Biochemical Pharmacology, Vol. 42, No. 12, pp. R21–R24, 1991.
Printed in Great Britain.

0006–2952/91 $3.00 + 0.00

RAPID COMMUNICATION

ELEVATED LEVELS OF ALL-*TRANS*-RETINOIC ACID IN CULTURED RAT EMBRYOS 1.5 HR AFTER INTRAAMNIOTIC MICROINJECTIONS WITH 13-*CIS*-RETINOIC ACID OR RETINOL AND CORRELATIONS WITH DYSMORPHOGENESIS

Joan Creech Kraft*, Tot Bui and Mont R. Juchau
Department of Pharmacology, School of Medicine, University of Washington, Seattle, WA 98195, U.S.A.

(Accepted 14 October 1991)

Accutane (isotretinoin, 13-*cis*-retinoic acid, 13-*cis*-RA) is a potent human teratogen [1-3], and it has been proposed that high doses of retinol taken during pregnancy also present a human teratogenic risk [4,5].

Recent studies *in vitro*, with a rat whole embryo culture system, compared concentration-effect relationships for all-*trans*-retinoic acid (all-*trans*-RA), 13-*cis*-RA and retinol after intraamniotic microinjections on day 10 of gestation [6]. The dysmorphogenic effects elicited were qualitatively similar for all three retinoids, but were produced by a low concentration of all-*trans*-RA (250 ng/mL), a 6-fold higher concentration of 13-*cis*-RA and an approximately 16-fold higher concentration of retinol. The present study uses this unique method of intraamniotic microinjection of cultured rat conceptuses, followed by HPLC analyses of the pertinent retinoids in conceptal tissues 1.5 hr after microinjections of all-*trans*-RA (250 ng/mL), 13-*cis*-RA (1500 ng/mL) or retinol (4000 ng/mL) on day 10 of gestation. The objective of this study was to determine whether conceptal conversion of 13-*cis*-RA and retinol to all-*trans*-RA may have been a prerequisite for the dysmorphogenic activity of the latter two chemicals.

MATERIALS AND METHODS

The whole embryo culture system with conceptuses from time-mated pregnant rats (Sprague-Dawley, Wistar-derived) has been described in detail previously [7,8]. Explanted conceptuses were cultured for 16 hr in 50% heat-inactivated (56°, 30 min) female rat serum and 50% Waymouth's medium (saturated with a gas mixture of O_2:CO_2:N_2 ; 5:5:90) in roller bottles at 37° and in total darkness, prior to exposure to the retinoid under study. The microinjection procedure was carried out as described earlier [7,8] but in a darkened room with yellow light to prevent photoisomerization. Amniotic fluid concentrations of all-*trans*-RA (250 ng/mL), 13-*cis*-RA (1500 ng/mL) and retinol (4000 ng/mL) produce qualitatively similar patterns and a roughly equal incidence of branchial arch dysmorphogenesis [6] and were therefore used in the present study. The retinoids were gifts from Hoffmann-La Roche, Inc. (Nutley, NJ). Solutions to be microinjected were prepared in dimethyl sulfoxide (DMSO) and were checked for correct concentration and purity with HPLC analysis immediately before each experiment. The microinjected conceptuses were incubated in freshly prepared culture medium (saturated with gas mixtures of O_2:$C0_2$:N_2; 20:5:75) for 1.5 hr in the dark. The yolk sacs and embryos were removed and pooled separately in polyethylene tubes which were kept on ice and stored at -70°. Litters from 16 pregnant rats (approximately 90-150 embryos proper or yolk sacs; 100-150 mg wet weight) were required for each HPLC analysis of the retinoids and each group was numbered as an individual experiment (see Table 1). The embryos proper or yolk sacs were treated with 2-3 vol. of isopropanol, vortexed for 1 min and homogenized at 4° using a Sonic-L converter for 10 sec at a setting of 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 [9]. The analytical eluents consisted of solvent A (methanol) and solvent B (40 nM ammonium acetate, pH 7.3, and methanol, 50% v/v). The pumps interfaced with a Shimadzu SPD-6AV UV-VIS detector (set at 354 nm) and a Shimadzu CRA Chromatopac data processor. The system was equipped with a Shimadzu mixing chamber and manual injector. The analytical column (120 x 4.6 mm) was slurry packed with Spherisorb 3 ODS II (3 μm). Cartridges

***Corresponding author. Tel. (206) 543-4274; FAX (206) 685-3822.**

(20 x 4.6 mm) prepacked with Lichrosorb RP 18 (10 μm) were used as precolumns.

RESULTS AND DISCUSSION

The tissue levels of retinoids found in untreated conceptuses (controls) on day 10 of gestation are shown in Table 1. Endogenous levels of all-*trans*-RA were 20 ng/g in embryos proper and 18 ng/g in yolk sacs and were lower for 13-*cis*-RA, 3 ng/g in both the embryos proper and yolk sacs. The endogenous levels of retinol were 194 ng/g in the embryos proper and 255 ng/g in the yolk sacs on day 10 of gestation.

The levels of retinoids in conceptal tissues 1.5 hr after microinjections of all-*trans*-RA (250 ng/mL), 13-*cis*-RA (1500 ng/ml) or retinol (4000 ng/mL) into the amniotic cavity in rat whole embryo culture on day 10 of gestation are shown in Table 1. Relatively high levels of all-*trans*-RA and retinol were found in the embryos proper by 1.5 hr (all-*trans*-RA, 247 ng/g and retinol, 3280 ng/g). Measured levels of the microinjected retinoids in the yolk sacs were 2- to 3-fold lower than these. The 4-oxo metabolites and β-glucuronides could not be detected in the conceptal tissues at this time point. Interestingly, only a small percentage of the microinjected 13-*cis*-RA was detected in embryos proper or yolk sacs at 1.5 hr, in two experiments. This indicated that 13-*cis*-RA had not accumulated in the embryos proper or yolk sacs at this time point, as did both all-*trans*-RA and retinol, but may have remained predominantly in the amniotic cavity or may have undergone rapid biotransformation. (Because of the minute quantities present, measurements of retinoids in the amniotic cavity were not possible.) The all-*trans* isomer was found in the embryos proper at higher levels than 13-*cis*-RA after microinjections of 13-*cis*-RA.

TABLE 1. Levels of retinoids in control conceptal tissues and 1.5 hr after microinjections of all-*trans*-RA, 13-*cis*-RA or retinol into the amniotic cavity in rat whole embryo cultures on day 10 of gestation

	Control			all-*trans*-RA (250 ng/mL)			13-*cis*-RA (1500 ng/mL)			Retinol (4000 ng/mL)		
Metabolite (ng/g)	**Expt. No.**	**Embryo proper**	**Yolk sac**	**Expt. No.**	**Embryo proper**	**Yolk sac**	**Expt. No.**	**Embryo proper**	**Yolk sac**	**Expt. No.**	**Embryo proper**	**Yolk sac**
13-*cis*-RA	*1**	3†	3	*2*	47	27	*3* *4*	14 56	>1 21	*5*	>1	22
all-*trans*-RA	*1*	20	18	*2*	247	102	*3* *4*	64 108	22 27	*5*	72	36
Retinol	*1*	194	255	*2*	239	360	*3* *4*	300 342	318 285	*5*	3280	900

* The following number of embryos proper or visceral yolk sacs were used: experiment 1, 90; experiment 2, 148; experiment 3, 119; experiment 4, 142; experiment 5, 135.

† Duplicate or triplicate HPLC analyses (not shown) were carried out in each experiment and confirmed the data of the first HPLC analysis carried out immediately after collection of the samples.

These findings relate directly to observations *in vivo* in which 13-*cis*-RA was detected at very low levels in mouse embryo tissues after the oral administration of marginally teratogenic doses of 13-*cis*-RA (10 and 100 mg/kg; embryo/ maternal plasma concentration ratios of ~0.1 and 0.03, respectively, at 2 hr) [10,11]. *In vivo*, as *in vitro*, the all-*trans* isomer was detected at higher concentrations than 13-*cis*-RA in the embryo after oral administration [10,11] or intraamniotic microinjection of 13-*cis*-RA. Creech Kraft *et al.* [11] have also shown with experiments *in vivo* that all-*trans*-RA readily accumulates in tissues of the mouse embryo proper at levels nearly equivalent to measured plasma concentrations after oral administration of teratogenic doses (10 mg/kg; embryo/maternal plasma concentration ratios of ~0.9 at 2 hr) [11]. This finding is reflected in the experiments *in vitro* presented here.The same approximate concentration of all-*trans*-RA (250 ng/mL) placed in the amniotic cavity was detected in tissues of the embryos proper after 1.5 hr.

Possibly the most important observation of this study is that all-*trans*-RA was detected in the embryos proper at levels between 64 and 108 ng/g after the microinjections of 13-*cis*-RA (1500 ng/mL), or retinol

(4000 ng/mL). These levels of all-*trans*-RA were 3- to 5-fold higher than those detected in untreated embryos on day 10 of gestation and almost certainly contributed to the dysmorphogenesis observed [8]. Tissue levels of 13-*cis*-RA measured after microinjections of either all-*trans*-RA or 13-*cis*-RA were similar and always lower than the levels of all-*trans*-RA. Therefore, 13-*cis*-RA appeared unlikely to contribute substantially to the observed dysmorphogenic effects. 13-*cis*-RA was only marginally detectable in conceptal tissues after microinjections of retinol and appeared similarly unlikely to play a major direct role in the dysmorphogenic activity of that retinoid. Whether elevated levels of retinol were contributing to the observed dysmorphogenesis, however, cannot be ruled out completely.

Our reported data strongly suggest that conceptal tissues are capable of biotransformation of 13-*cis*-RA and retinol and that all-*trans*-RA likely is ultimately responsible for the dysmorphogenic effects produced by 13-*cis*-RA and retinol in the embryo culture system. It should be noted that, although 4-oxo-all-*trans*-RA was not detected in the conceptal tissues at the time point measured, it is a dysmorphogen, nearly as potent as all-*trans*-RA in our culture system [unpublished results] and most likely is playing a role in human 13-*cis*-RA teratogenicity. From the results presented here, using our culture system, we conclude that isomerization of 13-*cis*-RA and oxidative biotransformation of retinol to all-*trans*-RA within embryonic tissues seem to play a crucial role for the dysmorphogenic activities of 13-*cis*-RA and retinol, respectively.

The fact that all-*trans*-RA, but not 13-*cis*-RA, binds with high affinity to both the cellular retinoic acid binding protein (CRABP) [12,13] and the α,β,γ RAR receptors [14] also makes our findings less surprising. All-*trans*-RA can be easily stabilized in the embryos proper through protein binding. More importantly, and of particular relevance to our studies on dysmorphogenesis is that RAR-α and RAR-β reportedly occur in mouse embryos during early morphogenesis in both the hindbrain and in the spinal cord. The distribution of CRABP correlated well with the known target tissues of retinoid teratogenesis [15].

Acknowledgements This work was supported by NIEHS Grants ES-04041 and ES-07032. We thank Hoffmann-La Roche for their contribution to buy HPLC equipment.

REFERENCES

1. Rosa FW, Teratogenicity of isotretinoin. *Lancet* **2**: 513, 1983.

2. Braun JT, Franciosi RA, Mastri AR, Drake RM and O'Niel BL, Isotretinoin dysmorphic syndrome. *Lancet* **1**: 506-507, 1984.

3. Lammer EJ, Chen DT, Hoar RM, Agnish ND, Benke PJ, Braun JT, Curry CJ, Fernhoff PM, Grix AW Jr., Lott IT, Richard JM and Sun SC, Retinoic acid embryopathy. *N Engl J Med* **313**: 837-841, 1985.

4. Rosa FW, Wilk AL and Kelsey FO, Teratogen update: Vitamin A congeners. *Teratology* **33:** 355-364, 1986.

5. Anonymous, Teratology Society position paper: Recommendations for vitamin A use during pregnancy. *Teratology* **35**: 269-275, 1987.

6. Lee QP, Juchau MR and Creech Kraft J, Microinjection of cultured rat embryos: Studies with retinol, 13-*cis*- and all-*trans*-retinoic acid. *Teratology* **44**: 313-323, 1991.

7. Stark KL and Juchau MR, Microinjection of cultured rat embryos: A new technique for studies in chemical dysmorphogenesis. *Toxicol Appl Pharmacol* **100:** 411-416, 1989.

8. Stark KL, Lee QP, Namkung MJ, Harris C and Juchau MR, Dysmorphogenesis elicited by microinjectioned acetaminophen analogues. *J Pharm Exp Ther* **255**: 74-83, 1990.

9. Creech Kraft J, Eckhoff Chr, Kuhnz W, Loefberg B and Nau H, Automated determination of 13-*cis* and all-*trans*-retinoic acid, their 4-oxo-metabolites and retinol in plasma, amniotic fluid and embryo by reversed-

phase high-performance liquid chromatography with a precolumn switching technique. *J Liq Chrom* **11**: 2051-2069, 1988.

10. Creech Kraft J, Kochhar DM, Scott WJ and Nau H, Low teratogenicity of 13-*cis*-retinoic acid (isotretinoin) in the mouse corresponds to low embryo concentrations during organogenesis: Comparison to the all-*trans* isomer. *Toxicol Appl Pharmacol* **87**: 474-482, 1987.

11. Creech Kraft J, Loefberg B, Chahoud I, Bochert G and Nau H, Teratogenicity and placental transfer of all-*trans*-, 13-*cis*-, 4-oxo-all-*trans*, and 4-oxo-13-*cis*-retinoic acid after administration of a low oral dose during organogenesis in mice. *Toxicol Appl Pharmacol* **100**: 162-176, 1989.

12. Kwarta RF, Kimmel CA, Kimmel GL and Slikker W Jr., Identification of the cellular retinoic acid binding protein (CRABP) within the embryonic mouse (CD-1) limb bud. *Teratology* **32**: 103-111, 1985.

13. Howard WB, Sharma RP, Willhite CC and Dawson MI, Binding affinities of retinoids to fetal cellular retinoic acid-binding protein (CRABP) in relation to their teratogenic potency in hamsters. *Biochem Pharmacol* **40**: 643-648, 1990.

14. Apfel C, Crettaz M and LeMotte P, Differential binding and activation of synthetic retinoids to retinoic receptors. In: *Retinoids in Normal Development and Teratogenesis* (Ed. Morriss-Kay G). Oxford University Press, Oxford, in press.

15. Ruberte E, Dolle P, Chambon P and Morriss-Kay G, Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryos. *Development* **111**: 45-60, 1991.