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Original Article

The use of a retinoid receptor antagonist in a new model to study vitamin A-dependent developmental events

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ABSTRACT Multiple fetal anomalies occur in vitamin A deficient animals as well as in retinoic acid receptor gene *'knockout'* mice, indicating that retinoic acid (an active metabolite of vitamin A) performs some essential functions in normal development. Additional approaches are needed to probe directly the stages and sites in the embryo where a presence of endogenous retinoic acid is indispensable. We have employed a new strategy for this purpose which involved an intervention in retinoic acid receptor (RAR)-dependent functions at specific developmental stages by means of a highly effective RAR antagonist, AGN 193109. We report that in an *in vitro* cell differentiation bioassay, AGN 193109 completely reversed the inhibitory action of a potent RAR agonist, AGN 190121. In pregnant mice, a single oral 1mg/kg dose of the antagonist given on 8 day *post coitum* (dpc) produced a severe craniofacial anomaly (median cleft face or frontonasal dysplasia) and eye malformations in virtually all exposed fetuses. On the other hand, treatment on 11 dpc, a time in development when RARs are strategically expressed in the limb bud primordium, no limb anomalies could be induced by the antagonist. Even after a high dose of 100 mg/kg, limb development progressed normally in spite of the fact that measurable concentrations of the antagonist were present. Because retinoids are long known to influence skin morphology, we next monitored the effects of the antagonist on skin development. When given late in gestation, on 14 dpc, we found that the antagonist delayed differentiation and maturation of the fetal skin and hair follicles. We conclude that this model provides a convenient and pertinent system which enables us to seek and clarify true functions of retinoic acid and its cognate receptors in embryogenesis and in adult animals.

KEY WORDS: *retinoic acid, RAR antagonist, craniofacial, eye, dysmorphogenesis*

Introduction

Young animals raised on vitamin A-deficient diet grow poorly, become blind and have a low survival rate (Underwood, 1984; Wolf, 1996). Although severely deficient animals are infertile, less severe deficiency in various species (rat, pig, rabbit, monkey) produces a spectrum of fetal malformations affecting the eyes, brain, heart and blood vessels, and the urogenital system (Wilson and Warkany, 1950; Wilson *et al.*, 1953; Underwood, 1984; Wolf, 1996). These observations have underscored an essential role of retinoic acid (RA) –an active metabolite of vitamin A– in adult physiology and in embryonic development. It is now known that RA action within the cell is mediated by a group of specific nuclear receptors termed retinoic acid receptors (RARα, β, and γ) and retinoid X receptors (RXRα, β, and γ) (Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Mangelsdorf *et al.*, 1990; Chambon, 1996). As a first step in the search for molecular functions of vitamin A in embryogenesis, recent investigations have used the gene "knock-out" approach with the expectation that null mutations in individual receptor genes would lead to alterations in fetal phenotype. This approach has turned out to be successful but complex, since null mutations in multiple RAR genes had to be introduced in individual mice to obtain phenocopies of the vitamin A deficiency (VAD) syndrome (Lohnes *et al.*, 1994,1995; Mendelsohn *et al.*, 1994; Chambon, 1996).

Abbreviations used in this paper: AGN, Allergan; FN, frontonasal; RAR, retinoic acid receptor; RXR, retinoic X receptor; VAD, vitamin A deficiency.

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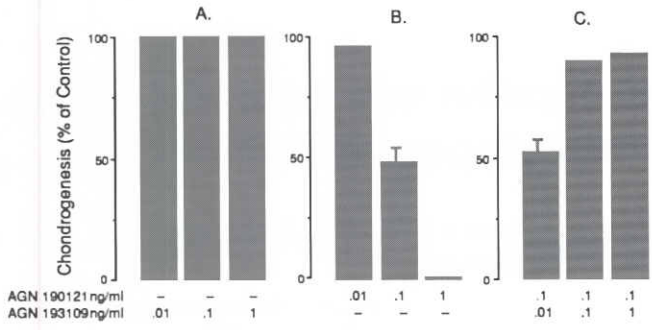


Fig. 1. Inhibition of chondrogenic differentiation in micromass cultures of mouse embryo limb bud cells by RAR agonist AGN 190121, and restoration of normal differentiation by AGN 193109.

As a model in resolving the developmental role of vitamin A these null mutant animals are invaluable, yet a few problems have been encountered. Frequently, the affected fetuses die in utero and are lost (Lohnes *et al.*, 1993; Lufkin *et al.*, 1993). It may prove difficult to isolate primary target developmental events from secondary changes associated with the in utero non-viability of the affected embryos. Additionally, compensatory mechanisms in response to receptor ablation can potentially further obscure the physiological role of RAR activation in the normal animal (Taneja *et al.*, 1996).

In this report we present results of an attempt to intervene in mouse embryogenesis by means of pulse administration of an RAR antagonist at several different developmental stages. The antagonist, AGN 193109, has been shown to bind *in vitro* to each of the RARs (α, β, γ) with a very high affinity yet it is completely inactive in transactivating RARs in transfected CV-1 cells (Johnson *et al.*, 1995). Also AGN 193109 potently inhibited transactivation mediated by RA in these cells (Johnson *et al.*, 1995). AGN 193109 antagonizes the effects of RAR agonists in non-transfected cells as well as *in vivo* (Agarwal *et al.*, 1996; Standeven *et al.*, 1996a,b,c). We show that the antagonist-induced defects in the mouse embryo were mostly confined to the craniofacial region and the eyes, indicating that vitamin A (i.e., RA) is critical for development of the head structures. In contrast, limb development was found to be insensitive to the effects of the antagonist. This unexpected finding raises the likelihood that the actions of RARs were either not fully disrupted in our experiments or that there are additional compensatory factors in the embryo which permit normal development to proceed even in the absence of RARs.

Results

Properties of the antagonist

In a previous study, we compared binding constants (K_d values) of AGN 193109 with those of retinoic acid (RA) and another synthetic retinoid, AGN 190121, using baculovirus-expressed retinoic acid receptors (RAR α, $-\beta$, and $-\gamma$) and retinoid X receptors (RXR α, $-\beta$, and $-\gamma$) as described (Cheng and Prusoff, 1973; Christensen *et al.*, 1991; Heyman *et al.*, 1992). AGN 193109 bound with higher affinities (K_d=2-3 nM) to all three RAR subtypes than RA or AGN 190121 [(Johnson *et al.*, 1995) and data not shown]. In transactivation assays using CV-1 cells transfected with RAR-holoreceptors and a $TREp_{al}$-luciferase reporter gene (Umesono *et al.*, 1988), AGN 193109 was completely inactive at all three RARs while both RA and AGN 190121 were effective activators of transcription (Table 1). Neither AGN 193109 nor AGN 190121 bound to or transactivated any of the RXRs (data not shown).

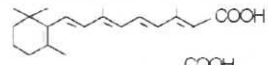
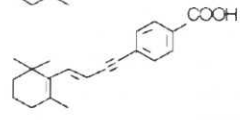
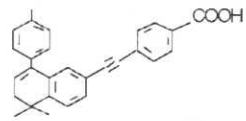
We have previously reported that AGN 193109 is able to antagonize RAR agonist-induced gene transcription in CV-1 and human ectocervical epithelial ECE 16-1 cells (Johnson *et al.*, 1995; Agarwal *et al.*, 1996). Here we examined the ability of AGN 193109 to reverse the inhibitory effects of AGN 190121 on chondrocyte differentiation in mouse embryo mesenchymal cells. Mouse embryo limb bud mesenchymal cells spontaneously differentiate into chondrocytes when explanted at high cell density as micromass cultures in droplets of enriched medium (Ahrens *et al.*, 1977). RA and other RAR agonists consistently inhibit this differentiation process which can be readily detected and quantified (Kistler, 1987; Kochhar and Penner, 1992). As expected, the addition of AGN 190121 to the culture medium inhibited chondrogenesis and reduced the number of chondrogenic nodules in a dose-dependent fashion as compared to control, vehicle-treated cultures (Fig. 1B). The antagonistic action of AGN 193109 on cell differentiation was assayed by its introduction at various concentrations into the agonist-treated cultures. The inhibitory effects of the agonist were efficiently mitigated by AGN 193109 at equimolar concentrations (Fig. 1C). The antagonist alone produced no discernible effects on limb bud cell differentiation (Fig. 1A).

The antagonist behaves as a specific teratogen

Five mice given a daily oral 1 mg/kg dose on 7-11 dpc yielded 52 live fetuses along with 16% resorptions. All 52 were abnormal with obvious craniofacial defects (Table 2). All fetuses had a blunted snout with a median cleft of variable widths separating the two bilaterally symmetrical frontonasal prominences (Fig. 2). The two halves of the frontonasal mass overhung the mandible which was of normal shape but reduced in length. The tongue was visible through the broad oronasal opening, and the secondary palate was missing. The top of the cranium harbored a protrusion of the brain covered by the skin, topped by a single or double hematoma. About 75% of the fetuses showed either no evidence of eyes (no palpe-

TABLE 1

HALF-MAXIMAL CONCENTRATIONS (EC 50) FOR RAR AGONISTS AND AGN193109 IN ACTIVATING TRANSCRIPTION[a]

Retinoid	nM RARα	RARβ	RARγ
RA 	7	1	0.7
AGN190121 	598	2	10
AGN193109 	NA	NA	NA

[a] Transactivation assays were performed in CV-1 cells contransfected with the luciferase reported plasmid Δ MTV-TREp-Luc and an expression vector of the indicated retinoid receptor. NA= not active (Johnson *et al.*, 1995).

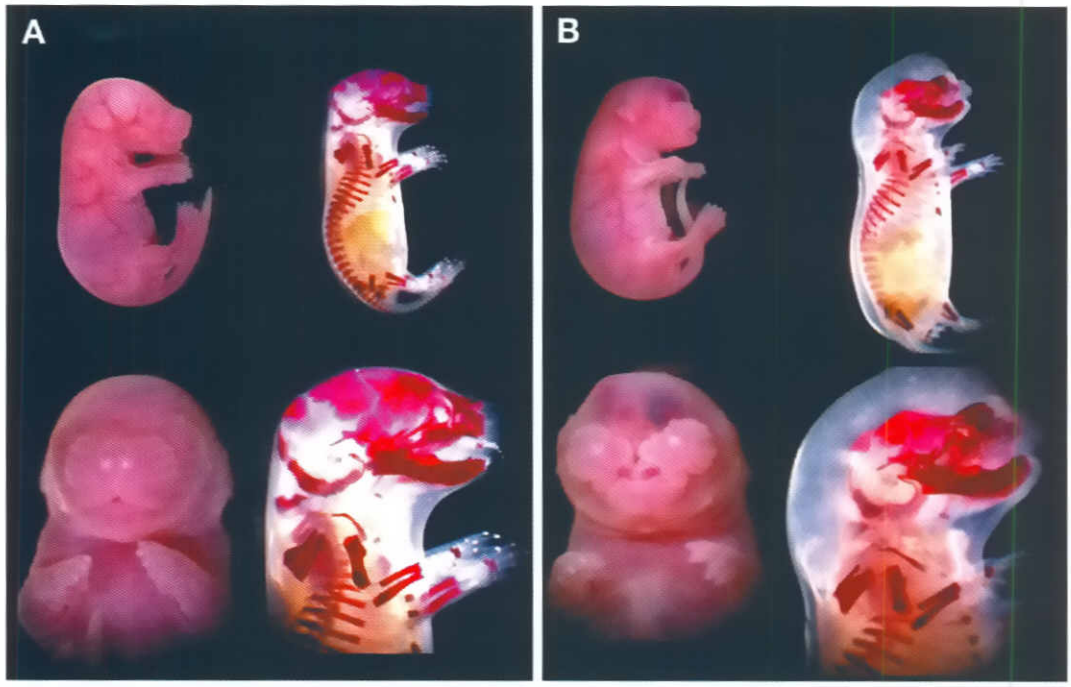


Fig. 2. Phenotypes of normal and antagonist-treated 17 dpc fetuses. ***(A)*** *Normal, live fetus. Alizarin red-stained skeleton is on the right. The lower panels are magnified images of the same preparations.* ***(B)*** *Antagonist-treated fetus and its skeleton, showing median facial cleft, cerebral hematoma, and deficient frontal bones. The limbs and rest of the skeleton are not affected.*

bral protuberance) or the eyes were much smaller than normal. The limbs appeared to be normal and there were no digital defects.

In alizarin red-stained bone preparations, the whole skeleton was normal except that of the face, skull, and the cervical vertebrae (Fig. 2). Consistent with the external findings about the lack of midfacial structures, the nasal bones were deficient and widely separated, and the maxillary bones were distorted and deficient. In the skull, the medial portions of the frontal bones were absent in all fetuses resulting in protrusion of the frontal lobes of the brain. The calvaria posterior to the frontal bones were normal except that 50% of the fetuses had occipital bones reduced in size. One or more abnormalities in first two cervical vertebrae were found in 40% of the fetuses; these were, bifid atlas, fusion between the neural arches of the atlas and the axis, or a change in the shape of one or both. Bony eye sockets were present in all fetuses except that they were reduced in size, either unilaterally or bilaterally.

Internal (visceral) structures were examined in cross-sections of the trunk made by hand-held razor blade under a dissection microscope. Out of 21 fetuses thus examined, only one showed an incomplete inter-atrial septum of the heart. None of the remaining fetuses showed any evidence of overt developmental deviation in the diaphragm or in the cervical, thoracic, abdominal or pelvic organs.

None of the 122 fetuses obtained from 10 vehicle-treated mice showed any external, internal or skeletal malformations.

Mouse development between 7 dpc and 11 dpc encompasses almost the entire period of organogenesis, hence it was surprising that the antagonist-induced anomalies were confined only to the head and neck structures. In order to determine if there was a developmental stage or stages most sensitive to the antagonist for the origin of frontonasal (FN) and eye defects, we administered single doses to different groups of animals at 12 h intervals beginning on 6 dpc and ending on 11 dpc. All animals were killed and the fetuses examined on 17 dpc. The antagonist did not impair viability of litters in any treatment group; the resorption frequencies varied 2-5%, and the fetuses on 17 dpc had body weight within the normal range of 890±48 mg each. None of the embryos exposed to the antagonist prior to 8 dpc showed any FN or eye defects. The only skeletal anomaly was in the cervical vertebrae which affected 16% of embryos treated on 7 dpc and 11% on 7.5 dpc (Fig. 3). On 8 dpc, 86% of the embryos developed the FN syndrome, and these also had eye defects. The only other anomaly on this day was an occasional exencephaly; 3 out of 106 embryos examined on this day were affected. The susceptible period for FN defects was brief, since within 12 h, on 8.5 dpc, only 37% harbored the FN syndrome and within 24h, on 9 dpc, the syndrome was completely absent. The eye defects were still preponderant on 8.5 dpc; in addition to the 37% embryos with the FN syndrome, a further 30% embryos showed eye defects which were otherwise without any other craniofacial defects. On 9 dpc, and subsequently on 10 and 11 dpc, the FN and eye defects were not found. The embryos exposed to the antagonist at these stages were entirely normal with no skeletal or visceral defects. No limb defects were encountered either in the multiple dose regimen (7-11 dpc) or in the single dose groups.

There is considerable evidence that RARs modulate pattern formation in the limb bud during organogenesis (Summerbell,

TABLE 2

DEVELOPMENTAL EFFECTS OF DAILY SINGLE 1MG/KG DOSE OF AGN 193109 OR VEHICLE ALONE GIVEN ORALLY ON EACH OF THE 7-11 DPC. THE FETUSES WERE EXAMINED ON 17 DPC

	Number of litters treated	Number of implantation sites	Number resorbed	Total number of fetuses		
				Examined	Normal	Abnormal
AGN 193109	5	62	10 (16%)	52	0	52
Vehicle	10	125	3 (2.4%)	122	0	

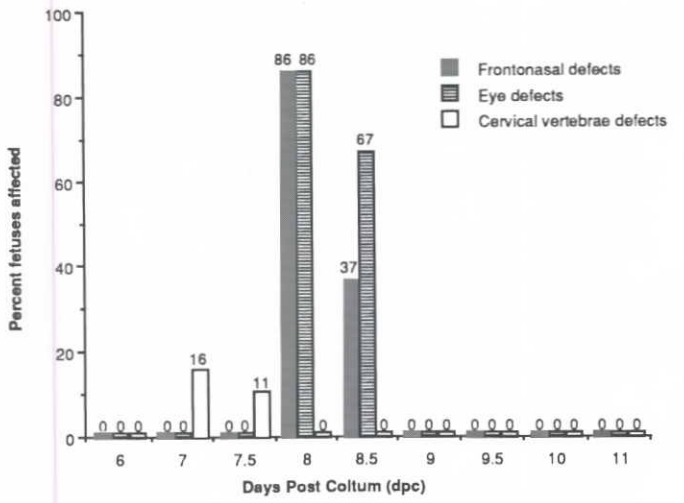


Fig. 3. The frequencies of fetal defects produced by a single, 1 mg/kg, dose of the antagonist given on one of the 6-11 dpc. *Mice were killed on 17 dpc. The frontonasal and eye defects occurred only after treatment on 8-8.5 dpc.*

1983; Tickle *et al.*, 1985; Mendelsohn *et al.*, 1992; Helms *et al.*, 1996) , and that an excess of RA on 11 dpc produces severe reduction deformities in the mouse limbs (Kochhar, 1973; Satre and Kochhar, 1989). We treated the next group of animals with a large dose of the antagonist, 100 mg/kg, on 11 dpc. We found that the embryos remained totally insensitive to the antagonist. This high dose induced no other symptoms in the resultant fetuses.

We sought evidence that the observed phenotypic changes were directly associated with the presence of the antagonist in the embryo by monitoring the distribution of AGN 193109 to the embryo soon after maternal dosing. The use of 1 mg/kg dose in this analysis was unsuccessful because in 8 out of 10 mice thus treated in a pilot study, the level of the antagonist in the tiny 8 dpc embryos was just above the limit of detection making quantification unreliable. We also chose to include the whole conceptus (embryo undissected from the extra embryonic membranes) in the analysis to improve quantification. After an oral dose of 10 mg/kg on 8 dpc, the drug was detected simultaneously in the maternal plasma and the conceptus within 1h (Table 3). The drug levels peaked in both compartments by 4h after the dose, and then gradually declined over the next 20h. A similar pharmacokinetic profile was obtained on 11 dpc, indicating that the placental transfer of the drug was similar at both the early and the late stages of organogenesis (Table 3, Fig. 4A). On 11 dpc, a comparison of pharmacokinetic profiles between two different doses, 10 mg/g and 100 mg/kg, yielded several instructive points. Firstly, the absorption of the antagonist into the maternal plasma was already near the saturation point after the lower dose of 10 mg/kg, since the concentration peak increased by only two-fold after the ten fold higher dose of 100 mg/kg (Fig. 4B). Secondly, the distribution of the antagonist to the limb bud was considerable; the amount in the limb bud was not only proportional to its level in the rest of the embryo, but it also persisted for a longer duration after the 100 mg/kg dose (Fig. 4B). These results are consistent with the view that the limb bud development was intrinsically insensitive to the antagonist.

Epidermal changes in the skin

Wolbach and Howe (1925) first reported that vitamin A depletion causes squamous metaplasia in the epithelial lining of the viscera. In transgenic mice where RAR function was disrupted late in gestation by means of an expression of dominant negative RARs, dramatic alterations were found in the skin (Andersen and Rosenfeld, 1995; Imakado *et al.*, 1995; Saitou *et al.*, 1995). The skin was dry and shiny, and it was thin and fragile. Keratinocyte differentiation and maturation were suppressed, and hair follicle formation was delayed. These mutant animals died within 24 h after birth, presumably due to an aberrant barrier function of the affected skin (Imakado *et al.*, 1995; Saitou *et al.*, 1995).

Following the lead of transgenic animal studies, we gave a single large dose, 100 mg/kg of the antagonist to mice on 14 dpc and obtained near-term fetuses on 18 dpc. All treated fetuses were entirely normal except that they had shiny, translucent skin (Fig. 5). In histological sections, the antagonist-treated skin displayed normal layering of the epidermis except that stratum corneum was more compact than normal, indicating delayed maturation. The hair follicles in the affected skin consisted of loosely aggregated cells rather than the encapsulated appearance in normal skin (Fig. 5). These results suggest a role for hormonal RAR-dependent pathways in the normal differentiation of the skin during embryogenesis. They also indicate that the more dramatic morphology observed in the dominant negative RAR transgenic mice is due to a disruption in more than the RAR-mediated pathways alone (Imakado *et al.*, 1995; Saitou *et al.*, 1995). This is not surprising since the dominant negative RAR is believed to function by sequestering RXRs as inactive heterodimers, thereby disrupting all RXR-dependent heterodimeric pathways (Andersen and Rosenfeld, 1995).

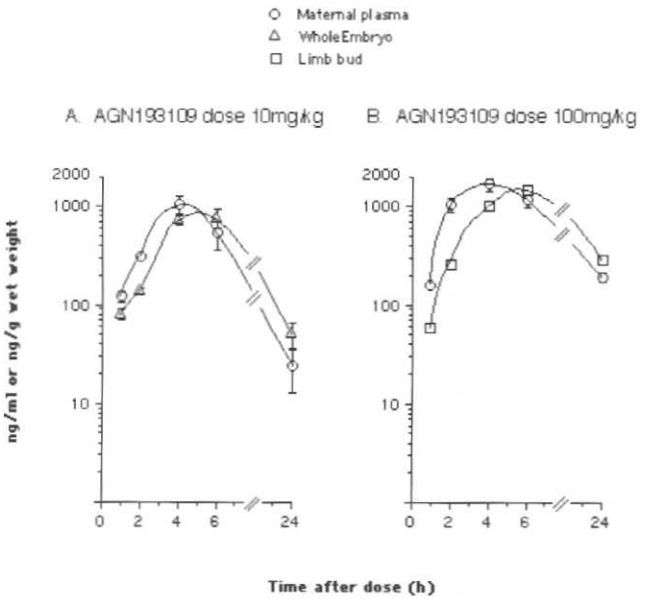


Fig. 4. Levels of the antagonist in the maternal plasma, whole embryo, or the limb buds after treatment on 11 dpc. (A) *After 10 mg/kg dose.* ***(B)*** *After 100 mg/kg dose.*

Discussion

The median facial cleft and frontonasal deficiency induced by the antagonist are strikingly similar to the craniofacial defects which occurred in double null mutants lacking both RARα and RARγ (Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994), attesting to the physiological roles of RAR-mediated gene transcription in normal craniofacial development and the ability of AGN 193109 to antagonize this function. It should be noted, however, that the mutants also exhibited other skeletal and visceral malformations, including the limbs, not found in the antagonist-treated embryos (Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994). Also, in contrast to the antagonist-treated fetuses, all RAR null mutants suffered from growth retardation and almost 50% died in utero (Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994).

To reconcile our results with those reported for the RAR "knockout" mice, it may be necessary to differentiate between retinoid hormone (i.e., RA) -mediated RAR effects and hormone-independent RAR effects on the development of the embryo. Recent findings suggest that the gene regulatory effects of RARs are dependent on their interactions with various co-activator (Chakravarti *et al.*, 1996; Kamei *et al.*, 1996) and co-repressor proteins (Chen and Evans, 1995; Horlein *et al.*, 1995; Kurokawa *et al.*, 1995) which are also shared by other nuclear hormone receptors and transcription factors (O'Donnell *et al.*, 1991; Arias *et al.*, 1994; Burris *et al.*, 1995; Cavailles *et al.*, 1995; Onate *et al.*, 1995). Thus, a deletion of RARs through gene inactivation can result in a significant change in the stoichiometry that exists between several nuclear hormone receptors/transcription factors and co-activators/co-repressors. This disruption of a delicate balance can possibly lead to an alteration in not only RAR gene regulatory pathways but in several other pathways mediated by multiple nuclear receptors and transcription factors. Moreover, the absence of RARs persists throughout gestation in the null mutant which presumably results in progressively widespread anomalies as the development proceeds. On the other hand, the use of an antagonist provides a selective interdiction of RAR pathways in a temporally specific manner. Thus, it is not surprising that the phenotype produced by the RAR antagonist was more restricted than that produced by the double RAR "knockouts".

In view of the above, our results imply that RA actions through RARs are essential exclusively in the normal development of the eyes, facial processes, and craniofacial and cervical skeleton. It is possible that a great variety of other anomalies found in receptor null mutants, such as that found in the limbs, are secondary to an overall growth retardation prevalent in such embryos.

TABLE 3

LEVELS OF AGN 193109 AFTER 10 MG/KG ORAL DOSE ON 8DPC

Time after dose	Maternal Plasma ng/ml ± S.E.	Conceptous* ng/g wet weight ± S.E.
1h	106 ± 30	70 ± 8
2h	416 ± 120	549 ± 45
4h	438 ± 110	742 ± 90
6h	388 ± 140	556 ± 180
24h	49 ± 14	94 ± 35

* Includes embryo and extra embryonic menbranes and fluids.

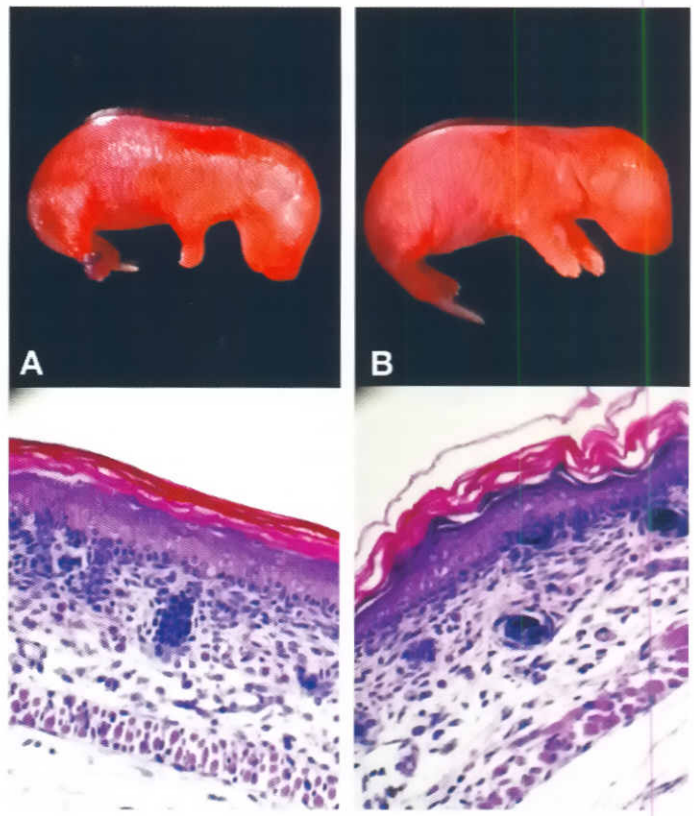


Fig. 5. Changes in the fetal skin produced by a single 100 mg/kg dose of the antagonist given on 14 dpc. (A) *Live, antagonist-treated fetus. The lower panel shows a section of its flank skin.* ***(B)*** *Live, vehicle-treated fetus and a section of its skin.*

Previous evidence strengthens our proposal that the median facial cleft and eye defects have arisen due to abrogation by AGN193109 of some of the vitamin A-dependent/RAR-mediated functions. Although microphthalmia and other eye defects were common among fetuses of vitamin A-deficient animals produced by nutritional deprivation in earlier studies, facial defects had not been observed (Wilson and Warkany, 1950; Wilson *et al.*, 1953). However, in a recent review Morriss-Kay and Sokolova (1996) reported that mouse embryos with midline facial clefts were seen among litters after a prolonged vitamin A deprivation lasting over two generations; these embryos were non-viable and died in utero by day 16 of pregnancy. Using a unique nutritional protocol, Dickman *et al.* (1997) recently reported that severe cranial, neural, eye and other anomalies affected a majority of early rat embryos obtained from vitamin A-deprived females. Some of the embryos in this study showed frontonasal phenotype similar to the antagonist-treated fetuses. Since the embryos were observed only on 13.5 dpc, viability of the deficient embryos remained undetermined. All embryos were growth retarded raising the possibility that some anomalies, such as abnormally-shaped limbs, may have resulted secondarily from growth insufficiency (Dickman *et al.*, 1997).

Mechanisms of action

The craniofacial anomalies such as those produced by AGN 193109 have been reported in humans and other species. A

nongenetic, congenital disorder in a number of human babies was reviewed by DeMeyer (DeMeyer, 1967) who termed such phenotype as the median cleft face syndrome; additional cases were characterized as frontonasal syndrome (Sedano *et al.*, 1970; Robin *et al.*, 1996). In this syndrome the manifestations are limited to the head and include ocular hypertelorism, median facial cleft affecting both the nose and the upper lip, and at times, the palate. There is a protrusion of the brain because frontal bones do not form (anterior cranium bifidum occultum). In one of the two new cases described by Sedano *et al.* (Sedano *et al.*, 1970), the child had right anophthalmia and left microphthalmia. No causal associations are as yet apparent.

No direct leads are available at present as to the cellular mechanisms by which the presence of the antagonist compromises craniofacial development. Some clues as to the early tissue changes associated with the median facial clefts have been suggested in teratological studies. Cell death in the frontonasal mesenchyme was observed within 12 h after treatment of mice on 11 dpc with a glutamine analog DON (Greene and Kochhar, 1975; Burk and Sadler, 1983). Cell death was not implicated in median facial clefts in mice and hamsters exposed to various other teratogens at earlier stages of development such as 8 or 9 dpc (Tassinari and Long, 1982; Darab *et al.*, 1987). Tassinari and Long (1982) raised the likelihood that a marked deficiency of the frontonasal mesenchyme in hamster embryos exposed to cadmium chloride may have resulted from a disruption of neural crest cell development. In general, teratogen-treated embryos show other anomalies and may be growth-retarded, making it difficult to assign distinctive causal roles to individual cellular events such as cell proliferation, cell death, or cell migrations.

Since RARs are ligand-activated transcription factors, there is a strong likelihood that expression of some genes of critical importance in craniofacial morphogenesis and eye development is altered in the antagonist-treated embryos. The phenotype of mice homozygous for *AP-2* null mutation shows a considerable degree of overlap with the craniofacial defects in mice treated on 8 dpc with AGN193109 (Schorle *et al.*, 1996; Zhang *et al.*, 1996). AP-2 is a retinoic acid-inducible transcription factor which is specifically expressed in cephalic ectoderm and neural crest cells migrating from the neural folds (Williams *et al.*, 1988; Luscher *et al.*, 1989; Mitchell *et al.*, 1991). Apart from midfacial cleft, the embryos also showed exencephaly and umbilical hernia (abdominoschisis). The skeletal defects occurred in the head and trunk region where many bones were either absent or deformed. In eye development, one of the most interesting and well studied is the dominant mutation known as small eye (*sey*). Heterozygous *sey/+* mice typically have small eyes, while homozygous *sey/sey* animals completely lack eyes and nose. A similar phenotype has been observed in rats (Matsuo *et al.*, 1993; Fujiwara *et al.*, 1994) and in humans (Glaser *et al.*, 1994). Interestingly, the mutation has been recently demonstrated in the mouse, rat and human to be caused by defects in *Pax-6*, implicating *Pax-6* as an important factor in craniofacial development (Grindley *et al.*, 1995). It will be rewarding in further studies to examine if the expression and activity of *AP-2* and *Pax-6* are altered in AGN193109-treated embryos, particularly during stages of neural tube closure and neural crest migration (Morriss-Kay, 1996).

In conclusion, we have demonstrated that the RAR antagonist model is a convenient and pertinent model for delineating RAR hormone-dependent effects on the normal development of the embryo. Using this model, we demonstrate that RA is critical for the development of the craniofacial and cervical regions during a narrow and specific (8-8.5 dpc) temporal window. Surprisingly, we find no evidence of abnormal limb morphogenesis in RAR antagonist-treated fetuses. Further studies utilizing more frequent applications of the antagonist and the use of RAR subtype-specific antagonists will help in elucidating the roles of RAR-mediated pathways in normal development.

Materials and Methods

Retinoids

Both retinoids used in this study (Table 1) were synthesized in the Department of Chemistry, Retinoid Research, Allergan, Inc. [see review of synthetic methods in (Chandraratna *et al.*, 1995; Johnson *et al.*, 1995) and references therein]. All procedures involving manipulation of the retinoids were performed in the dark under dim yellow light to retard photodegradation. The analogs were dissolved in DMSO (10 or 100 mg/ml) from which fresh dilutions were made before use. For oral intubation, the dilutions were made in vegetable (soybean) oil; for tissue culture studies the DMSO-dissolved retinoids were diluted with the tissue culture medium.

Cell culture bioassay

The bioassay employed high density micromass cultures of 11 dpc embryonic mouse limb bud cells as described (Kochhar and Penner, 1992; Jiang *et al.*, 1995). Briefly, forelimb buds were dissociated in a trypsin-EDTA solution and the resultant single-cell suspension was plated as 20 µl spots (200,000 cells/spot) on plastic culture dishes. Retinoid concentrations ranging from 0.01 ng/ml to 10mg/ml (0.03 mM-30mM) were added to the culture medium (Eagle's MEM+10% fetal bovine serum, GIBCO) 24h after initial plating. Control cultures received only the vehicle (DMSO, concentration <1% by vol.). The cultures were terminated 96 h after plating, at which time the medium was removed and the cells were fixed for 1 h in 10% formalin containing 0.5% cetylpyrdinium chloride. The cultures were rinsed in acetic acid, and stained for 1 h in 0.5% Alcian blue solution at pH 1.0, differentiated in 3% acetic acid and then dehydrated in ethanol and scored for chondrogenesis under the microscope. An absence or reduction in the number of cartilage nodules in stained cultures as compared with control cultures was taken as a measure of suppression of chondrogenesis. The number of cartilage nodules stained in the whole spot were counted by automated image scan using the N.I.H. Image-1.52 application. Mean number of nodules and standard deviations were calculated for four replicate cultures per concentration. The median concentration of each retinoid causing 50% inhibition of chondrogenesis compared with controls (IC50) was calculated by logarithmic curve fitting of the dose-response data.

Developmental anomalies

Timed pregnant ICR mice were used in the whole animal study essentially as described previously (Kochhar, 1973; Kochhar and Penner, 1992). The day of vaginal plug detection was designated as 0 dpc. A syringe fitted with blunt-ended needle was used to orally intubate groups of mice with AGN 193109 dissolved in the DMSO:oil vehicle (1:19, vol/vol). The volume of the vehicle was held constant at 5 ml/kg. The animals in the control group were given an identical volume of the vehicle alone. The first group of 5 mice was given a daily 1 mg/kg dose for 5 days beginning at 10 AM on 7 dpc. To discover stage-specific effects of the antagonist, the next group was treated with a single dose at either 10 AM or 10 PM on one of 6-11 dpc. The third group of 5 mice were each given a single 100 mg/kg dose on 11 dpc to ascertain sensitivity of limb development to the antagonist. The last group of 3 mice were each treated on dpc 14 with a 100 mg/kg dose to elicit alteration in skin development.

All animals were killed on 17 dpc and the resultant fetuses examined for limb and craniofacial defects as described previously (Kochhar, 1973; Kochhar and Penner, 1992). Upon laparotomy, the fetuses were examined

for external malformations and weighed, then one-half of each litter was fixed in 95% ethanol and processed for staining of the skeleton by the rapid alizarin red S dye method (Kochhar, 1973). These preparations were examined under a dissection microscope. The other half of each litter was fixed in Bouin's solution and examined for anomalies of the craniofacial region by free-hand razor sectioning. Differences in dose-related incidence of malformations were assessed by computing percentages of affected conceptuses among total implantation sites. The groups were compared statistically by a method based on t tests of arcsin square root transformed percentages. Values at 0.05 probability level were considered significant.

For histological analysis, skin dissected from the flank of 18 dpc fetuses was fixed in Bouin's solution, embedded in paraffin and 5-10 mm sections stained with either hematoxylin/eosin or Mallory's trichrome.

Quantification by HPLC

The levels of the antagonist reaching the embryo after maternal dosing were monitored on both 8 and 11 dpc. After an oral 10 mg/kg dose, 3 or 4 animals were killed for each time interval ranging from 1-24 h after the dose. An additional group of mice were given 100 mg/kg dose on 11 dpc. Maternal blood was drawn from the inferior vena cava and embryos removed from the uterus and processed for measurements by HPLC according to protocols described previously (Kochhar and Penner, 1992), with some modifications. Whole conceptuses (embryo, extra-embryonic membranes including fluids, and ectoplacental cone) were used on 8 dpc, while on 11 dpc the embryos were removed from the placental membranes and processed alone. Limb buds were removed from the latter group and processed as a separate sample. The plasma and sonicated tissue samples were extracted in 2 volumes of ice-cold ethanol, vortexed and centrifuged, and supernatant injected directly into the HPLC system (Kochhar and Penner, 1992). The column was eluted by a non-linear gradient at pH 6.8 ranging from methanol, acetonitrile, 0.1 M ammonium acetate in a 46:33:21 ratio to 100% methanol, at a flow rate of 1 ml/min. The eluted fractions were detected by UV absorbance at 320 nm and identified by the retention times of known standards. The concentrations of AGN193109 were calculated from the integrated HPLC peak areas with reference to calibration curves (linear) obtained from the authentic compound. The detection limit in this system was 1 ng/ml.

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