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Short Contribution

The role of RXR-α in retinoic acid-induced cleft palate as assessed with the RXR-α knockout mouse

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ABSTRACT Treatment of pregnant mice with retinoic acid (RA) in mid-gestation produces cleft palate and limb defects in the fetuses. RXR-α has been previously shown to mediate the teratogenic effects of RA in the limb. In this study, we show that RXR-α is also involved in retinoid-induced palatal clefting. Treatment of RXR-α knockout mice with a teratogenic dose of RA on gestation day 11 or 12 induces cleft palate at a lower frequency than that seen in wild-type animals.

KEY WORDS: *RXR-α, retinoic acid, cleft palate*

The developing murine secondary palate is susceptible to the teratogenic effects of retinoic acid (RA) (Kochhar and Johnson, 1965). While the molecular mechanisms by which RA induces cleft palate are largely unknown, RA is thought to exert its effects on tissue development by alteration in the expression of genes essential for that development (Hofman and Eichele, 1994). RA appears to modulate gene expression primarily through a family of nuclear receptors that act as DNA-binding transcription factors capable of binding to specific sequences (called retinoic acid response elements, RAREs) in the promoters of target genes (Giguere, 1994). There are two subfamilies of such receptors: the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each comprising multiple isoforms derived from differential splicing and/or alternative promoter usage (Leid *et al.*, 1993; Mangelsdorf *et al.*, 1993). Heterodimers of RARs and RXRs are considered the functional units that activate RAREs in cell culture systems, and more recent genetic studies argue for RXR/RAR heterodimers as the active dimers *in vivo* (Kastner *et al.*, 1997; Mascrez *et al.*, 1998). Functional interactions between RXRs and RARs are also suggested by synergistic effects of limiting concentrations of RXR-specific and RAR-specific agonists in the activation of retinoid target genes in EC cells (Roy *et al.*, 1995).

To determine the role of these specific receptors in embryonic development, the corresponding genes have been mutated via homologous recombination to create mice that are null for receptor expression, i.e. "gene knockout mice" (reviewed in Chambon, 1995). RAR-α and RAR-γ (all isoforms) null mutants exhibit some congenital malformations, while mutation of RAR-α1, RAR-γ2 or RAR-β produce no defects. In general, knockout of two RAR genes (double knockout) produces embryos with reduced viability; furthermore, the different RAR double mutants exhibit all of the malformations seen in fetal Vitamin A Deficiency (VAD) Syndrome malformations (Chambon, 1995). Ablation of all RAR-α and all RAR-γ isoforms together produces, among other malformations, a cleft palate, although knockout of RAR-α1 with all RAR-γ isoforms yields a normal palate (Lohnes *et al.*, 1994). Interestingly, double RXR knockout mutants do not produce as severe a set of malformations as produced by the RAR double knockouts (Krezel *et al.*, 1996). That RXR-α is functionally the most important RXR during development is suggested by the observation that severe developmental defects are seen in double mutants in which a RAR mutation is associated with a RXR-α, but not a RXR-β or RXR-γ, mutation (Krezel *et al.*, 1996; Kastner *et al.*, 1997). Furthermore, mice doubly nullizygous for RXR-β and RXR-γ do not exhibit severe developmental defects. Also, mice heterozygous for RXR-α in a RXR-β and RXR-γ null background survive to adulthood, suggesting that a single RXR-α allele can perform most of the RXR functions during embryonic development (Krezel *et al.*, 1996).

Genetic evidence exists for a role for the RARs/RXRs in mediating the teratogenic effects of RA on embryonic development. Fetuses null for RAR-γ do not develop the spina bifida and truncations of the axial skeleton in the post-thoracic region normally seen in response to RA delivered on gestation day (gd) 8.5-9.0 (Lohnes *et al.*, 1993). Interestingly, RAR-γ heterozygotes are partially resistant to RA-induced truncations, suggesting that a critical level of RAR-γ is required to mediate these effects completely. RAR-γ has also has been implicated in the induction of severe craniofacial malformations following treatment with RA on gd 7.3, an effect that seems to be stage specific since no change in frequency or severity of defects was seen in mice treated on gd 11 (Iulianella and Lohnes, 1997).

Abbreviations used in this paper: RA, retinoic acid; RXR-α, retinid X receptor alpha; RAR, retinoic acid receptor; gd, gestation day; CP, cleft palate; FP, fused palate.

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Treatment of mice lacking all isoforms of RAR- β with a teratogenic dose of RA on gd 8.5 and 11.5 produced the same craniofacial and limb malformations as seen in wild-type fetuses, indicating that RAR-β is *not* necessary to mediate the teratogenic effects of RA on the skull and limbs *in vivo* (Mendelsohn *et al.*, 1994; Luo *et al.*, 1995). Also, embryos in which RAR-α1 or both RAR-α1 and RAR-β were deleted were susceptible to the teratogenic effects of RA, indicating that RAR-α1 and RAR- β singly or in combination do not play a major role in RA-induced craniofacial and limb malformations (Luo *et al.*, 1996). However, RAR-β2 may play a role in certain aspects of retinoid-induced limb malformations since antisense oligonucleotides to RAR- β2, but not RAR- β1/β3, RAR-α2, RAR-γ2, or RXR-β, reversed the inhibitory effect of RA on chondrogenesis *in vitro* (Jiang *et al.*, 1995). Evidence for a role for RXR-α in mediating the teratogenic effects of RA comes from the demonstration that treatment of RXR-α knockout mice with teratogenic doses of RA on gd 11.5 fails to produce the limb defects normally seen in treated wild-type mice (Sucov *et al.*, 1995). There is, however, no basal requirement for RXR-α in normal limb development, since RXR-α knockout embryos exhibit normal limb development (Sucov *et al.*, 1995)

We have used RXR-α knockout mice to examine the role of RXR-α in mediating the teratogenic effect of RA on palate development. Previous reports with wild-type ICR mice have documented that over 85% of embryos of mice treated on gd 11 with retinoic acid at a dose of 100 mg/kg body weight exhibit palatal clefting (Kochhar *et al.*, 1984, personal observation). Degree of palate development may be ascertained in embryo heads cleared with KOH and stained for cartilage and bone (Fig. 1). A cleft is evident in Figure 1B,D and E by the cartilaginous (blue) nasal septum being clearly visible (arrow); this view of the septum is occluded when the palatal shelves are fused (Fig. 1A,C). In the current study we have crossed mice heterozygous for the RXR-α mutation with either wildtype or RXR-α heterozygous partners, treated pregnant dams with retinoic acid at a dose of 100 mg/kg body weight, and evaluated embryos at gd 15.5 or beyond for the extent of palate fusion. The secondary palate fuses at gd 13.5-14.5 in normal mouse embryos. Surprisingly, only 45% of the live embryos exhibited a palatal cleft (Table 1a). These figures were approximately the same whether embryos were isolated from mice dosed on either gd 11.5 (48%) or gd 12.5 (35%). Genotype analysis revealed that only 46% of the heterozygous embryos exhibited a palatal cleft (Table 1b), versus 80% of the wildtype embryos, a number very close to that previously reported (Kochhar *et al.*, 1984). Thus, embryos in which at least one allele of RXR-α is deleted show a reduced incidence of clefting of the secondary palate in response to maternal treatment with a teratogenic dose of RA on gd 11.5 or gd 12.5. This represents the first demonstration of a role for a retinoid receptor in the induction of cleft palate by a retinoic acid insult during early palate organogenesis.

The homozygous null animals, in keeping with published reports (Sucov *et al.*, 1994, 1995; Dyson *et al.*, 1995, Kastner *et al.,* 1994), were either dead or severely compromised by gd 15. Two of 4 homozygous embryos which were alive at time of isolation had a fused palate; an additional 14 embryos were dead upon isolation and could not be analyzed. The failure of palate fusion in the two viable homozygous null embryos might be a non-specific consequence of the cardiac defects which will soon result in lethality, and which cause a general and progressive developmental delay prior to death. Thus, in terms of palate development, RXR-α homozygous null embryos are at least as refractory to RA challenge as are heterozygous embryos, and are likely to be even more so. A fuller analysis of the effect of RA on palate fusion in the RXR-α null mice awaits a model in which the embryos are rescued from the mid-gestational demise,

TABLE 1

PHENOTYPES AND GENOTYPES OF RA-TREATED EMBRYOS

(a) Incidence of cleft palate (CP), fused palate (FP) and death expressed as percentage of total number of embryos treated.

Day of dosing	Total (number)	CP (number, % of total)	FP (number, % of total)	Dead (number, % of total)
gd 11.5 and 12.5	104	47 (45%)	43 (41%)	14 (14%)
gd 11.5	81	39 (48%)	32 (40%)	2 (12%)
gd 12.5	23	8 (35%)	11 (48%)	4 (17%)

(b) Number of wild-type (+/+) or RXR-α heterozygous (+/-) embryos exhibiting cleft palate (CP), fused palate (FP), or death following treatment with RA on gd 11.5 or gd 12.5. Expt. #2 and 5 each had 1 homozygous null embryo that was CP; expt. # 4 and 5 each had 1 homozygous null embryo that was FP (data not shown). Results of five crosses in which both animals were heterozygous for RXR-α, and three crosses in which one was heterozygous and one wild-type.

Expt. #Genotype of cross	Total Fetuses (number)	Dose (gd)	Day examined (gd)	CP +/+ (number)	FP +/+ (number)	CP +/- (number)	FP +/- (number)	Dead
1 +/-X+/-	14	11.5	17.5	3	0	6	3	2
2 +/-X+/-	10	12.5	15.5	1	0	2	6	-
3 +/-X+/-	13	12.5	15.5	1	0	3	5	2
4 +/-X+/-	15	11.5	15.5	1	0	4	3	6
5 +/-X+/-	14	11.5	15.5	2	0	7	1	2
6 +/-X+/+	13	11.5	15.5	2	0	5	6	-
7 +/-X+/+	11	11.5	18.5	1	0	4	6	-
8 +/-X+/+	14	11.5	17.5	1	3	2	8	-
Total	104	-	-	12	3	33	38	12

allowing the palate phenotype to develop. Development of a transgenic model that will allow such analysis is underway.

The variable response amongst the heterozygous embryos in terms of palate fusion is similar to the lower incidence, though not complete abrogation, of limb defects seen in these mice in response to a teratogenic dose of RA (Sucov *et al*., 1995). RXR-α gene dosage, affected by genetic background and subtle differences in the expression of modifier genes that impinge on the RXR-α signal transduction pathway, may be an important factor in the manifestation of these phenotypes. Variability in the degree of severity of cardiac defects has been described in RXR-α knockout mice (Gruber *et al*., 1996). As well as exhibiting a phenotype intermediate between wild-type and nullizygous embryos, heterozygous embryos exhibited a considerable variability in the severity of particular cardiac defects. A variety of profound and subtle effects resulting from other gene knockout models have been described, some of which are attributable to genetic background of the mice. For example, the stage at which embryonic lethality occurs in the TGF-β1 knockout mouse depends on the strain of mouse on which the transgene is bred (Kallapur *et al*., 1999), while knockout of bone morphogenetic protein (BMP)-4 caused variability in the type of embryonic lethality within the same litter (Winnier *et al*., 1995). The TGF-β2 knockout mice display a wide variety of congenital defects, most of which are only partially penetrant (Sanford *et al*., 1997). In the case of the cleft palate seen in TGF-β3 knockout mice (Proetzel *et al*., 1995), breeding the mice on a mixed genetic background resulted in greater variability in expressivity of clefting compared with mice on a more homogeneous background (Doetschman, 1999). In the present study, although the RXR-α mutation has been bred for at least 7 generations on an ICR background, the RXR-α heterozygous embryos in a particular litter may exhibit variability in their genetic backgrounds resulting from complex interactions between their ICR and (residual) 129 strain genotypes. Furthermore, the ICR background is itself outbred, and is therefore not genetically homogeneous. When affecting the expression of a gene(s) important for palate development and in some way interacting with the RXR-α signaling system, this genetic variability may be sufficient to modify the response of the developing palatal shelves to retinoic acid. The mixed genetic background of these mice may be important in demonstrating the role of RXR-α in mediating the effect of RA on palate development. Doetschman has suggested that such genetic backgrounds can provide more information on gene function than obtained with congenic strains (Doetschman, 1999). It will be of interest to see if different genetic backgrounds will alter the expressivity of the phenotype described in this communication.

As indicated above, a RAR/RXR-α heterodimer probably mediates the effects of retinoids during embryonic development. It remains to be determined which RAR isotype partners with RXR-α to mediate the teratogenic effects of RA in palate development. A possible candidate is RAR-β, which is induced by RA in palate tissue (personal observation) and has been implicated in retinoid-induced limb defects (Kochhar, 1995; Soprano and Soprano 1995). Though RAR-β null mice are susceptible to the teratogenic effects of RA (Luo *et al*., 1995; *vide supra*), this susceptibility may change in the presence of a mutant RXR-α genotype. However, other RARs may first be required to heterodimerize with RXR-α to induce RAR-β via the RARE in its promoter, since RAR-β mRNA is often not constitutively expressed in many retinoid-sensitive tissues, including the developing palatal shelves (Mendelsohn *et al*., 1991; personal observations). It will also be of interest to ascertain the role of RXR-α in normal and abnormal (in the absence of an exogenous RA insult) development of the secondary palate. While embryos heterozygous for RXR-α exhibit normal palate development, homozygous null embryos are either dead or severely compromised at the time of palate morphogenesis (Sucov *et al*., 1994; this study). RXR-α-specific antagonists, in association with palate organ culture techniques, may circumvent some of these problems. Also, information on the relationship between endogenous RA production, RXR-α activation and the expression of downstream target genes important for palate development in wild-type and heterozygous embryos should contribute to our understanding of the molecular mechanisms involved in these processes.

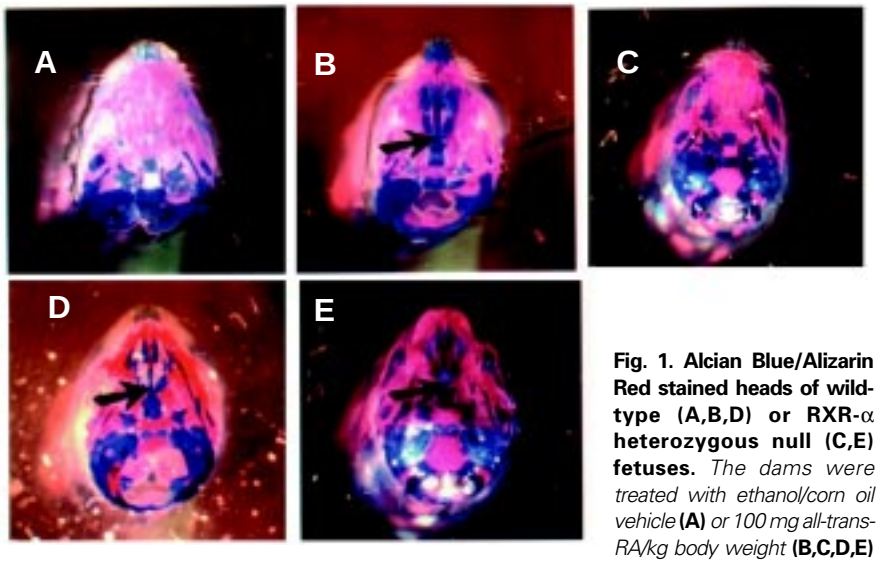

Fig. 1. Alcian Blue/Alizarin Red stained heads of wild-type (A,B,D) or RXR-α heterozygous null (C,E) fetuses. *The dams were treated with ethanol/corn oil vehicle* **(A)** *or 100 mg all-trans-RA/kg body weight* **(B,C,D,E)** *on gd 11. Heads of fetuses were cleared with KOH and stained with Alcian Blue and Alizarin Red to detect cartilage and bone, respectively. Arrow points to position of nasal septum (blue cartilaginous rod), either visible in cleft palate* **(B,D,E)**, *or not visible in fused palate* **(A,C)**. *Fetuses from wild-type mice* **(A,B)** *are shown as indication of normal teratogenic effect of RA on palate development in these mice. Fetuses shown in* **(C)**, **(D)** *and* **(E)** *were genotyped and found to be heterozygous ($^{+}/_{-}$), wild-type ($^{+}/_{+}$), and heterozygous ($^{+}/_{-}$) null for RXR-α, respectively.*

Experimental Procedures

Animals

The RXR-α knockout mouse strain used in these studies has been described (Sucov *et al.,* 1994). The mice had been crossed through at least 7 ICR generations before this study, and all mice used, both heterozygous and wild-type, were ICR strain.

RA treatment

Mice heterozygous for RXR-α were crossed with heterozygous or wild-type animals, and the pregnant animals dosed on gd 11.5 or gd 12.5 (gd 0.5= morning cervical plug was found) with 100 mg all-*trans*-retinoic acid (RA)/kg body weight. RA was suspended in 0.2 ml ethanol, 0.8 ml corn oil added, and the suspension mixed vigorously to obtain a uniform suspension. The mice were dosed by gavage with 0.2 ml of suspension, control animals treated with the same volume of ethanol/corn oil mixture without RA.

Genotyping of embryos

Genotype of the fetuses was determined by PCR analysis of DNA derived from fetal tails. DNA, prepared using the Wizard Genomic DNA kit (Promega Corp., Madison, WI.), was subjected to PCR amplification using RXR-α-specific primers (TGC CCA TCC CTC AGG AAA TAT GG; TAG AGG ATG GGT GAA CTT AAT GAC AA (Sucov *et al*., 1994), and Neomycin (Neo) cassette -specific primers (TCG GCC ATT GAA CAA GAT GG; sequences from Dr. Jaspal Khillan, Transgenic Facility, Thomas Jefferson University, Philadelphia, PA, USA., personal communication). PCR conditions: 94°C, 1 min / 55°C, 1 min / 72°C, 1.5 min; 50 cycles. Products of 600bp and 327bp, respectively, were obtained, their specificity being confirmed with genomic DNA from wild-type, heterozygous and homozygous null mice.

Identification of palate phenotype

Dams were sacrificed by cervical dislocation on gd 15 or gd 17. Degree of palate fusion was assessed by removing the lower jaw for direct visualization of the palate; development of palatal bones was also assessed after alcian blue/alizarin red staining of fetuses to visualize cartilage and bone (McLeod, 1980).

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