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SHORT COMMUNICATIONS

Conceptal biotransformation of 4-oxo-all-*trans*-retinoic acid, 4-oxo-13-*cis*-retinoic acid and all-*trans*-retinoyl-β-glucuronide in rat whole embryo culture

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Abstract—In cultured rat conceptuses, intraamniotic microinjections of 2500 ng/mL of 4-oxo-13-*cis*-retinoic acid, 600 ng/mL 4-oxo-all-*trans*-retinoic acid or 4000 ng/mL all-*trans*-retinoyl-β-glucuronide, produce qualitatively and quantitatively similar patterns of dysmorphogenesis as those reported after the intraamniotic microinjection of 250 ng/mL all-*trans*-retinoic acid [Lee *et al.*, *Teratology* **44**: 313–323, 1991; Creech Kraft *et al.*, *Teratology* **45**: 259–270, 1992]. In the present study, we utilized HPLC techniques to analyze retinoid levels in cultured rat conceptuses, 1.5 hr after intraamniotic microinjections of 4-oxo-13-*cis*-retinoic acid (2500 ng/mL), 4-oxo-all-*trans*-retinoic acid (600 ng/mL) or all-*trans*-retinoyl-β-glucuronide (4000 ng/mL). Our findings show that, after the microinjections of 4-oxo-all-*trans*-retinoic acid or 4-oxo-13-*cis*-retinoic acid (at these selected concentrations), 4-oxo-all-*trans*-retinoic acid was predominant in the embryos proper at concentrations of about 200 nM. This was roughly equivalent to the levels of all-*trans*-retinoic acid assayed after microinjections of all-*trans*-retinoyl-β-glucuronide (4000 ng/mL). We conclude from these studies that both 4-oxo-all-*trans*-retinoic acid and all-*trans*-retinoic acid behave as ultimate or proximate dysmorphogens.

13-*cis*-Retinoic acid (13-*cis*-RA,* Accutane®) is a recognized human teratogen [1, 2]. After its administration to humans at therapeutic doses, five metabolites have been identified in the serum [3]. These are 4-oxo-13-*cis*-retinoic acid (4-oxo-13-*cis*-RA), 4-oxo-all-*trans*-retinoic acid (4-oxo-all-*trans*-RA), 13-*cis*-retinoyl-β-glucuronide (13-*cis*-RAG), all-*trans*-retinoyl-β-glucuronide (all-*trans*-RAG) and all-*trans*-retinoic acid (all-*trans*-RA). Each of these metabolites produces qualitatively similar dysmorphogenic effects after microinjections into the amniotic sacs of cultured whole rat embryos on day 10 of gestation. However, concentration–effect relationships differ markedly for these metabolites [4, 5]. Four- to sixteen-fold higher concentrations of the glucuronides and *cis* isomers are required to elicit incidences and severities of malformations similar to those produced by all-*trans*-RA and 4-oxo-all-*trans*-RA. Recent evidence has been provided [6] to indicate that, in cultured whole rat embryos, conceptal biotransformation to all-*trans*-RA is most likely responsible for the dysmorphogenic effects elicited by intraamniotically microinjected 13-*cis*-RA (1500 ng/mL) or retinol (4000 ng/mL). In the present study, we utilized HPLC techniques to analyze retinoid levels in cultured rat conceptuses 1.5 hr after intraamniotic microinjections of 4-oxo-13-*cis*-RA (2500 ng/mL), 4-oxo-all-*trans*-RA (600 ng/mL) or all-*trans*-RAG (4000 ng/mL). Quantities chosen were selected because each elicits approximately equivalent patterns of dysmorphogenesis (qualitatively and quantitatively) to those produced by all-*trans*-RA (250 ng/mL) [5]. The purpose of the study was to investigate the concept that both 4-oxo-all-*trans*-RA and all-*trans*-RA, in contrast to 4-oxo-13-*cis*-RA or the glucuronide conjugates (all-*trans*-RAG and 13-*cis*-RAG), behave as ultimate or proximate dysmorphogens.

* Abbreviations: 13-*cis*-RA, 13-*cis*-retinoic acid; 4-oxo-13-*cis*-RA, 4-oxo-13-*cis*-retinoic acid; 4-oxo-all-*trans*-RA, 4-oxo-all-*trans*-retinoic acid; 13-*cis*-RAG, 13-*cis*-retinoyl-β-glucuronide; all-*trans*-RAG, all-*trans*-retinoyl-β-glucuronide; all-*trans*-RA, all-*trans*-retinoic acid; DMSO, dimethyl sulfoxide; and CRABP, cellular retinoic acid binding protein.

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 4-oxo-all-*trans*-RA (600 ng/mL), 4-oxo-13-*cis*-RA (2500 ng/mL) and all-*trans*-RAG (4000 ng/mL) produced qualitatively similar patterns and a roughly equal incidence of branchial arch dysmorphogenesis [5] and were therefore used in the present study. The 4-oxo metabolites were gifts from Hoffmann-La Roche, Inc. (Nutley, NJ). All-*trans*-RAG was a gift from Drs. James Olson and Arun Barua of Iowa State University, Ames, IA. 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:CO_2:N_2; 20:5:75) in 1.5 hr in the dark. Yolk sacs and embryos were removed and pooled separately in polyethylene tubes which were kept on ice and stored at −70°. Litters from 16–24 pregnant rats (approximately 90–190 embryos proper or yolk sacs; 100–180 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 mL) 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

Table 1. Levels of retinoids in control conceptal tissues and 1.5 hr after microinjections of 4-oxo-13-*cis*-RA, 4-oxo-all-*trans*-RA or all-*trans*-RAG into the amniotic cavity in rat whole embryo cultures on day 10 of gestation*

	Control†			4-oxo-all-*trans*-RA (600 ng/mL)			4-oxo-13-*cis*-RA (2500 ng/mL)			all-*trans*-RAG (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
4-oxo-13-*cis*-RA	1	0	0	2	0	0	4	78	0	6	0	0
				3	78	0	5	53	0			
4-oxo-all-*trans*-RA	1	0	0	2	39	0	4	114	54	6	0	0
				3	81	0	5	57	52			
13-*cis*-RAG	1	0	0	2	0	0	4	0	0	6	796	520
				3	0		5	0	0			
all-*trans*-RAG	1	0	0	2	0	0	4	0	0	6	896	232
				3	0		5	0	0			
13-*cis*-RA	1	3	3	2	0	0	4	3	0	6	20	12
				3	0	0	5	0	3			
all-*trans*-RA	1	20	18	2	16	6	4	18	4	6	60	32
				3	20	15	5	16	11			
Retinol	1	194	255	2	296	240	4	576	380	6	476	480
				3	330	345	5	380	420			

* The following numbers of embryos proper or visceral yolk sacs were used: Expt. 1, 90; Expt. 2, 135; Expt. 3, 187; Expt. 4, 135; Expt. 5, 109; and Expt. 6, 159.

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 measurements were always within the reported day-to-day coefficients of variations of <5% reported in Ref. 9.)

† These values have been reported in Ref. 6.

analytical column (120 × 4.6 mm) was slurry packed with Spherisorb 3 ODS II (3 μM). Cartridges (20 × 4.6 mm) prepacked with Lichrosorb RP 18 (10 μm) were used as precolumns.

Results

Retinoid levels in tissues of untreated conceptuses (controls) have been reported recently [6] and are included again in Table 1 (Expt. 1) for purposes of direct comparison.

Levels of retinoids in conceptal tissues measured 1.5 hr after microinjections on day 10 of 4-oxo-all-*trans*-RA (600 ng/mL) are shown in Table 1 (Expts. 2 and 3). In Expt. 2, a total of 135 microinjected conceptuses (litters from 16 rats) were extracted and analyzed. In the embryos proper, only 39 ng/g of 4-oxo-all-*trans*-RA was detected. A second experiment, using the litters of 24 rats (187 embryos), was carried out in order to verify these results. In both Expts. 2 and 3, levels of all-*trans*-RA and retinol were quantitatively similar to those measured in controls in the embryos proper as well as in the yolk sacs.

Levels of retinoids in conceptal tissues assessed 1.5 hr after microinjections of 4-oxo-13-*cis*-RA (2500 ng/mL) into conceptal amniotic fluid are shown in Table 1 (Expts. 4 and 5). In both embryos proper and visceral yolk sacs, tissue levels of all-*trans*-RA and 13-*cis*-RA were quantitatively similar to levels of the same retinoids assayed in untreated controls. Levels of retinol were higher than those measured in controls (for reasons currently unexplained). In experiments 4 and 5, 4-oxo-13-*cis*-RA (78 and 53 ng/g) and 4-oxo-all-*trans*-RA (114 and 57 ng/g) were detected in the embryos proper.

Levels of retinoids in conceptal tissues after intraamniotic microinjections of all-*trans*-RAG (4000 ng/mL) are shown in Table 1 (Expt. 6). After microinjections of all-*trans*-RAG (a total of 159 conceptuses from the litters of 24 rats), tissue levels of retinol were higher in both embryos proper and yolk sacs, than in those of untreated controls. There was a 3-fold increase in levels of all-*trans*-RA in the embryos proper (60 ng/g) as well as a 2-fold increase of this metabolite in yolk sacs (32 ng/g). Approximately 40% of microinjected glucuronide (all-*trans*-RAG, 896 ng/g: 13-*cis*-RAG, 796 ng/g) was detected in the embryos proper.

Discussion

Our HPLC analyses showed that following intraamniotic microinjection of 4-oxo-all-*trans*-RA, the 4-oxo-13-*cis*-RA isomer was present in the embryo proper. Similarly, after microinjections of 4-oxo-13-*cis*-RA, the 4-oxo-all-*trans*-RA isomer could be detected in both the embryo proper and yolk sac, and after microinjections of all-*trans*-RAG, the 13-*cis*-RAG isomer was detected in the yolk sac and embryo. These results indicate that isomerization of retinoids in conceptal tissue occurred under our experimental conditions. Moreover, after microinjection of all-*trans*-RAG, there was a 2- to 3-fold increase of all-*trans*-RA in the yolk sac and embryo as compared to controls, which indicated that hydrolysis of the glucuronide conjugate had taken place. Comparable levels of the all-*trans*-4-oxo-metabolite were measured in embryos proper after intraamniotic microinjections of a large quantity of 4-oxo-13-*cis*-RA (2500 ng/mL) or a 4-fold lower concentration of 4-oxo-all-*trans*-RA (600 ng/mL). Levels of 4-oxo-13-*cis*-RA in the embryos proper and yolk sacs after microinjection of 4-oxo-13-*cis*- or 4-oxo-all-*trans*-RA were always lower than concentrations of 4-oxo-all-*trans*-RA. Therefore, 4-oxo-all-*trans*-RA is most likely a direct acting dysmorphogen.

After intraamniotic microinjection of all-*trans*-RAG, the 3-fold increase in levels of all-*trans*-RA, compared to controls, makes it seem probable that all-*trans*-RA is largely responsible for the dysmorphogenesis elicited by all-*trans*-RAG. Whether increases in the embryo proper of all-*trans*-RAG and 13-*cis*-RAG contribute to the dysmorphogenic effect cannot be concluded from these studies, but the available data from these and previous studies [5, 10] do not provide support for that concept. Interestingly, the reported embryonic levels of all-*trans*-RA (60 ng/g) 1.5 hr after intraamniotic microinjections in rat whole embryo culture of 4000 ng/mL all-*trans*-RAG were very similar to the embryonic concentrations of all-*trans*-RA (60–100 ng/g) 1.5 hr after microinjection of 4000 ng/mL retinol or 1500 ng/mL 13-*cis*-RA [6]. Since all-*trans*-RA, but not 13-*cis*-RA, binds with high affinity to both cellular retinoic acid binding protein (CRABP) [11, 12] and α, β and γ RAR receptors [13], our detected amounts of all-*trans*-RA may have been stabilized in the embryos proper through protein binding. Even 24 hr after intraamniotic microinjections of all-*trans*-RA (250 ng/mL), 13-*cis*-RA (1500 ng/mL), all-*trans*-RAG (4000 ng/mL) or retinol (4000 ng/mL) in rat whole embryo culture, embryonic levels of all-*trans*-RA were always 10-fold higher than those of the untreated controls [10].

Binding studies have shown that 4-oxo-all-*trans*-RA binds with only slightly less affinity to CRABP than all-*trans*-RA and that 4-oxo-13-*cis*-RA binds with 10-fold less affinity to CRABP than all-*trans*-RA [12, 14]. However, 4-oxo-all-*trans*-RA has somewhat less binding affinity to the RAR α, β and γ receptors than does all-*trans*-RA [13], and this may explain the 2-fold differences between levels of all-*trans*-RA and 4-oxo-all-*trans*-RA needed to produce dysmorphogenesis in our culture system *in vitro*.

We conclude from these studies with intraamniotic microinjections in rat whole embryo culture that conceptal biotransformation of 4-oxo-13-*cis*-RA and all-*trans*-retinoyl-β-glucuronide to 4-oxo-all-*trans*-RA and all-*trans*-RA, respectively, is playing a major role in the dysmorphogenic effects elicited by the latter two compounds. Since both all-*trans*-RA and 4-oxo-all-*trans*-RA were also present in the serum of a woman who was being treated with Accutane® (13-*cis*-RA) [3], it is tempting to speculate that they are likewise playing a major role in the human teratogenic activity of 13-*cis*-RA.

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Effects of 1,25-dihydroxyvitamin D_3 analogue 1,24(OH)2-22-ene-24-cyclopropyl D_3 on proliferation and differentiation of a human megakaryoblastic leukemia cell line

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Abstract—The novel analogue of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$ D_3), 1,24(OH)2-22-ene-24-cyclopropyl D_3 (calcipotriol, MC903), exhibits similar effects on cell proliferation and cell differentiation in a newly established human megakaryoblastic leukemia cell line (HIMeg). MC903 was found to inhibit cell proliferation and induce cell differentiation in a liquid culture system at concentrations comparable to those of 1,25$(OH)_2$ D_3. Colony formation assay showed that MC903 or 1,25$(OH)_2$ D_3 markedly diminished the colony-forming ability of HIMeg cells at concentrations of 10^{-6} M to 10^{-10} M. Cell cycle analysis demonstrated that, as seen with 1,25$(OH)_2$ D_3, MC903 also altered the cell cycle distribution; the fraction of cells in $G_0 + G_1$ increased while those in S and G_2 + M decreased. It can be concluded from these findings that 1,25$(OH)_2$ D_3 and its analogue MC903 have approximately equipotent effects on cells of megakaryoblastic lineage and are potentially useful in studying the cellular processes that are responsible for megakaryocytopoiesis.

Vitamin D_3 is a secosteroid; sequential hydroxylation in the liver and kidney converts it to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3*] which is the active form. 1,25$(OH)_2$ D_3 has profound effects on the regulation of bone and mineral metabolism [1]. Most, if not all, of its actions are mediated like steroid hormones via binding and activation of high affinity nuclear receptors. The activated receptors then modulate gene expression by binding to hormone response elements of the target genes [2]. In classical target tissues, 1,25$(OH)_2$ D_3 enhances the expression of the calcium-binding protein, alkaline phosphatase, and the 24-hydroxylase [1–3]. Extremely sensitive radioligand receptor binding assays have shown that 1,25$(OH)_2$ D_3 receptors are present not only in the classical target tissues such as bone, intestinal mucosa and kidney, but also in the skin, endocrine glands, thymus, brain, bone marrow, and activated or transformed lymphopoietic cells, among other tissues [4, 5]. The presence of high affinity 1,25$(OH)_2$ D_3

* Abbreviations: MC903, calcipotriol, 1,24(OH)2-22-ene-24-cyclopropyl D_3; 1,25$(OH)_2$ D_3, 1,25-dihydroxyvitamin D_3; HIMeg, human megakaryoblastic leukemia cell line; IMDM, Iscove's modified Dulbecco's medium; FCS, fetal calf serum.