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Comparative molecular pathology of cadmium- and all-*trans*-retinoic acid-induced postaxial forelimb ectrodactyly ☆

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Abstract

Cadmium chloride ($CdCl_2$) and all-*trans*-retinoic acid (RA) induce postaxial forelimb ectrodactyly in C57BL/6N mice when administered during early limb development, and co-administration yields a synergistic response suggesting a common final pathway to the defect. In the current study, forelimb buds from embryos given high maternal teratogenic doses of $CdCl_2$ or RA, or the combination of both agents at low doses were collected at various time points after treatment on GD 9.5 and examined for cellular apoptosis, proliferation, and patterning genes. Some cellular perturbations detected in the developing limb bud were similar for both teratogens, whereas other alterations were unique to each agent. For example, at 12 and 18 h, $CdCl_2$ treatment increased apoptotic cells in the mesenchyme underneath the apical ectodermal ridge (AER), whereas RA caused apoptosis in the AER and proximal mesenchyme. Further, the combined low-dose treatment increased cell death synergistically in all three regions. $CdCl_2$ and the low-dose combined treatment inhibited mesenchymal proliferation at 12 h, which was associated with induction of $p21^{cip1}$ and inhibition of phospho-c-Jun. In contrast, RA did not inhibit mesenchymal proliferation and did not induce $p21^{cip1}$ expression or change c-Jun phosphorylation. All three treatment groups showed a delay in the patterning of distal chondrogenesis centers as indicated by *Sox9* expression. There was also common inhibition in the expression of AER markers, *Fgf8* and *Fgf4*, and the mesenchymal marker *Msx1* involved in the maintenance of epithelial–mesenchymal interactions. Collectively, a model is hypothesized where limb patterning can be perturbed by insults to both ectoderm and mesoderm.

Keywords: Ectrodactyly; Apical ectodermal ridge; Progress zone; Epithelial–mesenchymal interaction; Cadmium; Retinoic acid

Introduction

A number of teratogenic agents, including acetazolamide (Layton and Hallesy, 1965), ethanol (Webster et al., 1983), all-*trans*-retinoic acid (RA) (Collins et al., 2006), 13-*cis*-RA (Sulik and Dehart, 1988) and cadmium (Layton and Layton, 1979), are known to cause a relatively specific postaxial forelimb ectrodactyly in the C57BL/6N(J) mouse strain when administered during early limb development (gestational day [GD] 9.0–10.0). Since the induced ectrodactyly is a limb reduction defect with axial specificity caused by teratogens during early limb development, it is plausible that the malformation could result from an increase in cell death, a decrease in cellular proliferation, or an alteration of limb patterning. Administration of 13-*cis*-RA, an agent presumed to function through the retinoid pathway in a similar way to all-*trans*-RA, on gestational day 9.5 to C57BL/6J mice was shown to induce various preaxial and postaxial limb autopod defects that were accompanied by excessive cell death in the apical ectodermal ridge (AER) (Sulik and Dehart, 1988). Administration of a dose of cadmium chloride ($CdCl_2$) on gestational day 9 and 1 h to C57BL/6J mice was shown to induce cell death in the mesoderm subjacent to the ectoderm and to a lesser extent in the ectoderm (Messerle and Webster, 1982). Thus, it may be predicted that both $CdCl_2$ and RA would induce cell death with different primary target cells. Although there are no known

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studies associating ectrodactyly induced by these two teratogens with alterations in cellular proliferation, there was a study that found marginally reduced proliferation rates in postaxial limb bud regions following administration of acetazolamide, another agent that produces the same defect (Schreiner et al., 1995). Alterations in patterning gene pathways, manifested as a truncation of the AER and/or reduced expression or signaling of sonic hedgehog (Shh) in the zone of polarizing activity (ZPA), have been demonstrated following administration of either $CdSO_4$ (Scott et al., 2005) or RA (Shimuzu et al., 2007), as well as other teratogens that induce this specific malformation (Chrisman et al., 2004; Bell et al., 2005).

These three parameters, cell death, cell proliferation and limb morphologic patterning, may all contribute to the induction of forelimb ectrodactyly. Studies in limb development have shown an association between growth, which integrates the parameters of cellular proliferation and death, and patterning (reviewed in Conlon, 1995). Additionally, digital pattern is affected by the number of mesenchymal cells in the limb field (Summerbell, 1981a, b; Alberch and Gale, 1983; Niswander et al., 1994). Alternatively, patterning defects in the limb are associated with an alteration in limb bud size, which may be a determinant of the number of digits formed (Niswander et al., 1994; Parr and McMahon, 1995; Barrow et al., 2003). Thus, chemicals that alter growth and/or morphologic patterning could potentially interact in the induction of such a limb reduction defect.

Limb patterning is an orderly process that is precisely regulated by many signaling molecules. Both AER and ZPA are important signaling centers during early limb development. The AER is a multilayered columnar epithelium that lies along the distal boundary between the dorsal and ventral limb ectoderm, and expresses several fibroblast growth factors (Fgf-2, -4, -8, -9, -17) that promote proximal–distal (P–D) outgrowth (Fallon et al., 1994; Niswander et al., 1993; Sun et al., 2000; Lewandoski et al., 2000; Sun et al., 2002). The ZPA is located in the distal posterior mesenchyme that patterns the limb along the anterior–posterior (A–P) axis and regulates digit identity through the Shh signaling pathway (Riddle et al., 1993; Ahn and Joyner, 2004). A positive feedback loop between Fgf4 in the posterior AER and Shh in the ZPA plays a major role in limb patterning (Helms et al., 1994; Niswander et al., 1994). A positive interaction between AER and its underlying mesenchyme, the progress zone (PZ), is also important for proper limb outgrowth. Signals from the PZ are required to induce the AER while reciprocal interactions from the AER maintain the PZ in an undifferentiated and proliferative state (reviewed in Johnson and Tabin, 1997). A variety of factors are expressed in the PZ including Fgf10 (Ohuchi et al., 1997), c-Rel (Kanegae et al., 1998), Wnt5a (Yamaguchi et al., 1999), Twist (Zuniga et al., 2002), Evx-1 (Niswander and Martin, 1993), Msx1 and Msx2 (Hill et al., 1989; Davidson et al., 1991). The Msx genes encoding homeodomain transcription factors are involved in the epithelial–mesenchymal interactions in the developing limb bud (Ros et al., 1992; Wang and Sassoon, 1995; Hara and Ide, 1997; Bushdid et al., 2001) and play a important role in AER formation, as ectopic expression of Msx1 results in the formation of an ectopic ridge expressing Fgf8 (Ahn et al., 2001), and *Msx1;Msx2* double mutants failed to form AER anteriorly and delayed AER maturation posteriorly (Lallemand et al., 2005). However, the expression pattern of Msx in teratogen-treated forelimbs has not been previously examined.

When RA was given simultaneously with $CdCl_2$ to pregnant C57BL/6N on GD 9.5, a significant increase in the incidence and severity of postaxial forelimb ectrodactyly was observed in the fetuses, compared to corresponding doses of $CdCl_2$ or RA alone (Lee et al., 2006). Thus, it is possible that both agents perturb some common molecular targets or pathways to induce teratogenesis. In the current study, the molecular pathogenesis of this specific limb malformation was investigated by markers for the aforementioned cellular processes (apoptosis, proliferation and pattern formation) following maternal treatment with $CdCl_2$ (3.5 mg/kg), RA (50 mg/kg) or a combination of low doses of $CdCl_2$ (1 mg/kg) and RA (5 mg/kg). The doses used are based on the previous teratological studies: 3.5 mg/kg $CdCl_2$ and 50 mg/kg RA induced a similar rate of embryolethality (approximately 50% rate of resorption) and a comparable frequency of postaxial forelimb ectrodactyly (60–70%), while the combination of 1 mg/kg $CdCl_2$ and 5 mg/kg RA had a lower resorption rate (22%) but a very high percentage of the limb defect (92%) (Lee et al., 2006; Collins et al., 2006). The goal of this study was to determine similarities and differences in the pathogenesis of postaxial forelimb ectrodactyly after exposure to teratogenic doses of the two independent teratogens and their combination. It was found that although $CdCl_2$ and RA caused the same anatomical digital phenotype when administered during early limb development, the anatomical pattern of cellular apoptosis and proliferation were different. Alternatively, the perturbations in specific pattern formation genes and proteins following administration of either of the teratogenic agents or the combination of both were found to be variable, where both similarities and differences were detected.

Materials and methods

Animals and treatments. C57BL/6NCrlBR (C57BL/6N) mice were obtained from Charles River Laboratories (Hollister, CA). Animal housing and chemical treatments followed the same protocol as previously reported (Lee et al., 2006). Briefly, the animals were maintained in a climate-controlled room (23±2 °C) under an alternating 12-h light/dark cycle (09:00 to 21:00 light cycle). They were maintained on Purina Formulab 5008 Lab Chow (St. Louis, MO) and water *ad libitum*. Timed matings were produced by placing individual males into cages containing multiple females for 2 h between 07:00 and 09:00 a.m. At 09:00 a.m. on the day a vaginal plug was detected, GD 0 was designated to initiate. On GD 9.5, pregnant mice were injected intraperitoneally with a dose of 3.5 mg/kg $CdCl_2$, 50 mg/kg RA, or a combination of 1 mg/kg $CdCl_2$ and 5 mg/kg RA at a dosing volume of 10 ml/kg body weight. Individual doses of 1 mg/kg $CdCl_2$ and 5 mg/kg RA were also administered but only in several litters of mice for the study of cell death. All procedures involving RA were performed under dim yellow light to prevent photoisomerization. Control animals were treated either with the vehicle for $CdCl_2$ (deionized water), or the vehicle for RA (8% ethanol +92% soybean oil) on GD 9.5.

Free-floating and cryostat limb bud sections. Concepti were removed from the uterus and whole embryos were dissected out at a variety of time points post-treatment in ice-cold phosphate-buffered saline (PBS) under a dissecting microscope, washed in sterile PBS and fixed immediately in 4% PBS-buffered paraformaldehyde (PFA) at 4 °C overnight. Embryos were dehydrated with