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9-*cis*-RETINOIC ACID: A DIRECT-ACTING DYSMORPHOGEN

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Abstract—Experiments *in vitro* with cultured rat conceptuses demonstrated that 9-*cis*-retinoic acid (9-*cis*-RA) (300 ng/mL amniotic fluid) produced branchial arch and somite defects similar to those elicited by equal concentrations of all-*trans*-retinoic acid (all-*trans*-RA), but with an increase in cephalic defects that included missing optic vesicles. After conceptuses were intraamniotically microinjected with 600 ng 9-*cis*-RA/mL amniotic fluid on day 10 of gestation, an unusual heart defect was also observed. HPLC analyses indicated that 9-*cis*-RA readily underwent conversion to the less active metabolite, 13-*cis*-retinoic acid (13-*cis*-RA), in cultured conceptuses during the first 4 hr after treatment but only after 6 hr could elevated levels of the potent dysmorphogen all-*trans*-RA be detected. In separate experiments, conversion of 13-*cis*-RA or of all-*trans*-RA to 9-*cis*-RA could not be detected during a 6-hr embryo culture period. Endogenous levels of 9-*cis*-RA in whole rat embryos also were below limits of detection but small quantities of this isomer could be detected in neonatal rat eye and human embryonic brain. Our present study strongly suggests that 9-*cis*-RA is a direct-acting dysmorphogen with probable specific target sites of action.

At endogenous tissue levels, retinoids appear to have an active role in normal embryonic development [1–3], whereas either abnormally elevated or abnormally low retinoid concentrations in target tissues during sensitive stages can induce terata [4–6]. It has been proposed that two families of nuclear receptors, the retinoic acid receptors (RARs†) and the retinoid X receptors (RXRs), are directly involved in normal as well as abnormal development [7, 8]. These receptors are part of the larger superfamily of steroid/thyroid receptors.

It is now known that RARs directly bind all-*trans*-retinoic acid (all-*trans*-RA) with high affinity resulting in receptor activation but that the RXRs are incapable of binding all-*trans*-RA with high affinity [9, 10]. The RXRs, however, bind and are activated by the 9-*cis* stereoisomer of all-*trans*-RA [11, 12]. 13-*cis*-Retinoic acid (13-*cis*-RA) does not bind to either receptor with high affinity, but 9-*cis*-retinoic acid (9-*cis*-RA) binds with high affinity to both RAR and RXR receptor families [13].

Experiments *in vitro* with cultured rat conceptuses have shown that all-*trans*-RA is 7–10 times more potent than 13-*cis*-RA, either when added directly to the culture medium or when microinjected intraamniotically [14, 15]. Major defects produced by the 13-*cis* and *trans* isomers were qualitatively similar and particularly involved the branchial arches and somites. Using HPLC analyses, results indicated that all-*trans*-RA was ultimately responsible for the dysmorphogenic effects produced by 13-*cis*-RA, all-*trans*-retinoyl-β-glucuronide (all-*trans*-RAG) or retinol [16–18]. The latter three retinoids undergo conversion to all-*trans*-RA within tissues of the conceptus. As yet, no *in vivo* or *in vitro* investigations of the dysmorphogenic or teratogenic effects of the 9-*cis* isomer have appeared in the literature.

Using rat whole embryo culture, the present study was carried out to investigate: (1) whether 9-*cis*-RA, a ligand for RXR, produces similar or different defects than the RAR ligand, all-*trans*-RA, when added to the culture medium or microinjected intraamniotically; (2) whether 9-*cis*-RA is a direct-acting dysmorphogen; (3) whether 9-*cis*-RA can be converted to other retinoid metabolites within tissues of the embryo or yolk sac; and (4) whether 9-*cis*-RA is endogenously present in rat embryos.

MATERIALS AND METHODS

Chemicals. 9-*cis*-RA, 13-*cis*-RA and all-*trans*-RA were gifts from Hoffmann-La Roche, Inc. (Nutley, NJ). Spectrophotochemical grade dimethyl sulfoxide (DMSO) and D-mannose[U-^{14}C] (45–55 mCi/mmol) were purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ) and New England Nuclear (Wilmington, DE), respectively. All other chemicals used were of the highest purity commercially available.

Whole embryo culture. Time-mated rats (Sprague–Dawley, Wistar-derived) were obtained from Tyler Laboratories (Bellevue, WA) on day 4 or 5 of gestation. All animals were allowed free access to food and water and were housed in polycarbonate cages with crushed corncob material for bedding. The morning after copulation was designated as day

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† Abbreviations: all-*trans*-RA, all-*trans*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; all-*trans*-RAG, all-*trans*-retinoyl-β-glucuronide; RARs, retinoic acid receptors; and RXRs, retinoid X receptors.

0 of gestation. Explantation of conceptuses was as described previously [19]. The conceptuses (day 9.5) were removed from ether-anesthetized dams and transferred into culture medium (50% heat-inactivated female rat serum/50% Waymouth's medium) saturated with O_2:CO_2:N_2 (5:5:90). For microinjections, the embryos were cultured for 16 hr in roller bottles at 37° prior to treatment. Embryos were selected on the morning of day 10 for intraamniotic microinjection of 9-*cis*-RA or for additions of 9-*cis*-RA to the culture medium. The criteria utilized for staging the embryos were as follows: partially elevated neural folds (~45°), 8–10 somites and full dorsoflexion of the caudal region. Conceptuses were pooled from separate culture bottles and randomized prior to treatment or transferral to fresh medium for the metabolic studies. During microinjections, only microscopic light and a yellow desk lamp were used in order to prevent retinoid isomerization. The treated or (in the case of the metabolic studies) untreated conceptuses were then incubated in freshly prepared medium saturated with O_2:CO_2:N_2 (20:5:75) for 24 hr in the dark. For the metabolic studies, 9-*cis*-RA was added on day 11 of gestation to the culture medium and the embryos and yolk sacs were collected for HPLC studies 2, 3.5, 4.5 and 6 hr after treatment. Stock solutions of retinoids were prepared under yellow light by dissolution in absolute ethanol, stored in glassware at −20°, and were diluted in DMSO immediately before microinjections or additions to the culture medium. Control conceptuses were injected with an equal volume of vehicle. Medium was regassed in the dark with O_2:CO_2 (95:5) 24 hr after the beginning of the culture period and conceptuses were cultured for an additional 4–5 hr. On day 11.5, conceptuses were examined and evaluated for abnormalities under a binocular dissecting microscope by individuals who had no knowledge of the treatment under study.

Microinjection. Microinjections of chemicals into the amniotic compartments were performed according to modifications of the methods previously described [20]. Conceptuses received intraamniotic microinjections via a pressure injection system (model PLI-100, Medical Systems, Inc., Greenvale, NY) at 8 psi. Each glass pipette had a tip diameter (O.D.) of 10–15 μm and the injection volume was calibrated with radiolabeled D-mannose. The volume of amniotic fluid (mean = 1.0 μL: N = 10) was measured by injection of D-mannose[U-^{14}C] into the amniotic cavity and 30 min were allowed for solution equilibration; a small measured volume of fluid was then counted (Beckman Scintillation Counter) and the concentration calculated [21]. These volumetric measurements permitted an estimate of the final quantity of compounds injected into amniotic space and the resulting concentration of the compound in the amniotic fluid compartment [20, 21]. Confirmatory experiments were conducted to assure that there was no significant backflow of the injected material from the amniotic cavity into the micropipette upon withdrawal. A modification of the method of Bradford [22], adapted for the microplate reader, was utilized to quantify the concentrations of protein.

Studies of metabolism and endogenous retinoid levels. Prior to HPLC analysis, the treated or untreated yolk sacs and embryos were removed and pooled separately in polyethylene tubes that were kept on ice and stored at −70°. Approximately 30–40 embryos or yolk sacs were needed for each HPLC analysis, which was carried out in duplicate. Rat eye tissue (100 mg) was collected from day 8 post-partum rats. Human embryonic brains were obtained from embryos subsequent to dilatation and curettage and were acquired through the Central Laboratory of Human Embryology at the University of Washington. Studies were approved by the University of Washington Human Subjects Review Committee. Human gestational ages were estimated by crown-rump and foot lengths. All samples were treated with 2 vol. of isopropanol, vortexed for 1 min, and homogenized at 4° using a Sonic-L converter for 10 sec, at setting 2 (Branson Sonic Power Co.). The homogenate was centrifuged at 4° for 20 min at 4000 *g*. A portion (100–200 μL) of the supernatant fraction was injected into the HPLC system. The HPLC apparatus consisted of two model 600 dual piston Shimadzu pumps linked together to form a binary gradient as described earlier [23]. In our present study, the flow rate was reduced from 2 mL/min previously reported to 1.6 mL/min. This procedure effected the definitive separation of 9-*cis*-RA from 13-*cis*-RA and all-*trans*-RA as well as from other retinoids (see Fig. 2a). The analytical column (120 × 4.6 mm) was slurry packed with Spherisorb 3 ODS II (3 μm). Cartridges (20 × 4.6 mm) prepacked with Lichrosorb RB 18 (10 μm) were used as precolumns.

Statistics. Statistical analyses of conceptal growth and differentiation data were performed using analysis of variance followed by Dunnett's multiple comparison test. The level of significance chosen was $P < 0.05$. The Chi Square test was utilized for statistical analyses of differences in incidences of defects.

RESULTS

When 9-*cis*-RA was microinjected to produce a final intraamniotic concentration of 150 ng/mL, 11 of 38 (29%) of the embryos exhibited abnormal branchial arch development (Table 1, Fig. 1a and 1c). All of these embryos were afflicted with multiple abnormalities. None of the embryos microinjected with an equivalent volume of vehicle exhibited such defects. When 9-*cis*-RA was intraamniotically microinjected to produce a final concentration of 300 ng/mL in the amniotic fluid, we observed an increase in branchial arch defects (42%) and in cephalic defects (33%) which included missing optic vesicles and shortened prosencephalon (Fig. 1b, 1d, and 1e). At 600 ng 9-*cis*-RA/mL amniotic fluid, a 53% incidence of cardiac defects, involving gross enlargement of the heart, was recorded.

Additions of 150 or 300 ng/mL 9-*cis*-RA to the culture medium produced virtually identical effects compared to those elicited after intraamniotic microinjections but were accompanied by a slight increase in the incidence of most defects (Table 2). As the concentration of 9-*cis*-RA increased in the

Table 1. Dysmorphogenic/embryotoxic concentration–effect relationships of microinjected 9-*cis*-retinoic acid in cultured whole rat embryos

Parameter	Control	Dose* (ng/mL) 150	300	600
Embryos cultured	40	38	48	31
Survival (%)	40 (100)	38 (100)	48 (100)	30 (97)
Any defects (%)	3 (8)	19† (50)	29† (60)	27† (90)
Multiple defects (%)	0 (0)	12† (32)	11† (23)	12† (40)
Anterior schisis (%)	0 (0)	2 (5)	7† (15)	2 (7)
Cephalic defects‡ (%)	0 (0)	3 (8)	16† (33)	6† (20)
Rhombencephalic schisis (%)	0 (0)	2 (5)	3 (6)	4 (13)
Branchial arch defects (%)	0 (0)	11† (29)	20† (42)	10† (33)
Irregular somites (%)	3 (8)	12† (32)	10 (21)	13† (43)
Abnormal rotation (%)	0 (0)	6† (16)	8† (17)	8† (27)
Open optic vesicles (%)	0 (0)	8† (21)	13† (27)	2 (7)
Cardiac defects (%)	0 (0)	0 (0)	0 (0)	16† (53)
Mean yolk sac diameter (mm)	3.5 ± 0.3	3.4 ± 0.3	3.3 ± 0.5	3.1 ± 0.3†
Mean embryonic length (mm)	3.0 ± 0.3	2.9 ± 0.3	2.8 ± 0.3†	2.9 ± 0.4
Mean somite number	28 ± 3	28 ± 3	29 ± 1	23 ± 1†
Mean yolk sac protein (μg)	395 ± 82	535 ± 138†	478 ± 150†	241 ± 85†
Mean embryonic protein (μg)	381 ± 83	537 ± 55†	467 ± 100†	299 ± 50†

Statistical comparisons were carried out for defects by Chi square analyses and for quantitative parameters by ANOVA and Dunnett's multiple comparison test.

* 9-*cis*-RA (dissolved in DMSO) or an identical volume of the vehicle was microinjected into the amniotic fluid of cultured whole rat embryos on day 10 of gestation. Numbers in the top section of the table indicate numbers of surviving embryos exhibiting the defects indicated, with percentages in parentheses. Numbers in the bottom section of the table are means (± SD) for surviving embryos for each of the growth parameters indicated.

† Indicates a statistically significant difference ($P < 0.05$) relative to the corresponding vehicle control value.

‡ "Cephalic defects" include missing optic vesicles and shortened prosencephalons.

amniotic fluid or culture medium, the percentages of abnormal embryos increased.

The addition of 9-*cis*-RA to the culture medium caused a decrease in some of the growth parameters, whereas after intraamniotic microinjections there was an increase in certain ones. Only after microinjections of the highest concentration (600 ng/mL) were decreases in growth parameters observed.

Table 3 shows data suggesting a conversion of 9-*cis*-RA to 13-*cis*-RA in the embryos but not yolk sacs at 2, 3.5, 4.5 and 6 hr after additions of 300 ng/mL 9-*cis*-RA to the culture medium. A less polar metabolite than all-*trans*-RA (Fig. 2b) was also observed in the embryos but not yolk sacs at all time points measured, but this unidentified metabolite was not quantified. After 6 hr, elevated levels of all-*trans*-RA were also detected in the embryos but not the yolk sacs.

Table 4 shows that after additions of dysmorphogenic concentrations of 13-*cis*-RA (500 ng/mL) to the culture medium, all-*trans*-RA and 13-*cis*-RAG were detected in the embryo at each time point measured. Additions of a dysmorphogenic quantity of all-*trans*-RA (300 ng/mL) to the culture medium (Table 5) resulted in readily detectable levels of 13-*cis*-RA after 2, 3.5, 4.5 and 6 hr and the 4-oxo metabolite was detected at relatively high levels after 4.5 hr. No 9-*cis*-RA could be detected at the time points measured after the additions of either all-*trans*-RA or 13-*cis*-RA to the culture medium.

Endogenous retinoid levels of rat embryos, although reported earlier [16–18], were reassessed using our present HPLC separation method (Fig. 2a). On day 11 of gestation (in two separate investigations) the retinoid concentrations were as follows: 13-*cis*-RA, 0 ng/g, 8 ng/g; 9-*cis*-RA, 0 ng/g, 0 ng/g; all-*trans*-RA, 23 ng/g, 20 ng/g; retinol, 208 ng/g, 211 ng/g. For comparative purposes, levels of retinoids were also asssessed in the eyes of rats on day 8 post-partum and in the human embryonic brain on day 47 of gestation. Both of these tissues are target sites of retinoid dysmorphogenesis. The values are presented in Fig. 2c and 2d. One should note the presence of both all-*trans*-RA and small amounts of 9-*cis*-RA.

DISCUSSION

Previous experiments *in vitro* with cultured rat conceptuses have shown that all-*trans*-RA is 7- to 10-fold more potent than 13-*cis*-RA when added to the culture medium (300 ng all-*trans*-RA/mL culture medium) [14] or when microinjected (300 ng all-*trans*-RA/mL amniotic fluid) [15]. The major defects involved the branchial arches and somites. Using HPLC analyses, evidence was presented that all-*trans*-RA is ultimately responsible for the

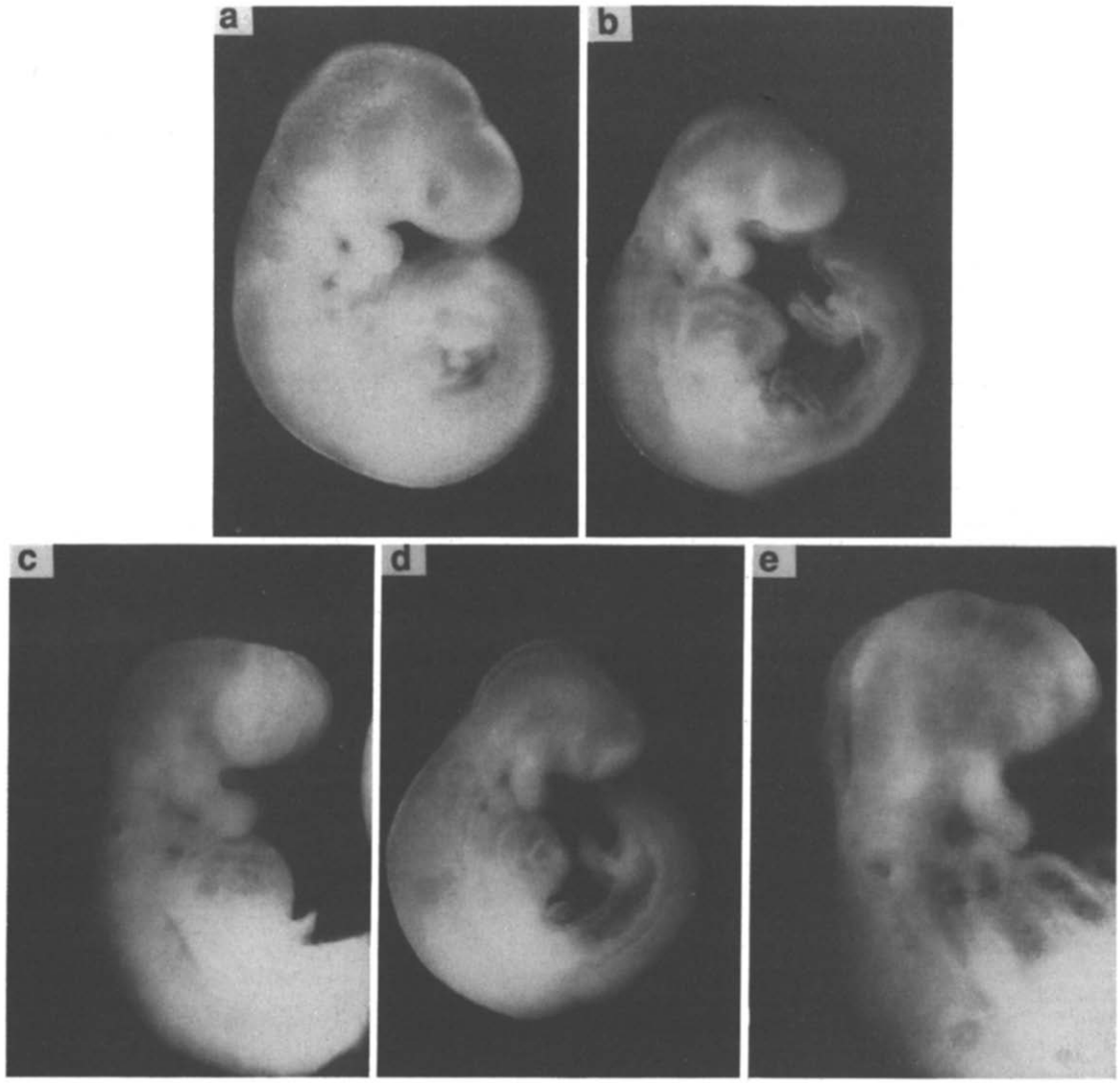

Fig. 1. (a) Photograph of a gestational day 11.5 rat embryo microinjected on day 10 of gestation with 10 nL DMSO. Magnification: ×20 × 1.5. (b) Photograph of a gestational day 11.5 rat embryo microinjected with 300 ng 9-*cis*-RA/mL amniotic fluid on day 10 of gestation. Note the typical cephalic abnormalities (missing optic vesicle, shortened prosencephalon) and underdeveloped second branchial arch). Magnification: ×25. (c) Photograph of a gestational day 11.5 rat embryo microinjected with 150 ng 9-*cis*-RA/mL amniotic fluid on day 10 of gestation. Note the prosencephalic schisis, missing optic vesicle, underdeveloped second branchial arch and open optic vesicle. Magnification: ×38. (d) Photograph of a gestational day 11.5 rat embryo microinjected with 300 ng 9-*cis*-RA/mL amniotic fluid on day 10 of gestation. Note the mescencephalic shisis, missing optic vesicle, and underdeveloped second branchial arch. Magnification: ×27. (e) Photograph of a gestational day 11.5 rat embryo microinjected with 300 ng 9-*cis*-RA/mL amniotic fluid on day 10 of gestation. Note the rhombencephalic schisis, missing optic vesicle, underdeveloped second branchial arch, and open otic vesicle. Magnification: ×45.

dysmorphogenic effects produced by 13-*cis*-RA [16, 17]. In the present study, we have shown that intraamniotic microinjections of 9-*cis*-RA (300 ng/mL amniotic fluid) elicited branchial arch and somite defects qualitatively and quantitatively similar to those elicited by all-*trans*-RA (300 ng/mL amniotic fluid) but with an increase (5–44%) in incidence of cephalic defects including missing optic vesicles [15].

These studies represent the first reported investigations of the dysmorphogenic and embryotoxic effects of the 9-*cis* isomer. When 600 ng/mL 9-*cis*-RA was microinjected, an increase in cardiac abnormalities occurred that had not been observed previously after microinjections of several other retinoids or all-*trans*-RA [15, 24]. This may represent a specific defect producible by high concentrations of 9-*cis*-RA on day 10 of gestation. Additional dysmorphogenic effects of the 9-*cis* isomer might be

Table 2. Dysmorphogenic concentration–effect relationships of 9-*cis*-retinoic acid added to the culture medium

		9-*cis*-RA*	
	Control	150 ng/mL	300 ng/mL
Embryos cultured	19	18	46
Survival (%)	19 (100)	18 (100)	45 (98)
Any defects (%)	1 (5)	13† (72)	30† (67)
Multiple defects (%)	0 (0)	7† (39)	22† (49)
Anterior schisis (%)	0 (0)	2 (11)	4 (1)
Cephalic defects‡ (%)	0 (0)	3 (16)	20† (44)
Rhombencephalic schisis (%)	0 (0)	4† (22)	8† (18)
Branchial arch defects (%)	0 (0)	9† (50)	23† (51)
Irregular somites (%)	1 (5)	6† (33)	17† (38)
Abnormal rotation (%)	0 (0)	11† (61)	11† (24)
Open otic vesicles (%)	0 (0)	1 (5)	14† (31)
Cardiac defects (%)	0 (0)	1 (5)	2 (4)
Mean yolk sac diameter (mm)	3.8 ± 0.2	3.2 ± 0.5†	3.2 ± 0.4†
Mean embryonic length (mm)	3.3 ± 0.4	2.9 ± 0.2†	2.8 ± 0.3†
Mean somite number	30 ± 1	24 ± 1†	28 ± 3
Mean yolk sac protein (μg)	580 ± 100	277 ± 68†	561 ± 49
Mean embryonic protein (μg)	475 ± 88	382 ± 57	473 ± 98

Statistical comparisons were carried out for defects by Chi square analyses and for quantitative parameters by ANOVA and Dunnett's multiple comparison test.

* 9-*cis*-RA (dissolved in DMSO) or an identical volume of the vehicle was added to the culture medium of cultured whole rat embryos on day 10 of gestation. Numbers in the top section of the table indicate surviving embryos exhibiting the effects indicated, with percentages in parentheses. Numbers in the bottom section of the table are means (± SD) for surviving embryos for each of the parameters indicated.

† Indicates a statistically significant difference ($P < 0.05$) relative to vehicle control value.

‡ "Cephalic defects" include missing optic vesicles and shortened prosencephalons.

Table 3. Levels of retinoids in conceptal tissue after addition of 300 ng/mL 9-*cis*-RA to the culture medium in rat whole embryo cultures on day 11 of gestation*

Metabolite† (ng/g)	2 hr		3.5 hr		4.5 hr		6 hr		6.5 hr	
	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac
13-*cis*-RA	78	0	50	0	78	0	130	0	51	0
9-*cis*-RA	312	429	376	400	221	247	160	246	152	120
all-*trans*-RA	3	0	14	0	18	0	96	0	127	0

* One hundred milligrams of tissue (approximately 30 embryos or yolk sacs) was needed for each measurement. Numbers in the table are ng retinoid/g (wet weight) of conceptal tissue. The results shown were verified in duplicate experiments for each time point and duplicate or triplicate HPLC analyses for each experiment.

† The three metabolites listed were the only ones detectable except retinol, which was reported previously [16–18], and one unknown.

expected by virtue of its capacity to bind with high affinity to RXRs. Additions of 9-*cis*-RA to the culture medium at the same concentrations that were microinjected produced virtually identical defects except that the incidence of multiple defects more than doubled and there was a decrease in some of the growth parameters. These observations suggest that 9-*cis*-RA may produce non-specific embryotoxicity via deleterious effects on yolk sac function when placed in the culture medium.

HPLC analyses indicated that biotransformation of retinoids occurred during the first 6 hr after additions to the culture medium. In two of the studies presented, 13-*cis*- and all-*trans*-RA were compared at concentrations that produced qualitatively and quantitatively similar dysmorphogenesis [14, 25, 26]. HPLC analyses indicated that all-*trans*-RA was readily converted to 13-*cis*-RA and the 4-oxo metabolite (Table 5) and that 13-*cis*-RA was converted to 13-*cis*-RAG and all-*trans*-RA in the embryos and yolk sacs (Table 4). Importantly, 9-*cis*-RA was below the limits of detectability. The mean

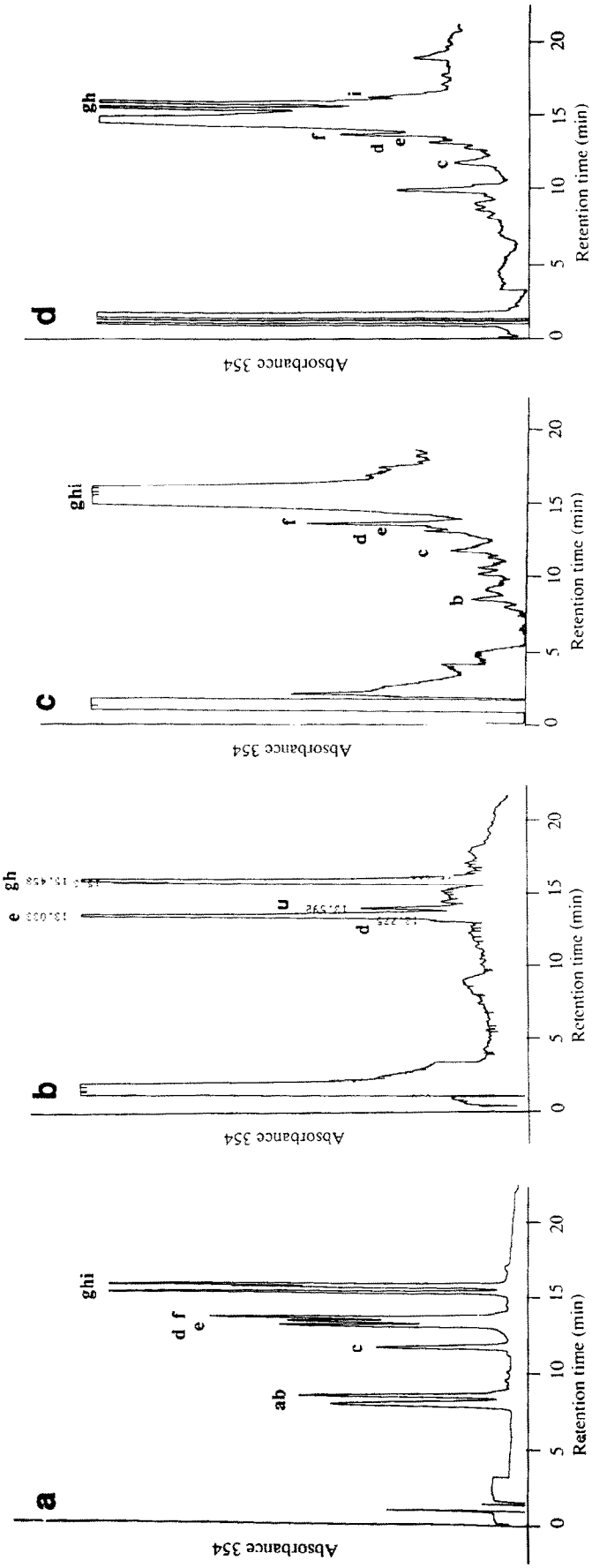

Fig. 2. (a) Chromatogram of retinoid standards. Peaks: a = 4-oxo-all-*trans*-RA (612 ng/mL), b = 4-oxo-13-*cis*-RA (567 ng/mL), c = all-*trans*-RAG (37 ng/mL), d = 13-*cis*-RA (576 ng/mL), e = 9-*cis*-RA (481 ng/mL), f = all-*trans*-RA (715 ng/mL), g = didehydro-retinol (864 ng/mL), h = retinol (490 ng/mL), and i = retinal (739 ng/mL), 0.02 absorbance units full scale (AUFS). (b) Chromatogram of rat embryos, 2 hr after treatment with 300 ng 9-*cis*-RA/mL culture medium on day 11 of gestation. Peak d coeluted with authentic 13-*cis*-RA (67 ng/g), peak e coeluted with authentic 9-*cis*-RA (480 ng/g), peak u is an unknown, peak g coeluted with authentic retinol (280 ng/g), and peak h coeluted with authentic retinal (520 ng/g), 0.0025 AUFS. (c) Chromatogram of postnatal day 8 rat eye. Peak b coeluted with authentic 4-oxo-13-*cis*-RA (8 ng/g wet weight), peak c coeluted with authentic all-*trans*-RAG (26 ng/g), peak d coeluted with authentic 13-*cis*-RA (14 ng/g), peak e coeluted with authentic 9-*cis*-RA (11 ng/g), peak f coeluted with authentic all-*trans*-RA (56 ng/g), peak g coeluted with authentic didehydro-retinol (960 ng/g), peak h coeluted with authentic retinol (1100 ng/g), and peak i coeluted with authentic retinal (800 ng/g), 0.00125 AUFS. (d) Chromatogram of human brain tissue on day 47 of gestation. Peak c coeluted with authentic all-*trans*-RAG (124 ng/g), peak d coeluted with authentic 13-*cis*-RA (146 ng/g), peak e coeluted with authentic 9-*cis*-RA (66 ng/g), peak f coeluted with authentic all-*trans*-RA (166 ng/g), peak g coeluted with authentic didehydro-retinol (740 ng/g), peak h coeluted with authentic retinol (1120 ng/g), and peak i coeluted with authentic retinal (98 ng/g), 0.005 AUFS.

Table 4. Levels of retinoids in conceptal tissue after addition of 500 ng/mL 13-*cis*-RA to the culture medium in rat whole embryo cultures on day 11 of gestation*

Metabolite† (ng/g)	2 hr		3.5 hr		4.5 hr		6 hr	
	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac
13-*cis*-RAG	12	52	56	100	10	15	32	60
13-*cis*-RA	575	390	502	680	392	165	414	454
all-*trans*-RA	92	70	100	142	85	58	52	50

* One hundred milligrams of tissue (approximately 30 embryos or yolk sacs) was needed for each measurement. Numbers in the table are ng retinoid/g (wet weight) of conceptal tissue. The results shown were verified in duplicate experiments for each time point and duplicate or triplicate HPLC analyses for each experiment.

† The three metabolites listed were the only ones detectable except retinol, which was reported previously [16–18]. 9-*cis*-RA was below the limits of detectability.

Table 5. Levels of retinoids in conceptal tissue after addition of 300 ng/mL all-*trans*-RA to the culture medium in rat whole embryo cultures on day 11 of gestation*

Metabolite† (ng/g)	2 hr		3.5 hr		4.5 hr		6 hr	
	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac	Embryo	Yolk sac
4-oxo-RA	0	0	0	0	0	116	108	94
13-*cis*-RA	92	88	90	110	96	112	94	110
all-*trans*-RA	134	120	280	254	254	306	242	342

* One hundred milligrams of tissue (approximately 30 embryos or yolk sacs) was needed for each experiment. Numbers in the table are ng retinoid/g (wet weight) of conceptal tissue. The results shown were verified in duplicate experiments for each time point and duplicate or triplicate HPLC analyses for each experiment.

† The three metabolites listed were the only ones detectable except retinol, which was reported previously [16–18]. 9-*cis*-RA was below the limits of detectability.

embryonic concentrations of all-*trans*-RA during the two studies were surprisingly similar, supporting the concept that all-*trans*-RA is the ultimate dysmorphogen for 13-*cis*-RA [16, 17]. Biotransformation of 9-*cis*-RA (Table 1) appeared to be restricted to the embryos in which 13-*cis*-RA and an unknown, less polar metabolite were detected at all time points measured (Fig. 2b). Significantly elevated levels of all-*trans*-RA were observed for the first time after 6 hr. 9-*cis*-RA was converted predominantly to a less active metabolite when placed in the culture medium at concentrations that elicited dysmorphogenesis with roughly the same incidence as that elicited by all-*trans*-RA at an identical concentration. This observation suggests that 9-*cis*-RA is a direct-acting dysmorphogen, approximately equipotent to all-*trans*-RA. This suggestion is consistent with the finding that both of these retinoids have a high affinity for nuclear receptors, the RXRs and RARs for 9-*cis*-RA and the RARs for all-*trans*-RA [11–13].

Endogenous all-*trans*-RA has been detected in whole rat embryos on days 10–12 of gestation [16–18] at levels between 3 and 20 ng/g and in human embryonic tissues at levels between 10 and 70 ng/g [27]. Using our present HPLC method which allows for separation of 9-*cis*-RA from 13-*cis*-RA and all-*trans*-RA, we were now able to show the presence of small amounts of 9-*cis*-RA in human embryonic brain and fetal rat eye (Fig. 2c and 2d), both target tissues of 9-*cis*-RA dysmorphogenesis, but none could be detected in whole rat embryos. These data suggest that only very low levels are involved in normal organogenesis and fetal development and may be localized or synthesized in specific tissues. Elevation of these levels would be expected to interfere with mechanisms involved in normal morphogenesis.

In conclusion, our present study has shown that: (1) 9-*cis*-RA produces defects similar to those elicited by equal concentrations of all-*trans*-RA after embryonic exposure on day 10 of gestation, except that 9-*cis*-RA caused an increased incidence of cephalic defects including missing optic vesicles and, at somewhat higher concentrations, an unusual heart defect not produced by other studied retinoids; (2) 9-*cis*-RA is readily converted in conceptal tissues to an unknown metabolite as well as to the less active isomer, 13-*cis*-RA, and only after 6 hr to levels of all-*trans*-RA that were considerably higher than endogenous levels; and (3) endogenous 9-*cis*-RA could not be detected in whole rat embryos but was detected in two target tissues of dysmorphogenesis, eye and brain. In view of our findings, we suggest

that 9-*cis*-RA is a direct-acting dysmorphogen and that the additional cephalic and cardiac defects observed may have been mediated via one or more of the nuclear RXR receptors.

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