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Receptor-Dependent Mechanisms of Glucocorticoid and Dioxin-Induced Cleft Palate

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Glucocorticoids (triamcinolone) and dioxins (TCDD) are highly specific teratogens in the mouse, in that cleft palate is the major malformation observed. Glucocorticoids and TCDD both readily cross the yolk sac and placenta and appear in the developing secondary palate. Structure-activity relationships for glucocorticoid- and TCDD-induced cleft palate suggest a receptor involvement. Receptors for glucocorticoids and TCDD are present in the palate and their levels in various mouse strains are highly correlated with their sensitivity to cleft palate induction. Receptors for glucocorticoids appear to be more prevalent in the palatal mesenchymal cells whereas those for TCDD are probably located in the palatal epithelial cells. Glucocorticoids exert their teratogenic effect on the palate by inhibiting the growth of the palatal mesenchymal cells whereas TCDD alters the terminal cell differentiation of the medial palatal epithelial cells.

Glucocorticoid-Induced Cleft Palate

Glucocorticoids administered to several species of experimental animals at midgestation inhibit complete formation of the secondary palate (*1-4*). Development of the mammalian secondary palate is a complex process that depends upon the presence of various hormones and growth factors (*5*). The palatal shelves first appear as outgrowths from the maxillary process and subsequently grow in a vertical position alongside the tongue. The shelves undergo a rapid reorientation to the horizontal position above the tongue which brings the apposing epithelia into contact at the midline. The medial epithelial cells then undergo a programmed cell death with resorption of the basement membrane and cell remnants, and the two shelves fuse into a single tissue, the secondary palate, which separates the oral and nasal cavities (*6,7*).

Different strains of inbred mice exhibit different degrees of susceptibility to glucocorticoid-induced cleft palate (*1,8*). All of the surviving offspring of A/J mice treated with glucocorticoids between days 11 and 15 of gestation have cleft palate, whereas C57BL/6J mice treated with the same dose produce 20 to 25% offspring with cleft palate (*8-10*). A number of mechanisms have been proposed to explain the different responses of various strains of mice to the teratogenic action of glucocorticoids (*5,11,12*). Walker and Fraser (*6*) showed that during normal development, the palatal shelves became horizontal later in the A/J than the C57BL/6J strain and suggested that this difference makes A/J mice more susceptible to cortisone than C57BL/6J mice (*13*). A recent study utilizing frozen sections (*14*) showed that, in addition to delaying palatal shelf elevation, cortisone treatment severely reduced the extent of contact between the palatal shelves in A/J mice. Although contact was present in 20% of the cortisone-treated fetuses, the mean area of the shelf contact was only 20% of that in control palates. Therefore, the net shelf contact in cortisone-treated litters was only 4% of the potential contact in control litters. Other studies in which shelf contact was assessed in glucocorticoid-treated fetuses reported contact in 27-30% of the fetuses. However, this limited contact, observed between the shelves in the midpalate region, was considered adequate for complete palatal fusion, and a failure in the fusion mechanism was proposed as an alternative (*4,15*). Diewert and Pratt (*14*) also observed shelf contact in the midpalate region; however, the shelves remained separated in the anterior and posterior regions. Since contact between the shelves normally occurs first in the midpalate region, some contact in this region would be expected with cortisone treatment; the presence of a small area of contact in some cortisone-treated palates cannot be considered equivalent to the extensive contact present in controls.

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Studies of effects of glucocorticoids on the palatal epithelium suggested that there may be significant differences between tissue responses *in vitro* and *in vivo*. Recent investigations of the *in vivo* effects of triamcinolone on palatal epithelium in the sensitive CF1 mouse line showed that degenerative events were delayed, but not prevented (*16,17*). These results suggested that effects of glucocorticoid on degenerative changes in the epithelium were not responsible for palatal clefting *in vivo*. However, studies of glucocorticoids *in vitro* were reported to cause changes in the medial epithelium. Cortisol was reported to prevent medial epithelial breakdown in the sensitive A/J strain, but not in the resistant C57BL/6J strain (*18*). Based on the results of previous *in vitro* studies showing palatal fusion in the presence of hydrocortisone (*19,20*), differences in culture conditions appear to have significant effects on the results. Therefore, although the palatal epithelium may be affected by teratogens *in vitro*, these effects are not similar to the major effect contributing to development of cleft palate *in vivo*.

Other possible explanations of strain differences in response to glucocorticoid-induced cleft palate are metabolism, level of endogenous steroids, and differential distribution of the steroid in maternal and fetal tissues. Additionally, there might be differences in sensitivity of embryonic facial structures toward the steroid. Variations in the endogenous levels of glucocorticoids in sensitive and resistant strains were not correlated with the sensitivity of the strains to steroids; there were not significant differences between A/J and C57BL/6J mice in the concentration of maternal or fetal corticosterone during midgestation (*21*). Glucocorticoids can cross the rodent placenta without being metabolized. When administered in pharmacologic doses to pregnant mice, a large fraction of the administered steroid reached embryos unmetabolized and appeared to function as the active teratogenic agent (*22*).

Glucocorticoid effects in adult and fetal tissues are mediated through the interaction of the hormone with specific intracellular receptor proteins in the cytoplasmic fraction of target cells. Schmidt and Litwack (*23*) proposed a five-step model for activation of the glucocorticoid-receptor complex. The receptor, which is a protein of 87,000 molecular weight (*24*) is present in the cytoplasm in a form that is phosphorylated, contains reduced sulfhydryl groups, and is capable of binding glucocorticoid. The unbound receptor may have associated with it a low molecular weight, heat-stable factor that maintains the protein in a conformation favorable for steroid binding and inhibits the subsequent activation. The phosphorylated receptor protein binds glucocorticoid to form an unactivated complex. This complex then undergoes a conformational change (activation) that results in the exposure of positively charged amino acid residues and hence an increased affinity for polyanions like DNA. During the activation step, the receptor or another component becomes dephosphorylated. Likewise, activation also may involve the dissociation of a low molecular weight, heat-stable factor which, when associated with the receptor, blocks activation. The activated glucocorticoid-receptor complex then translocates to the nucleus where it binds to chromatin acceptor sites and alters the transcription of specific genes. The steroid eventually dissociates from the nuclear receptor protein, and may then be recycled to the cytoplasm where it can be either degraded or reactivated with the heat-stable factor.

The appearance in specific embryonic tissues of cytoplasmic glucocorticoid receptors at discrete developmental stages has been implicated as one factor that determines the appearance of hormonal responsiveness in these tissues (*25*). Likewise, the presence of functional nuclear acceptor proteins for the steroid-receptor complex is also required for embryonic target tissues about to undergo hormone-dependent differentiation (*26, 27*).

Most evidence suggests that glucocorticoid teratogenicity is due to direct action on the embryo. The total binding of labeled glucocorticoid to palatal and other embryonic tissue proteins varied in different strains of mice and correlated with the teratologic responsiveness of the strains to glucocorticoids. Salomon and Pratt (*5,28-30*) demonstrated that mouse embryonic palatal mesenchyme cells possess high affinity, specific receptor proteins for glucocorticoid. The synthetic glucocorticoids dexamethasone and triamcinolone acetonide (TA) have a higher affinity for these receptors than the natural glucocorticoid hydrocortisone (HC), and a structure-activity study has shown that their affinity correlated well with their teratogenicity (*31*); TA was found to be 40 times as potent as HC and twice as potent as DEX (*32*).

Although the glucocorticoid receptors in embryonic mouse palatal shelves have not been biochemically characterized, various properties of receptors prepared from whole day-13 A/J and C57BL/6J mouse embryos have been studied (*32*). Embryonic cytosols from both strains were found to contain high affinity, limited capacity binding proteins for dexamethasone and triamcinolone acetonide, which bind both natural and synthetic glucocorticoids, and can be distinguished from serum corticosteroid binding globulin (transcortin) by their heat sensitivity, sedimentation, gel filtration properties, and sensitivity to sulfhydryl reagents. These embryonic receptor proteins appear to be indistinguishable from adult glucocorticoid receptor protein found in the liver.

Embryonic A/J facial mesenchyme cells, both freshly isolated and in primary culture, possessed two to three times more cytoplasmic receptors than C57BL/6J mesenchyme cells (*28*). Strain differences in the concentration of cytoplasmic receptors for glucocorticoids in the day-14 embryo were generally restricted to the orofacial region (*5*). Several investigators (*5,33,34*) showed that the levels of cytoplasmic glucocorticoid receptor in fetal palate and adult liver cytosols were correlated with sensitivity to glucocorticoid-induced cleft palate. In one case these results were not corroborated. Utilizing whole

mouse fetal heads and an unconventional method of cytosol preparation, Hackney (*35*) did not find a positive correlation between triamcinolone acetonide binding and cleft palate sensitivity in A/J, C3H and C57BL/6J mice.

Salomon and Pratt (*5*) reported that the concentration of glucocorticoid receptors in day-14 Swiss Webster mouse embryos was higher in the orofacial region than that observed in the liver, brain or forelimb. The biochemical basis for the increased level of receptors in the palate is not understood but may indicate that the developing secondary palate is dependent on a high level of glucocorticoid receptors for normal development. The location of these glucocorticoid receptors in the developing palate appears to be predominantly in the mesenchymal cells as revealed by autoradiographic and immunocytochemical methods (*35*).

Information concerning the level of glucocorticoid receptors present in the developing human palatal shelf is of great interest. Yoneda and Pratt (*37*) have established a line of human embryonic palatal mesenchymal cells (HEPM) from a human abortus in the prefusion stage of palatal development and found that these cells contained approximately tenfold higher levels of glucocorticoid receptors than primary cell cultures from mouse palatal shelves. However, it should be pointed out that comparison of established cell lines and primary cell cultures may not be valid. These HEPM cells have proved to be useful for understanding the complex hormonal regulation of palatal growth and differentiation.

Yoneda et al. (*38*) recently reported a study in which various fibroblastic cell lines, from tissues of individuals with cleft lip and/or cleft palate, were examined for level of glucocorticoid receptors. Skin punches were obtained from various affected and normal individuals in addition to oral tissue obtained during surgery from sites of lip or palate repair. A significant deficiency in glucocorticoid receptors was noted in the cell lines from clefted individuals. Although these tissues are older than the developing embryonic primary and secondary palate, the results were interpreted as indicating that a defect in the complex hormonal regulatory mechanism for embryonic growth may have occurred and persisted into various neonatal or adult tissues. Since this was a preliminary study with only a limited number of cell lines, this work must be expanded before definitive conclusions can be drawn.

Experimental modulation of receptor number or activity would provide further evidence of involvement in glucocorticoid-induced cleft palate. Yoneda and Pratt (*39*) have reported that the level of vitamin B_6 exerted a large influence of glucocorticoid-induced cleft palate in Swiss Webster mice. When the maternal vitamin B_6 level was increased by administration in the drinking water, cortisone-induced cleft palate decreased, in contrast with the increase in the cleft palate frequency observed when animals were placed on a vitamin B_6-deficient diet. This effect was interpreted as being due to the direct interaction in the palatal shelf of vitamin B_6 with the DNA binding site of the glucocorticoid receptor (*40*), thereby strongly influencing the amount of steroid-receptor complex that enters the nucleus and therefore also the resultant biological response.

The effects of glucocorticoids on primary cultures of mouse embryonic palatal mesenchyme cells have been studied *in vitro* (*29,41*). Glucocorticoid (at 10^{-6} to 10^{-8} M) caused a reduction in cell number and a simultaneous decrease in the incorporation of labeled thymidine in DNA in both A/J, C57BL/6J and human embryonic palatal mesenchyme cells. These cells synthesize collagen which constitutes approximately 7 to 10% of the total protein. Cells cultured in the presence of dexamethasone synthesized decreased amounts of both collagen and noncollagen proteins (*5*), but to a greater extent in A/J than in comparably treated C57BL/6J cells. A/J cells, possessing a higher level of glucocorticoid receptors, were also more sensitive to the inhibition of growth and of thymidine incorporation than C57BL/6J cells.

Differential effects on DNA synthesis and growth were observed in organ cultures of palatal shelves from A/J and C57BL/6J mice in the presence of dexamethasone or hydrocortisone (*42,43*). These results are in accord with observations that dexamethasone and cortisone inhibited the *in vivo* proliferation of mesenchymal cells of rat and mouse palatal processes (*3,44,45*). This inhibition of mesenchymal cell growth is a key factor in producing glucocorticoid-induced cleft palate (*14*) which is mediated by a receptor-dependent mechanism, presumably involving altered transcription of a key enzyme or protein in the palate (*46*). Recently, Grove et al. (*47*) provided evidence for a correlation between alteration in phosphatidylinositol turnover at the cell surface and growth inhibition induced by dexamethasone in human palatal mesenchymal cells.

Physiological levels of glucocorticoids (10^{-9} M) promote DNA synthesis and stimulate human and mouse palatal mesenchyme cell growth. Therefore, glucocorticoids at physiological concentrations either alone or through interactions with other hormones or growth factors may control certain stages of normal palatogenesis. Dexamethasone administered *in vivo* to A/J and C57BL/6J mice causes an increase in palatal glutamine synthetase which may be essential for expression of various mesenchymal or epithelial cell surface proteins (*48*).

Glucocorticoids are important regulators of carbohydrate, protein, and fat metabolism in adult tissues (*49*). Glucocorticoids also perform important functions during various stages of embryonic and fetal development (*50*). Glucocorticoids, either alone or with other hormones such as thyroxine, insulin, and growth factors, control the morphological and biochemical differentiation of a variety of fetal and postnatal tissues.

Dioxins and Their Receptors in Teratology

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) serves as the prototype for a large group of halogenated aroma-

tic hydrocarbons [chlorinated dibenzo-*p*-dioxin, dibenzofuran, azo(xy)benzene, biphenyl, and naphthalene isomers and brominated biphenyl isomers], which are all approximate isostereomers, produce a similar and characteristic pattern of toxic and biochemical responses, and are thought to act by a common mechanism (*51-53*).

The administration of TCDD (and congeners) to laboratory animals induces the coordinate expression of a battery of enzymes, including the activity of the cytochrome P-450-mediated microsomal monooxygenase aryl hydrocarbon hydroxylase in liver and a variety of other tissues (*54*). This pleiotropic response is initiated by the stereospecific, reversible binding of TCDD to a cytosolic protein, termed the induction receptor, and by the translocation of the ligand-receptor complex to the nucleus (*55-57*). In mice there is a genetic polymorphism at the locus that determines the receptor, the *Ah* locus (*58*). Inbred strains of mice carrying the Ah^b allele (e.g., C57BL/6J) have a high affinity receptor and are sensitive to the induction of AHH activity by TCDD; other inbred strains possessing the Ah^d allele (e.g., DBA/2) have a lower affinity receptor and are less sensitive to enzyme induction by TCDD. In crosses and backcrosses between C57BL/6J and DBA/2 mice, expression of the Ah^b allele is either dominant or codominant, depending on the assay used for phenotyping.

The toxic responses produced by halogenated aromatic hydrocarbons are quite characteristic; however, many of the lesions are highly species-specific (*59-61*). Following the administration of a lethal dose of TCDD or one of its congeners virtually all species experience a prolonged wasting syndrome prior to death, with loss of adipose tissue, involution of lymphoid organs, and degeneration of the seminiferous tubules of the testicle. In contrast, the more distinctive lesions involve proliferation and/or metaplasia of epithelial tissues such as skin, stomach, intestines, and urinary tract, and occur in a limited number of species. Similarly, the presence and severity of hepatic pathology vary considerably among species. The cause of death is unknown.

Two independent lines of evidence indicate that the toxic responses to TCDD and its congeners are mediated by their binding to the induction receptor. First, for a large number of halogenated aromatic hydrocarbons, the rank ordered structure-activity relation for their receptor affinity or their potency to induce AHH activity corresponds to their toxic potency (measured as the mean lethal dose, LD_{50}, or dose reported to produce a specific toxic response) (*51,53,62,63*). Second, toxic responses produced by TCDD in mice, e.g., thymic involution, cleft palate formation in the fetus, and hepatic porphyria, have been found to segregate with the *Ah* locus (*64,65*). In DBA/2J, RF/J, AKR/J, SWR/J, and 129/J, the five mouse strains with a low affinity receptor, TCDD produced 0 to 3% incidence of cleft palate. Four of the five strains with a high affinity receptor, C57BL/6J, A/J, BALB/cByJ and SEC/1REJ, developed a 54 to 95% incidence in response to TCDD. CBA/J was the only strain with a high affinity receptor that did not develop cleft palate and these mice are also resistant to cortisone-induced cleft palate (*65*). A recent study (*66*) has shown that β-naphthoflavone, which is another ligand of the *Ah*-receptor but which does not produce cleft palate by itself, was found to increase the frequency of TCDD-induced cleft palate when co-administered. In addition to the polysubstrate monooxygenase system, other enzyme systems not related to metabolic conversion of polycyclic hydrocarbons are considered to be structural gene products of the *Ah* locus. β-Naphthoflavone may induce a number of enzymes also stimulated by TCDD but in a different pattern and insufficient for cleft palate induction.

Galloway et al. (*67*) found that the inducible cytochrome P1-450 enzyme system is expressed in mouse embryos explanted from the uterus at day 7.5 or 8.5 of gestation. These results imply that the regulatory gene product (*Ah* receptor) and the structural gene product (cytochrome P1-450) are both functional from an early embryonic age. The *Ah* locus is most likely responsible for inducible benzo(a)pyrene metabolism and associated embryotoxicity and teratogenesis in the mouse embryo at or soon after day 7 of gestation.

Dencker and Pratt (*68*) showed that embryonic palatal shelves from C57BL/6J, a cleft palate-responsive mouse strain (*69*), have high levels of a specific cytoplasmic receptor for TCDD with similar biochemical characteristics as the adult liver. A nonresponsive strain, AKR/J, on the other hand, showed no specific TCDD binding in adult liver or any embryonic tissue and was resistant to the induction of cleft palate by TCDD (*67*). Studies using frozen sections (*70*) showed that TCDD-treated C57BL/6J palatal shelves made firm and extensive contact at their medial epithelium *in vivo*, in contrast with that found with A/J glucocorticoid-treated palatal shelves *in vivo* (*14*). TCDD administered on day 10 (*71*), at a teratogenic dose for the mouse palate, crossed the visceral yolk sac and/or chorioallantoic placenta and was present in the embryo at nanomolar concentrations, which is similar to the equilibrium binding constant (K_d) found in the palatal shelves (*68*).

Therefore, it appears that TCDD exerts a direct effect on the midgestation embryo to produce cleft palate. This effect appears to be limited to an alteration in the complex process of terminal cell differentiation of the medial epithelial cells. The known major effect of TCDD on human beings, i.e., chloracne, has also been ascribed to an effect on epidermal cell differentiation (*52*) and actually results in enhanced epidermal proliferation. It is surprising that *Ah* receptors would be present in embryos, since the known ligands, TCDD and benzo(a)pyrene, are not compatible with normal development. The *Ah* receptor-ligand complex may have once served in evolution as a hormone or growth factor for a variety of ectodermally derived tissues. If so, it is conceivable that the *Ah* receptor now exists in various

tissues without a natural ligand and is activated only when tissues are exposed to potent dioxins such as TCDD.

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