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Interactive Effects of Cadmium and Retinoic Acid on Mouse Limb Bud Development *In Vitro*

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Cadmium (Cd) is a wide-spread heavy metal which occurs both naturally and as a contaminant originating from a variety of industrial processes and from cigarette smoke (Friberg et al 1974). Retinoic acid (RA) is a major physiologically active metabolite of vitamin A (Kwasigroch et al, 1986), the *cis* form of RA being used for the treatment of cystic acne. Both Cd and RA are well-known teratogens in many species of animals resulting in a wide array of developmental abnormalities (Layton & Layton 1979; Sulik & Dehart 1988). The mechanism of perturbed development probably occurs via different mechanisms: Cd-induces reduction of hemoglobin content resulting in an acidotic embryonic environment (Feuston & Scott 1985), or Cd-induces cell necrosis and errors of induction (Messerle & Webster 1982); while RA, hypothesized to play a major role in limb pattern formation through positional information in vertebrates (Summerbell 1984), may disturb normal temporal-spatial processes. The current study was undertaken to determine whether Cd and RA have any interactive effects in limb buds cultured *in vitro*.

MATERIALS AND METHODS

A two-by-two factorial design was used to study the interactive effects of Cd and RA in submerged limb bud culture. One group of limbs served as control containing media, distilled water and 95% ethanol; a second was exposed to RA and vehicle only (RA); a third to cadmium chloride and vehicle only (Cd); and a fourth group was exposed to both agents (RA & Cd). One additional group of limbs exposed to media only was used as a negative control to determine any effects of the vehicle.

Single male CD-1 mice were placed in cages containing 2-5 females during the last hour of the dark cycle. Females were checked for the presence of a vaginal plug, which was considered as day 0 of pregnancy. Pregnant females were sacrificed on day 12 by cervical dislocation, the uterus removed aseptically and placed in sterile, cold Hank's balanced salt solution (HBSS). Embryos were dissected free of their placental membranes, placed in cold HBSS and both forelimb buds removed by microdissection from the body wall, lateral to the somites. Limb buds were then transferred to 50 ml Wheaton bottles with 5 ml of culture medium, consisting of 3.75 ml of BGJb culture medium, 1.25 ml cell-culture qualified, heat-inactivated fetal bovine serum (Gibco Canada Inc., Burlington, Ont.), and 50 µL of 8.5 mM vitamin C.

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Twenty µL of a stock solution of all-trans RA (Hoffman-LaRoche Inc.) dissolved in 95% ethanol (125 µg/ml) and 20 µL of a stock solution of cadmium chloride (Sigma Chemical Co.) dissolved in distilled water (1.25 mg/ml) was added to the culture medium to achieve a final concentration of 0.5 µg/ml and 5.0 µg/ml respectively. Doses were chosen to achieve a minimum teratogenic effect for each agent based on previous dose-response studies conducted in this lab (unpublished data) and other similar *in vitro* results (Kwasigroch et al 1984). Control vials contained equivalent volumes of distilled water and/or 95% ethanol. Cultures were gassed initially and at 24 hour intervals until termination with a 50% O_2, 5% CO_2, and 45% N_2 gas mixture for 30 seconds and incubated and rotated at 14 rpm for 72 hours at 37° C in a water-jacketed incubator.

Approximately 20 limbs per group reserved for biochemical analyses were frozen at -80° C until assayed. Following thawing, limbs were homogenized for 60 seconds at 24,000 r.p.m with a tissue sonicator in 2.0 mls of assay buffer consisting of 100 mM NaCl, 10 mM Trizma HCL (Sigma Chemical Co., St. Louis, Mo.), and 10 mM EDTA at pH 7.0. Total protein was determined using a bicinchoninic acid (BCA) reagent kit (Sigma Chemical Co., St. Louis, MO) using a procedure described by Smith et al. 1985. Extracellular matrix was assayed using a modified Alcian Blue staining method outlined by Hassell & Horigan 1982. One half ml of tissue homogenate was stained with 0.5% Alcian Blue 8GX in 6% acetic acid, centrifuged at 2500 g for 10 minutes, and washed and resuspended until a clear supernatant was obtained. The resulting pellet was digested in 4 M guanidine thiocyanate at 4° C overnight and the resulting suspension read in a Hewlett-Packard spectrophotometer at 600 nm. DNA was assayed fluorometrically by placing 200 µL of tissue homogenate in 300 µL of 0.33% Triton X-100 in 1.67 N NH_4OH for 30 minutes at 37° C. A 100 µL aliquot of this solution was centrifuged for 10 minutes at 13,000 r.p.m and 50 µL of the supernatant added to 3.0 ml of Hoechst bisbenzamide dye #33258 and the fluorescence read on a Turner Model 430 spectro-fluorometer with excitation of 360 nm and emission of 476 nm. DNA standards were prepared fresh daily from purified mouse DNA stock (50 µg/ml).

Approximately twelve limbs per treatment group were rinsed with 0.85% saline, fixed in Bouin's fixative, cleared in xylene and stored in cedarwood oil (Sigma Chemical Co., St. Louis, MO) for morphometrical analysis as described by Kwasigroch et al 1984. Area, perimeter, and form factor of cartilage anlagen and peripheral tissue was determined using an IBAS Image Analysis System (Carl Zeiss Inc.). Form factor is an index of bone shape and was calculated by the IBAS program using the formula: Form factor = $4\pi(\text{area}) \div (\text{perimeter})^2$. A value of 1.0 describes a perfect circle and values between 0 and 1 reflect varying degrees of irregularity, with more irregular-shaped bones having lower values.

Normally distributed interval continuous data were tested for interactions between Cd and RA using a General Linear Model (GLM) procedure designed by SAS Institute Inc., version 6.06, Cary, NC. Non-normal data were transformed using log or arcsine methods before analysis. Non-parametric data, such as the anlagen data seen in Table 2, were tested for significance using Kruskal-Wallis one-way analysis of variance and mean separations performed using Duncan's multiple range test. A significant treatment effect was assumed to exist when the probability of making a Type I error was ≤ 0.05.

RESULTS AND DISCUSSION

No difference was noted among limbs treated with the vehicles, 95% ethanol and distilled water, and limbs grown in media only for any of the parameters tested. However,

significant morphological effects were observed, with the major effect of both RA and Cd, separately and in combination, being a complete distal aphalangia (Table 1). RA treatment additionally resulted in a preaxial forelimb ectrodactyly and fusion of carpal bones, while Cd treatment only affected the distal phalanges. The effects of RA were very bone-specific, exhibiting markedly differing effects on various bone anlagen.

Table 1. Number of cartilage anlagen (Median ± Quartile) in forelimbs of D12 mouse limb buds cultured *in vitro* with cadmium chloride and RA.

Treatment	n	Metacarpals	Proximal phalanges	Distal phalanges	Carpals
CONTROL	18	5 ± 0	4 ± 0.5	2 ± 1.5	5 ± 0.5
Cd	17	5 ± 0	4 ± 1	0[a]	5 ± 0.5
RA	12	5 ± 0.5[a]	3 ± 0.5[a]	0[a]	4 ± 1[a]
Cd & RA	15	5 ± 0	3 ± 0.5[a]	0[a]	5 ± 1.5

[a] Significantly different ($P \leq 0.05$) from control values, based on Kruskal-Wallis test.

Table 2. Metacarpal and proximal phalanx form factors* (X ± SEM) of D12 mouse limbs cultured *in vitro* with cadmium chloride and RA for 72 h.

Treatment	n	MC 1	MC 2	MC 3	MC 4	MC 5	PP 1	PP 2	PP 3	PP 4	PP 5
CONTROL	22	0.87 ± 0.01	0.61 ± 0.01	0.57 ± 0.01	0.65 ± 0.01	0.84 ± 0.02	0.90 ± 0.02	0.80 ± 0.01	0.80 ± 0.01	0.80 ± 0.01	0.83 ± 0.02
Cd	20	0.87 ± 0.01	0.65 ± 0.03	0.65 ± 0.02	0.70 ± 0.02	0.85 ± 0.01	0.86 ± 0.03[a]	0.76 ± 0.03	0.80 ± 0.03	0.77 ± 0.04	0.79 ± 0.04
RA	27	0.84 ± 0.01[a]	0.64 ± 0.01	0.61 ± 0.01	0.66 ± 0.01[a]	0.71 ± 0.02[a]	ND	0.83 ± 0.02	0.84 ± 0.01	0.81 ± 0.03	0.86 ± 0.01
Cd & RA	23	0.81 ± 0.01	0.60 ± 0.02	0.57 ± 0.02[a]	0.63 ± 0.02[a]	0.68 ± 0.02	ND	0.81 ± 0.02	0.76 ± 0.02	0.74 ± 0.02	0.85 ± 0.02

Note: MC = Metacarpal, PP = Proximal phalanx.
* Form factor based on following formula: $4\pi(\text{area}) \div (\text{perimeter})^2$.
[a] Significant treatment effect ($P \leq 0.05$). † No data available.

Table 3. Carpal and long bone form factors (X ± SEM) of D12 mouse limbs cultured *in vitro* with cadmium chloride and RA for 72 h.

Treatment	n	Carpal	Ulna	Radius	Humerus	Scapula	Total
CONTROL	22	0.83 ± 0.02	0.30 ± 0.01	0.42 ± 0.02	0.43 ± 0.01	0.47 ± 0.01	0.56 ± 0.01
Cd	20	0.35 ± 0.01	0.42 ± 0.01	0.43 ± 0.01	0.55 ± 0.06	0.81 ± 0.02	0.56 ± 0.01
RA	27	0.35 ± 0.01	0.47 ± 0.02[a]	0.39 ± 0.01[a]	0.56 ± 0.03[a]	0.79 ± 0.03	0.57 ± 0.01
Cd & RA	23	0.32 ± 0.01[a]	0.51 ± 0.03	0.39 ± 0.01	0.47 ± 0.05	0.84 ± 0.01	0.60 ± 0.01

[a] Significant treatment effect ($P \leq 0.05$).

RA treatment also caused a partial ectrodactyly of digits 1,2, 3 & 5, resulting in proximal phalanges that were significantly smaller than controls, but with no significant effects on anlagen shape (Table 2). Preaxial reduction deficits of RA

treatment were exhibited in metacarpal 1, with a significant decrease in area. In contrast, the anlagen area of the other metacarpals was significantly increased as a result of RA treatment.

RA treatment also affected metacarpal and humerus shape, with metacarpals 1 and 5 and humeri becoming more angular in shape (Table 3) and humeri often appearing to have little or no cartilage development in the mid-diaphysis region as compared to control limbs. A significant rounding effect in contrast was observed in the humerus, radius and ulnar anlagen (Table 3). The ulna was the only long bone to be decreased in area by RA.

Cadmium treatment had relatively little effect compared to RA, but did result in proximal phalanges that exhibited areas of incomplete extracellular matrix formation, specifically in the centre and distal tips of the phalanges. This can been seen as a significant decrease in anlagen area of proximal phalanx 5 and decreased form factor of PP 1 (Table 2). Decreased scapular anlagen was another effect due solely to Cd.

The effects of combining Cd and RA together were morphologically attributable to RA treatment. The major effect of the combined treatment on metacarpal and phalangeal anlagen was skeletal hypertrophy. The combined treatment had considerably less morphological effect in the long bones with only significant effects on ulna and scapula anlagen area. Long bone form factor differences were mostly due to RA-induced deformities occurring mainly in the ulna, radius and scapula (Table 3). No combined treatment effects in these bones were observed. The exception was carpal anlagen which was significantly rounder in shape as a result of the combined Cd and RA treatment.

Table 4. Total protein, DNA, and ECM content (X ± SEM) of D12 mouse forelimb buds cultured *in vitro* with cadmium chloride and RA for 72 h.

Treatment	n	Protein (µg/mg)*	DNA(µg/mg)	DNA:Protein Ratio	ECM (µg/mg)	ECM:Protein Ratio
CONTROL	14	79.9 ± 3.3	27.5 ± 2.6	0.34 ± 0.03	6.7 ± 0.6	0.087 ± 0.041
Cd	6	79.4 ± 4.6	22.1 ± 3.2	0.27 ± 0.03	4.7 ± 0.4	0.060 ± 0.012
RA	7	77.4 ± 3.9	19.7 ± 2.8	0.25 ± 0.03	5.3 ± 0.5	0.067 ± 0.014
Cd & RA	7	69.5 ± 3.9	18.1 ± 2.3	0.25 ± 0.03	5.2 ± 0.4	0.076 ± 0.008

* Expressed as µg/mg limb bud wet weight.

No significant treatment effects were observed for relative DNA, protein or ECM content between limb buds cultured in RA, cadmium chloride, or both compounds together in the current study (Table 4), even though all three treatments significantly reduced total limb, anlagen and long bone area (Table 5) on a morphological basis. Total tissue area was only reduced by RA treatment and was reflected as a reduced tissue area with the combined treatment (Table 5). Although total tissue area was only affected by RA, the tissue:anlagen ratio was

significantly increased with Cd and combined treatments, suggesting Cd may have more of a total effect on bone anlagen while RA may have some overall tissue-specific effects in addition to loss of bone anlagen. The stimulatory effects of all three treatments were most evident in paw anlagen, while an opposing reduction of cartilage growth occurred in the long bones (Table 5).

Table 5. Total anlagen and tissue areas* (X ± SEM) of D12 mouse limbs cultured *in vitro* with cadmium chloride and RA for 72 h.

Treatment	n	Total Limb Area(mm^2)	Total Anlagen Area(mm^2)	Total Tissue Area(mm^2)	Tissue: Anlagen Ratio†	Long Bone Area(mm^2)	Paw Anlagen Area(mm^2)	Long Bone: Paw Ratio‡
CONTROL	22	2.46 ± 0.09	1.33 ± 0.05	1.21 ± 0.05	0.93 ± 0.04	1.05 ± 0.05	0.31 ± 0.01	3.46 ± 0.19
Cd	20	2.09 ± 0.08[a]	0.96 ± 0.04[a]	1.15 ± 0.06	1.21 ± 0.04[a]	0.71 ± 0.03[a]	0.27 ± 0.02	2.78 ± 0.15[a]
RA	27	2.12 ± 0.07[a]	1.08 ± 0.05[a]	1.04 ± 0.04[a]	0.98 ± 0.03	0.80 ± 0.03[a]	0.32 ± 0.01[a]	2.55 ± 0.10[a]
Cd & RA	23	2.09 ± 0.06[a]	1.03 ± 0.03[a]	1.06 ± 0.04	1.04 ± 0.03[a]	0.70 ± 0.02[a]	0.36 ± 0.01[a]	2.02 ± 0.10

* Areas expressed in mm^2.
† Ratio of soft tissue : limb anlagen area.
‡ Ratio of (ulnar + radial + humeral) : (Metacarpal + phalangeal + carpal) anlagen area.

Although not as severe as treatment on day 9, we have demonstrated that Cd causes morphological abnormalities in day 12 mouse limbs grown *in vitro*. Distal aphalangia and areas of incomplete extracellular matrix deposition resulted from Cd treatment alone, whereas day 9 treatment both *in vivo* and *in vitro* results in severe ectrodactyly and phocomelia (Messerle & Webster 1982, Feuston & Scott 1985). Cadmium and RA exhibited interactive effects on mouse forelimbs, with Cd having a sparing effect on RA-induced reduction deficits. Carpal bones were the most sensitive indicator of this mild interaction.

Cadmium causes postaxial forelimb ectrodactyly when administered on gestation day 9 *in vivo* (Messerle & Webster 1982) and phocomelia when administered *in vitro*. These defects have been attributed to direct effects on embryonic cells (Messerle & Webster 1982), or indirect extra-embryonic influences such as effects on the placenta, yolk sac and maternal kidney (Feuston & Scott 1985). The results of the current study suggest a direct effect on embryonic tissue, as maternal and extra-embryonic factors were removed. Mulvihill et al (1970) in studying Cd-induced cleft palate in golden hamsters suggested Cd may directly inhibit cartilage formation through derangement of embryonic mesenchymal cell activity. Whether the cartilage-deficient areas observed in the proximal phalanges of Cd treated limbs in this study result from a process of chondrogenic inhibition or from cell necrosis requires further study.

Retinoic Acid caused similar forelimb reduction defects in the current study as

reported by others (Kwasigroch et al 1984; Kwasigroch et al 1986). In contrast to the elongation of long bone anlagen seen in this study, Kwasigroch et al (1986) reported rounding or inhibited lengthening of bone anlagen as a result of treatment with RA at 0.5 µg/ml. Kwasigroch et al (1986) attributed this rounding to RA-mediated interference with distal movement of undifferentiated mesenchymal cells. The difference between their study and ours may be accounted for by slightly different culture conditions, the addition of fetal bovine serum, or different genotype of the mouse used in our study.

The reduction deficits associated with RA treatment was ameliorated somewhat by concurrent cadmium chloride treatment. Several mechanisms by which this effect occurs are possible. RA has been shown to cause cartilage degradation *in vitro* in fetal rat bone cultures (Kistler et al 1981), possibly as a result of RA-mediated induction of metalloproteases which specifically degrade cartilage. Since Zn^{2+} was found to reverse inhibition of cartilage degradation caused by EDTA (Kistler et al 1991), and Cd is able to inhibit many Zn^{2+} metalloenzymes (Scott & Feuston 1985), it is probable that Cd can inhibit these cartilage degrading metalloproteases. RA is postulated to directly affect gene transcription similar to thyroid and steroid hormones through a cellular retinoic acid binding protein which transports RA to the nucleus of target cells (Maden & Summerbell 1986). RA may selectively stimulate transcription of cartilage-degrading metalloproteases which cause proteoglycan release at the doses utilized in this study, and Cd may directly inhibit these enzymes when limbs were cultured in the presence of RA and Cd.

We have demonstrated that cadmium chloride is teratogenic in day 12 mouse limbs cultured *in vitro* with the major abnormalities being distal aphalangia and loss of extracellular matrix from the proximal phalanges. RA caused similar forelimb reduction deficits, as previously reported, which were ameliorated somewhat by interactions with Cd. Cadmium may inhibit the induction of cartilage degrading metalloproteases which result in proteoglycan release from embryonic limb bud cartilage. The *in vitro* limb bud cell culture system seems to be ideally suited to the future study of teratogenic interactions.

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