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Further Evidence for a Role of Arachidonic Acid in Glucocorticoid Teratogenic Action in the Palate (41745)

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Abstract. Arachidonic acid produces a significant reversal of the production of cleft palate by cortisone in the offspring of sensitive strains of mice *in vivo*. Arachidonic acid in nanogram per milliliter concentrations also produces a significant reversal of the cortisol inhibition of the programmed cell death of the medial edge epithelium of palatal shelves *in vitro*. This corrective action of arachidonic acid *in vitro* is significantly blocked by indomethacin at a nanogram per milliliter concentration which selectively inhibits the conversion of arachidonic acid to prostaglandins and/or thromboxanes at the level of cyclooxygenase. These results support the hypothesis that the inhibition of arachidonic acid release and subsequent prostaglandin and/or thromboxane production by glucocorticoids is involved in the teratogenic action of glucocorticoids and demonstrate that one site of this action is the inhibition of epithelial loss.

A positive correlation has been demonstrated between the anti-inflammatory potencies of glucocorticoids and their ability to inhibit prostaglandin synthesis (1). The mechanism of this inhibition involves a blockade of the release of the rate-limiting prostaglandin or thromboxane precursor, arachidonic acid, at the level of phospholipase A_2 which catalyzes the hydrolysis of arachidonic acid from phospholipids (2–4). Arachidonic acid is then converted into prostaglandins (and thromboxanes) by cyclooxygenase (5). These effects are mediated by glucocorticoid-induced polypeptides or proteins which inhibit the activity of phospholipase A_2 (6, 7). Exogenous arachidonic acid reverses the glucocorticoid-induced depression of prostaglandin production in anaphylactic lungs (2) and inflamed synovia (8), and reverses the inhibition by glucocorticoids of rat paw edema induced by carrageenin (9). Glucocorticoids are also well-known teratogens producing cleft palate in rodents in accordance with their anti-inflammatory potency (10, 11). Thus, we have hypothesized that glucocorticoids may produce their palatal teratogenic defect by blocking the release of arachidonic acid.

Our first study to test this hypothesis showed that arachidonic acid significantly reduces the production of cleft palate in rats by dexamethasone and that this corrective effect of arachidonic acid is blocked by indomethacin (12). By using [^{3}H]arachidonic acid as a tracer we also showed that dexamethasone treatment depressed significantly the free [^{3}H]arachidonic acid available to the microsomal cyclooxygenase in the fetal upper and lower jaws including the palate at the critical period of development.

The degeneration and loss of the medial edge epithelium is an important palatal change which permits fusion of the shelves *in vivo* after elevation of the embryonic palatal shelves from a vertical to horizontal position over the tongue (13). This epithelial change is mimicked in single palatal shelves *in vitro* and occurs at times corresponding to the same event *in vivo* (14–16). The breakdown of the epithelium *in vivo* and *in vitro* depends on the programmed appearance of hydrolytic enzymes from lysosomes (17, 18). Glucocorticoids prevent the loss of the epithelium *in vivo* and in single shelves *in vitro* by blocking the synthesis and/or release of these enzymes (19, 20). Thus, susceptibility to glucocorticoid-promoted palatal clefting is correlated with inhibition of programmed cell death in the medial edge epithelium *in vitro* and *in vivo*. All of these findings suggest that the single palatal shelf in culture provides a particularly convenient model for the examination of the role of arachidonic acid in the teratogenic action of glucocorticoids. The *in vitro* model obviously simplifies the problems of admin-

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istration of exogenous arachidonic acid in the intact pregnant animal. In this report we determine whether susceptibility to cleft palate produced in sensitive strains of mice by cortisol is reversed by arachidonic acid *in vivo* and whether the inhibition of programmed cell death by cortisol *in vitro* is reversed by arachidonic acid. We also determine whether such reversals may be prevented by the concurrent use of indomethacin *in vitro*.

Materials and Methods. Steroid-sensitive CD-1 (Crl; CD-1 (1CR)BR; Charles River Breeding Laboratory) and A/J (Jackson Laboratory) mice were maintained on 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.

For *in vivo* studies, pregnant females were injected subcutaneously on the 11th through 14th days of gestation. Cortisone (Sigma Chemical Co.) in dimethylsulfoxide (DMSO) was added in daily doses of 100 mg/kg for CD-1 mice and 50 mg/kg for A/J mice. Arachidonic acid (5,8,11,14-eicosatetraenoic acid (free acid); Sigma Chemical Company) was injected simultaneously with the steroid in a concentration of 200 mg/kg for both strains. Cortisone and arachidonic acid were injected at different subcutaneous sites. Controls received appropriate volumes of DMSO. Fetuses were collected and examined at the 17th day to determine clefting percentages.

For *in vitro* studies, single palatal shelves from 13-day (±2 hr) CD-1 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 black Millipore filter (0.45 μm; Millipore Corp.). The filter was supported by a glass ring in the 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 of each/ml; Flow Laboratories) in MEM. Serum stimulates prostaglandin synthesis in cultured cells (21). However, neither serum nor antibiotics affect epithelial breakdown in this *in vitro* model (unpublished data). Cortisol (cortisol-21-phosphate; Sigma Chemical Co.) was solubilized in MEM, sterilized by Millipore filtration, and included in the culture medium as a 1% addition. Arachidonic acid was diluted with ethanol and included as a 0.25% addition. Similarly, indomethacin (Sigma Chemical Co.) was dissolved in ethanol and included as a 0.25% addition. The ethanol concentration was adjusted to 0.5% in media containing only arachidonic acid or indomethacin. Controls received a 0.5% addition of ethanol. Ethanol as a 0.5% addition has no effect on control responses in cultured shelves (unpublished data). 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 to 60 hr of culture and fixed in a 5% formaldehyde–1% $CaCl_2$ solution (pH 7.1) for 12 to 24 hr. Thereafter, the shelves were dehydrated and then infiltrated and embedded in glycol methacrylate. Sections (approximately 2 μm) were prepared using glass knives. Sections were then stained with Lee's methylene blue–basic fuchsin which gives good differential staining of epithelial and mesenchymal cells in palatal shelves. The medial edge epithelium in the middle third of the palatal shelf shows maturational changes earlier than the medial edge epithelium at anterior and posterior borders. Therefore, sections were routinely cut through the middle third of both control and test shelves. Epithelial breakdown in the shelves was defined as the complete loss of epithelial lining at the medial edge. Previous studies (19, 20) have demonstrated that a cultured shelf with an epithelial-free edge is easily differentiated histologically from a shelf with an intact medial edge epithelium (see also Fig. 2 in the present report). The medial edge of each shelf was routinely scored as epithelial-free or epithelial-bound by at least two experienced observers working independently.

It has been argued convincingly that the litter rather than the fetus is the proper unit of measurement in teratology (22). Therefore all tests of significance *in vivo* used the Mann–Whitney *U* test. The significance of effects in palatal cultures was tested by chi-square.

Results. *Arachidonic acid reversal of cortisone-induced clefting in vivo.* Cortisone injected into pregnant CD-1 and A/J mice elicited fetal clefting percentages of 66 and 79%,

respectively (Table I). Simultaneous injection of arachidonic acid with cortisone reduced significantly palatal clefting to 45% for CD-1 fetuses and to 35% for A/J fetuses. Resorption percentages ranged from 32 to 44%. There was no consistent effect of arachidonic acid on the steroid-promoted resorption.

Arachidonic acid reversal of the cortisol inhibition of epithelial breakdown in single palatal shelves in vitro. The medial edge epithelium broke down in control shelves *in vitro* (Figs. 1 and 2A). In contrast, epithelial breakdown was inhibited in cortisol-treated shelves (Figs. 1 and 2B). Arachidonic acid reversed the cortisol inhibition of breakdown and did so in a dose–response manner (Figs. 1 and 2C). This reversal was statistically significant ($P < 0.01$; chi-square) for doses of arachidonic acid ranging from 0.1 to 10 μg/ml. Preliminary results with shelves from steroid-sensitive A/J mice show similar *in vitro* responses to cortisol and cortisol plus arachidonic acid. In CD-1 shelves, arachidonic acid alone inhibited some breakdown but without an evident dose–response pattern. Arachidonic acid in concentrations ranging from 0.0001 to 10 μg/ml permitted on average, only 44% of the shelves to undergo the normal developmental loss of the medial edge epithelium.

Indomethacin blockade of the arachidonic acid reversal of steroid-inhibited breakdown in vitro. In cultured shelves, indomethacin at a dose of 0.1 μg/ml overcame significantly ($P \simeq 0.03$; chi-square) the ability of arachidonic acid (0.1 μg/ml) to reverse the cortisol inhibition of breakdown (Figs. 1 and 2D); interestingly, higher as well as lower concentrations of indomethacin were less effective. Indomethacin in low doses (0.01, 0.001, and 0.0001 μg/ml) yielded breakdown percentages of 50, 60, and 71%, respectively. These percentages were not significantly different than the 62% breakdown elicited by cortisol and arachidonic acid (0.1 μg/ml) without indomethacin (Fig. 1). The ineffectiveness of indomethacin in the high-dose range may reflect its inhibitory effects on cyclic nucleotide phosphodiesterase (see Discussion). Indomethacin tested alone inhibited 50 to 57% breakdown in the concentration range of 0.1 to 10 μg/ml.

Discussion. The results presented here suggest that the single shelf in culture may be useful in examining the biochemical mechanisms underlying the teratogenic action of glucocorticoids in inhibiting programmed cell death in the absence of maternal influences. The culture model mirrors changes *in vivo* and thereby provides data which can be assumed, with some confidence, to have relevance for palatal development during the embryonic and fetal periods. Glucocorticoid injected into steroid-sensitive pregnant mice causes palatal clefting in their fetuses. Arachidonic acid injected simultaneously with the steroid significantly reduces fetal clefting, supporting our previous observations in rats (12).

TABLE I. EFFECT OF ARACHIDONIC ACID ON CORTISONE-INDUCED CLEFTING IN STEROID-SENSITIVE MICE IN VIVO

	CD-1		A/J	
	Cortisone (100 mg/kg)	Cortisone (100 mg/kg) plus arachidonic acid (200 mg/kg)	Cortisone (50 mg/kg)	Cortisone (50 mg/kg) plus arachidonic acid (200 mg/kg)
Total number of fetuses	219	153	78	79
Total number of litters	18	13	10	10
Number of viable fetuses	123	98	53	49
Number of viable fetuses with cleft palate	81	44	42	17
Percentage of viable fetuses with cleft palate	66	45[a]	79	35[b]
Number of resorbed fetuses	96	55	25	30
Percentage of resorbed fetuses	44	36	32	38

[a] Statistical difference, $P < 0.05$ (Mann–Whitney U test).
[b] Statistical difference, $P = 0.005$ (Mann–Whitney U test).

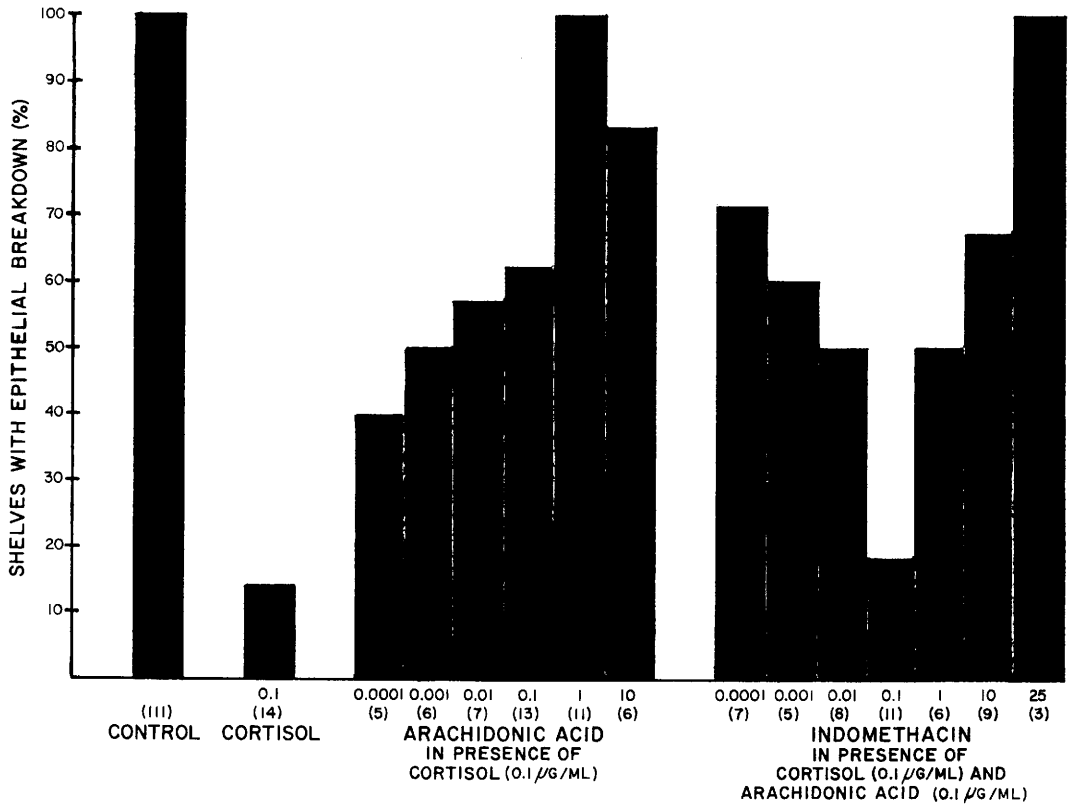

FIG. 1. Effects of cortisol, arachidonic acid, and indomethacin on the breakdown of the medial edge epithelium in single cultured shelves. Numbers immediately below bars indicate the concentrations (μg/ml) of additions (cortisol, arachidonic acid, or indomethacin) in the culture medium. Numbers in parentheses represent number of cultured shelves.

This reversal of glucocorticoid action is mimicked in the *in vitro* model using single palatal shelves from steroid-sensitive embryos. In culture, the medial edge epithelium of the palatal shelf normally undergoes autolysis and is lost. In the presence of cortisol, the epithelium is retained. With the addition of arachidonic acid to the cortisol-containing medium, the epithelial cells degenerate and are sloughed.

The culture model proved to be particularly useful for implicating the prostaglandin pathway in palatal development. In prostaglandin biosynthesis, arachidonic acid is transformed to prostaglandin precursors by the enzyme cyclooxygenase. Nonsteroidal anti-inflammatory drugs such as aspirin, phenylbutazone, and indomethacin inhibit cyclooxygenase and thereby block the synthesis of prostaglandins (5). Thromboxanes are also blocked. However, while mesenchymal cells from the embryonic palate have been shown to synthesize the prostaglandins PGE_2, $PFG_{2\alpha}$, and 6-KETO-$PGF_{1\alpha}$, the synthesis of thromboxanes remains to be confirmed (23, 24). When indomethacin is added to the culture medium in a concentration (0.1 μg/ml) that inhibits cyclooxygenase selectively (25), it significantly reverses the arachidonic acid correction of cortisol-inhibited breakdown. When a higher dose of indomethacin (10 μg/ml) is used under similar conditions, the reversal of the arachidonic acid correction is not significant. Indomethacin at 10 μg/ml has been shown to inhibit not only cyclooxygenase but cyclic nucleotide phosphodiesterase as well (25). Since cyclic nucleotide phosphodiesterase normally degrades cyclic AMP, the inhibition of phosphodiesterase could result in an accumulation of cyclic AMP levels in palatal cells. This possibility is of special interest since cyclic AMP levels normally increase in palatal shelves just prior to epithelial breakdown (26, 27). Preliminary

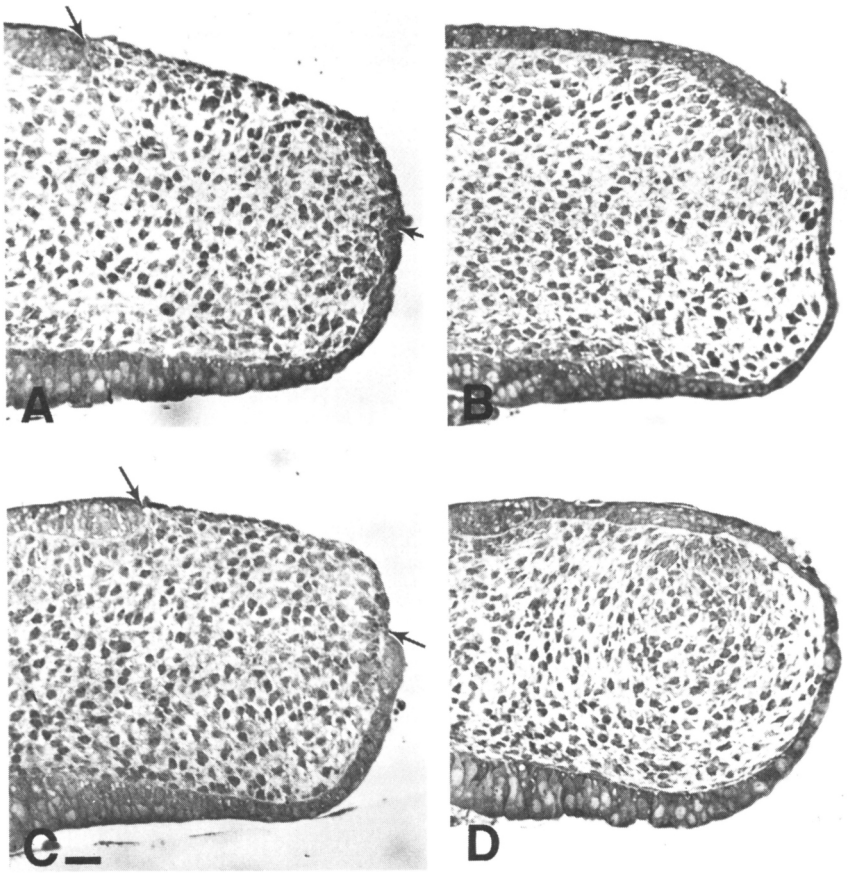

FIG. 2. Transverse sections of the medial edge of single palatal shelves cultured for 48–60 hr. In all figures, the upper surface is oral and the lower surface is nasal. (A) Control with an epithelial-free medial edge (between arrows). (B) Shelf treated with cortisol (0.1 μg/ml) which inhibits the breakdown of the medial edge epithelium. (C) Shelf in which the cortisol inhibition is reversed by arachidonic acid (0.1 μg/ml) resulting in the loss of epithelium at the medial edge (between arrows). As (A) and (C) illustrate, the palatal medial edge is not always maintained precisely in its normal anatomical position in single shelves in culture. (D) A shelf in which the arachidonic acid reversal of cortisol inhibition is blocked by indomethacin (0.1 μg/ml) resulting in the persistence of the medial edge epithelium. The magnification for all figures is the same; the bar in (C) represents 50 μm.

findings indicate that dibutyrylcyclic AMP reverses the inhibition of epithelial loss by steroid in cultured shelves (R. Piddington, R. Herold, and A. S. Goldman, unpublished data). Thus, indomethacin prevents the reversal of the clefting action of glucocorticoid by arachidonic acid *in vivo* (12) and *in vitro* (present report) at a concentration which selectively inhibits cyclooxygenase.

Phenylbutazone, another nonsteroidal anti-inflammatory drug, also acting at the level of cyclooxygenase promotes palatal clefting *in vivo* and has recently been shown to inhibit epithelial breakdown in cultured shelves (28). Indomethacin injected by itself *in vivo* does not produce cleft palate in fetal mice (29) probably due to its exceedingly low placental transfer (30). Indomethacin by itself in the *in vitro* model inhibits some breakdown. This inhibition, however, is less effective in preventing breakdown than indomethacin in the presence of cortisol and arachidonic acid. This suggests that either indomethacin may block cyclooxygenase less efficiently when endogenous arachidonic acid serves as substrate, or that pathways other than those implicating

prostaglandins or thromboxanes may also contribute to the loss of the medial edge epithelium. Cortisol inhibits phospholipase A_2 activity and thereby blocks arachidonic acid availability for the synthesis of leukotrienes as well as prostaglandins and thromboxanes. The possible involvement of leukotrienes in this developmental event remains uncertain pending their demonstration in fetal palatal cells, but it is possible that an indomethacin block of endogenous arachidonic acid may lead to excess leukotrienes.

The inhibition of epithelial breakdown by cortisol and reversal of this effect by arachidonic acid suggest that the steroid acts, as demonstrated elsewhere (2–4), by preventing the release of arachidonic acid from phospholipids. Further it suggests the possibility, as shown in other cells (5, 7), that this inhibition by cortisol is directed against phospholipase A_2 and mediated by a polypeptide or protein induced by the steroid. If these effects are true for the palate, the *in vitro* model could be used conveniently to monitor the actions of such a polypeptide or protein on epithelial breakdown.

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