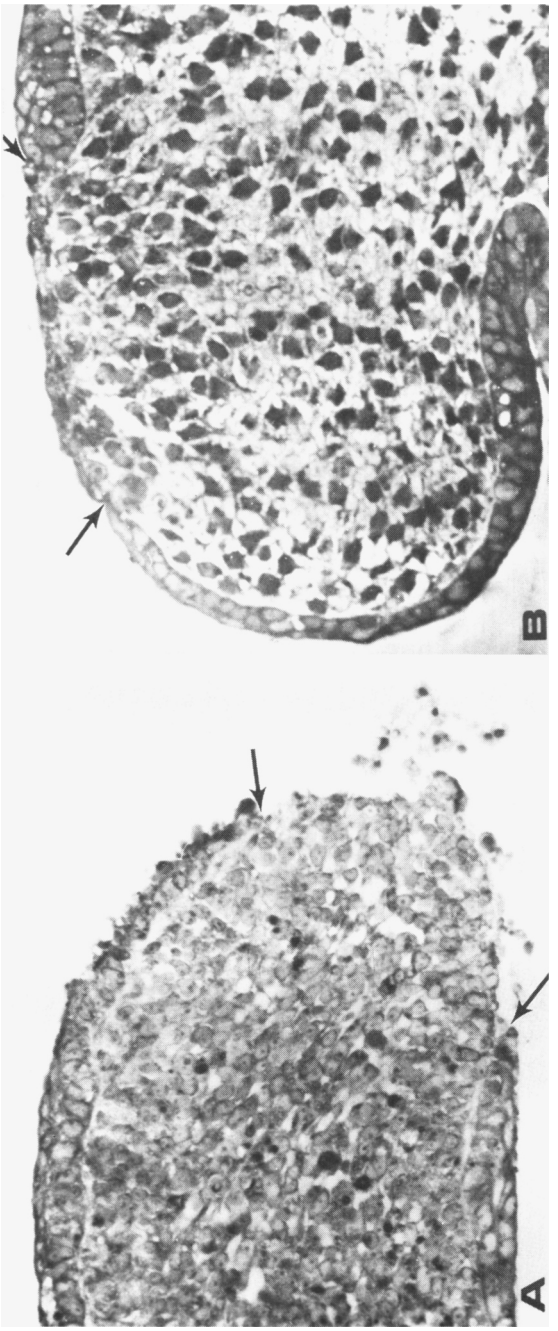

FIG. 1. This figure shows left (A) and right (B) shelves from a 13-day fetus cultured in medium with 2 μg cycloheximide/ml (A) or medium alone (B) for 56 hr. The epithelium at the medial edge (between arrows) has degenerated and been sloughed in both shelves: oral epithelium (above) and nasal epithelium (below) persist. As shown in the control (B), the medial edge is not always maintained precisely in its normal anatomical position in cultured shelves. The mesenchymal core of the cycloheximide-treated shelf has comparatively little extracellular matrix and thus shows crowding of the mesenchymal cells. There is also a relative increase in mesenchymal cell death and in number of macrophages in the cycloheximide-treated shelf. (A) and (B) typify the histological features observed in pairs of shelves from nine fetuses.

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1. Greene RM, Pratt RM. Developmental aspects of secondary palate formation. J Embryol Exp Morphol **36**:225–245, 1976.
2. Pourtois M. Onset of acquired potentiality for fusion in the palatal shelves of rats. J Embryol Exp Morphol **16**:171–182, 1966.
3. Vargas VI. Palatal fusion in vitro in the mouse. Arch Oral Biol **12**:1283–1288, 1967.
4. Smiley GR, Koch WS. An in vitro and in vivo study of single palatal processes. Anat Rec **173**:405–416, 1972.
5. Tyler MS, Koch WS. In vitro development of palatal tissues from embryonic mice. I. Differentiation of the secondary palate from 12 day mouse embryos. Anat Rec **182**:297–304, 1975.
6. Tyler MS, Koch WS. In vitro development of palatal tissues from embryonic mice. II. Tissue isolation and recombination studies. J. Embryol Exp Morphol **38**:19–36, 1977.
7. Mato M, Aikawa E, Katagira M. Alterations of the fine structure of the epithelium on the lateral palatine shelf during the secondary palate formation. Gunma J Med Sci **15**:79–99, 1967.
8. Angelici DR, Pourtois M. The role of acid phosphatase in the fusion of the secondary palate. J Embryol Exp Morphol **20**:15–23, 1968.
9. Greene RM, Pratt RM. Inhibition of epithelial cell death in the secondary palate in vitro by alteration of lysosome function. J Histochem Cytochem **26**:1109–1114, 1978.
10. Herold RC, Futran N. Effect of cortisol on medial edge epithelium of organ cultured single palatal shelves from steroid susceptible mouse strains. Arch Oral Biol **25**:423–429, 1980.
11. Goldman AS, Herold RC, Piddington RL. Inhibition of programmed cell death in the fetal palate by cortisol. Proc Soc Exp Biol Med **166**:418–424, 1981.
12. Greene RM, Kochhar DM. Spatial relations in the oral cavity of cortisone-treated mouse fetuses during the time of secondary palate closure. Teratology **8**:153–162, 1973.
13. Raspe G. Schering workshop on steroid hormone receptors. Advances in the Biosciences. Pergamon, Oxford, Vol 7, 1971.
14. Baxter JD, Rousseau GG, Benson MC, Garcea RL, Ito J, Tomkins GM. Role of DNA and specific cytoplasmic receptors in glucocorticoid action. Proc Natl Acad Sci USA **69**:1892–1896, 1972.
15. Kaiser N, Milholland RJ, Turnell RW, Rosen F. Cortexolone: Binding to glucocorticoid receptors in rat thymocytes and mechanism of its antiglucocorticoid action. Biochem Biophys Res Commun **49**:516–521, 1972.
16. Turnell RW, Kaiser N, Milholland RJ, Rosen F. Glucocorticoid receptors in rat thymocytes. Interaction with the antiglucocorticoid cortexolone and mechanism of its action. J Biol Chem **149**:1133–1138, 1974.
17. Hirata F, Schiffman E, Venkatasubramanian K, Salomon D, Axelrod J. A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. Proc Natl Acad Sci USA **77**:2533–2536, 1980.
18. Blackwell GJ, Carnuccio R, DiRosa M, Flower RJ, Parente L, Persico P. Macrocortin: A polypeptide causing the antiphospholipase effect of glucocorticoids. Nature (London) **287**:147–149, 1980.
19. Katsumata M, Gupta C, Baker MK, Sussdorf CE, Goldman, AS. Diphenylhydantoin: An alternative ligand of a glucocorticoid receptor affecting prostaglandin generation in A/J mice. Science **218**:1313–1315, 1982.
20. Fritz H. The effect of cortisone on the teratogenic action of acetylsalicylic acid and diphenylhydantoin in the mouse. Experientia **32**:721–722, 1976.
21. McDevitt JM, Gautieri RF, Mann DE Jr. Comparative teratogenicity of cortisone and phenytoin in mice. J Pharm Sci **70**:631–634, 1982.
22. Walker BE. Palate morphology and fetal movements after DPH. J Dent Res **58**:1740–1747, 1979.
23. Gasser DL, Mele L, Lees DD, Goldman AS. Genes in mice that affect susceptibility to cortisone-induced cleft palate are closely linked to Ir genes on chromosomes 2 and 17. Proc Natl Acad Sci USA **78**:3147–3150, 1981.
24. Goldman AS, Baker MK, Gasser DL. Susceptibility to phenytoin-induced cleft palate in mice is influenced by genes linked to *H-2* and *H-3*. Immunogenetics, **18**: 17–22, 1983.
25. Goldman AS, Shapiro BH, Katsumata M. Human foetal palatal corticoid receptors and teratogens for cleft palate. Nature (London) **272**:464–466, 1978.
26. Jacobs RM. Preliminary survey of the effects of cortisone upon palate formation, litter size and fetal weight in CD-1 strain of mice. J Dent Res **43**:715, 1964.
27. Pratt RM, Greene RM. Inhibition of palatal epithelial cell death by altered protein synthesis. Dev Biol **54**:135–145, 1976.
28. Zimmerman EF, Andrew F, Kalter H. Glucocorticoid inhibition of RNA synthesis responsible for cleft palate in mice; A model. Proc Natl Acad Sci USA **67**:779–785, 1970.
29. Goldman AS, Katsumata M, Yaffe SJ, Gasser DL. Palatal cytosol cortisol binding protein associated with cleft palate susceptibility and H-2 genotype. Nature (London) **265**:643–645, 1977.
30. Katsumata M, Baker MK, Goldman AS, Gasser GL. Influence of H-2-linked genes on glucocorticoid receptors in the fetal mouse palate. Immunogenetics **12**:443–450, 1981.
31. Gupta C, Goldman AS. H-2 histocompatibility region: Influence on the murine glucocorticoid receptor and its response. Science **216**:994–996, 1982.

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