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Msx1 and Msx2 gene expression is downregulated in the cadmium-induced omphalocele in the chick model

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Abstract

Purpose: The administration of cadmium (Cd) induces an omphalocele phenotype in the chick embryo. The molecular mechanism by which Cd acts still remains unclear. Msx1 and Msx2 are expressed in the developing body wall and regulate cellular proliferation and differentiation. It has been reported that Msx1/Msx2 double-mutant mice display an omphalocele phenotype. We hypothesized that gene expression levels of Msx1 and Msx2 are downregulated in the Cd chick model during the critical period of embryogenesis.

Methods: After 60 hours of incubation, chick embryos were exposed to either Cd or saline and harvested at 1 hour (1H), 4H, and 8H after treatment. Chicks were divided into 2 groups: control and Cd (n = 8 for each group at each time-point). Real-time polymerase chain reaction was performed to evaluate the messenger RNA levels of Msx1 and Msx2 in the Cd-induced omphalocele chick model and analyzed statistically. Immunohistochemistry was also performed to examine protein expression of Msx1 and Msx2 at each time-point.

Results: Messenger RNA expression levels of Msx1 and Msx2 at 1H were significantly decreased in the Cd group compared with controls ($P < .01$), whereas there were no significant differences at the other time-points. Immunoreactivity of Msx1 and Msx2 at 1H was remarkably decreased in Cd group compared with controls.

Conclusion: Downregulation of Msx1 and Msx2 gene expression during the narrow window of early embryogenesis may cause an omphalocele by disrupting cellular proliferation and differentiation in the developing body wall.

Omphalocele is a midline defect at the amnioectodermal transition, resulting in an enlarged umbilical ring through which organs such as the liver and bowel abnormally protrude into a sac of amniotic membrane. It occurs in approximately 1 of 3000 live human births [1,2] and results from failure of the intestine to return to the abdomen after the migration into the umbilical cord. Although experimental studies of ventral body wall defects (VBWDs) have relied on gene knockouts, surgery, and teratogenic chemicals to create models of these defects, there are still no reliable animal models of omphalocele. Induction of VBWD

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is a frequent but poorly reproducible effect of many common teratogens [3,4].

Cadmium (Cd) is a metallic element that has no nutritive function and is an established teratogen in a wide range of animal models, including rodent [5,6], xenopus [7], and avian [8-11] embryos. In the chick embryo, we have shown that administration of Cd at Hamilton-Hamburger stages 16 to 17 causes abnormal growth of the lateral plate mesoderm (LPM), resulting in the development of VBWD with similar characteristics to the human omphalocele [8]. In this model, Cd appears to be a specific teratogen, with a predictable and reproducible rate of omphalocele formation occurring in approximately 50% of chicks, with no other obvious multiple malformations. We have previously shown that the earliest detectable histologic changes in the Cd-induced VBWD are breakdown of junctions between peridermal cells, commencing 4 hours posttreatment with Cd, followed closely by increased apoptosis in the LPM, somites, and neuroepithelium [8-11]. These findings led us to hypothesize that the altered molecular signaling in the Cd-induced VBWD model occurs before the histologic changes first detected at 4 hours post–Cd administration.

The vertebrate Msx homeobox genes function as transcriptional regulators that control cellular proliferation and differentiation during embryonic development [12]. In the mouse, the Msx family comprises 3 members: Msx1, Msx2, and Msx3. Whereas Msx3 expression is confined to the neural tube, Msx1 and Msx2 are widely expressed especially at sites of development that depend on ectoderm-mesoderm interactions [13]. Both Msx1 and Msx2 are expressed in an overlapping manner at the level of the trunk in the developing body wall mesenchyme [14]. It has been reported that Msx1/Msx2 double-mutant mice display the omphalocele phenotype with disorganized abdominal muscle layers and connective tissues [14]. This knockout study indicates that Msx1 and Msx2 genes play an important role during embryonic ventral body wall formation. We designed this study to investigate the hypothesis that the gene expression of Msx1 and Msx2 is downregulated during the critical period of very early embryogenesis in the Cd-induced omphalocele chick model.

1. Materials and methods

1.1. Embryo growth and treatment

Fertilized unincubated eggs of the Ross strain were obtained from a local hatchery (Moyer's Farm, Celbridge, Ireland) and incubated at 38°C in a forced air incubator (SHEL LAB, Cornelius, OR) at 65% to 75% relative humidity. After 60 hours of incubation, chicks that had reached Hamburger-Hamilton stages 16 to 17 [15] were explanted into shell-less culture using a method adapted from Dugan et al [16] and given 50 μL of 50 μmol/L Cd or 50 μL chick saline (doses determined previously [8]). Test solutions were dropped onto the blastodisc using a micropipette and entered the embryo by a process of diffusion. The embryos were then returned to the incubator and harvested 1 hour (1H), 4H, and 8H after treatment. The embryos were divided into 2 groups: Cd group (n = 8 at each time-point) and controls (n = 8 at each time-point). The embryos exposed to Cd were defined as the Cd group, and the control group consisted of embryos that only received chick saline.

1.2. RNA isolation and real-time reverse transcription polymerase chain reaction

The whole chick embryos were dissected from their membranes, and total RNA of embryos was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the recommended protocol. For reverse transcription (RT), first-strand complementary DNA (cDNA) was synthesized from RNA by using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, United Kingdom) according to the manufacturer's instructions. After RT at 44°C for 60 minutes, polymerase chain reaction (PCR) was performed using a LightCycler 480 SYBR Green I Master (Roche Diagnostics) according to the manufacturer's protocol. The specific primer pairs used in this study are listed (Table 1). Serially diluted cDNA samples were used as standards. After an initial denaturation step of 5 minutes at 95°C, 36 cycles and 38 cycles of amplification for Msx1 and Msx2 primer pair, respectively, were carried out. Each cycle included a denaturation step, 10 seconds at 95°C; an annealing step, 15 seconds at 60°C; and an elongation step, 10 seconds at 72°C. Final elongation temperature was 65°C for 1 minute. Relative levels of gene expression were measured using a LightCycler 480 (Roche Diagnostics) according to the manufacturer's instructions. The expression levels of Msx1 and Msx2 gene were normalized to the level of GAPDH gene expression in each sample. Experiments were carried out at least in duplicate for each data point.

1.3. Immunohistochemistry

The paraffin-embedded chick embryos were sectioned transversely at a thickness of 5 μm, and the sections were deparaffinized with xylene and then rehydrated through

Table 1 Oligonucleotide primers used for real-time RT-PCR analysis

Gene		Sequences	Product size
GAPDH	Sense	5′cct ctc tgg caa agt cca ag3′	176
	Antisense	5′ggt cac gct cct gga aga ta3′	
Msx1	Sense	5′gga act gtg gca gag aaa gg3′	118
	Antisense	5′aat ggc cac agg tta aca gc3′	
Msx2	Sense	5′atc cag cct caa gaa tgc ag3′	108
	Antisense	5′gga tgc ctt gct ttg cta ac3′	