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ORIGINAL ARTICLE

Downregulation of Midkine gene expression and its response to retinoic acid treatment in the nitrofen-induced hypoplastic lung

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Abstract
Purpose Nitrofen-induced congenital diaphragmatic hernia (CDH) model has been widely used to investigate the pathogenesis of pulmonary hypoplasia (PH) in CDH. Recent studies have suggested that retinoids may be involved in the molecular mechanisms of PH in CDH. Prenatal treatment with retinoic acid (RA) has been reported to improve the growth of hypoplastic lung in the nitrofen CDH model. Midkine (MK), a RA-responsive growth factor, plays key roles in various organogenesis including lung development. In fetal lung, MK mRNA expression has its peak at E13.5–E16.5 and is markedly decreased during mid-to-late gestation, indicating its important role in early lung morphogenesis. We designed this study to investigate the hypothesis that the pulmonary MK gene expression is downregulated in the early lung morphogenesis in the nitrofen-induced PH, and to evaluate the effect of prenatal RA treatment on pulmonary MK gene expression in the nitrofen-induced CDH model.
Methods Pregnant rats were exposed to either olive oil or nitrofen on day 9 of gestation (D9). Fetal lungs were harvested on D15, D18, and D21 and divided into control, nitrofen with or without CDH [CDH(+) or CDH(−)]. In addition, RA was given on days D18, D19, and D20 and fetal lungs were harvested on D21, and then divided into control + RA and nitrofen + RA. The pulmonary gene expression levels of MK were evaluated by real-time RT-PCR and statistically analyzed. Immunohistochemistry was also performed to examine protein expression/distribution of MK in fetal lung.
Results The relative mRNA expression levels of MK were significantly downregulated in nitrofen group compared to controls at D15 ($^{\S}p < 0.01$), whereas there were no significant differences at D18 and D21. MK gene expression levels were significantly upregulated in nitrofen + RA (0.71 ± 0.17) compared to the control (0.35 ± 0.16), CDH(−) (0.24 ± 0.15), CDH(+) (0.39 ± 0.19) and control + RA (0.47 ± 0.13) ($^{*}p < 0.05$). Immunoreactivity of MK was also markedly decreased in nitrofen lungs compared to controls on D15, and increased in nitrofen + RA lungs compared to the other lungs on D21.
Conclusion Downregulation of MK gene on D15 may contribute to primary PH in the nitrofen CDH model by disrupting early lung morphogenesis. Upregulation of MK gene after RA treatment in the nitrofen-induced hypoplastic lung suggests that RA may have a therapeutic potential to rescue PH in CDH through RA-responsive growth factor signaling.

Keywords Retinoic acid · Pulmonary hypoplasia · Nitrofen · Midkine · Congenital diaphragmatic hernia

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Introduction

Pulmonary hypoplasia in congenital diaphragmatic hernia (CDH), characterized by immaturity and small lung size, produces respiratory failure which is one of the principle contributors to the high mortality in patients with CDH [1, 2]. Maternal exposure of nitrofen (2,4-dichlorophenyl-

p-nitrophenyl ether) in rodents during specific time in gestation results in pulmonary hypoplasia associated with CDH in the offspring, which is strikingly similar to the human condition [3]. Nitrofen-induced CDH rodent model has been widely used to investigate the pathogenesis of pulmonary hypoplasia in CDH, and recent studies from our laboratory using this model have provided insights into the molecular mechanisms of pulmonary hypoplasia associated with CDH [4–6]. However, the exact molecular mechanisms by which nitrofen induces hypoplastic lung in this model still remain unclear.

Retinoids, the family of molecules derived from vitamin A, are essential for normal development of various organs including the lungs and diaphragm [7]. Retinoic acid (RA), the active metabolite of vitamin A, is necessary for fetal lung development [8]. In the nitrofen-induced hypoplastic lung, several studies have suggested that the retinoid signaling pathway (RSP) may be involved in the pathogenic mechanisms [9, 10]. We have recently shown that prenatal treatment with RA rescues lung hypoplasia in nitrofen-induced hypoplastic lung [11, 12], and upregulates candidate genes involved in fetal lung development [13, 14].

Midkine (MK), a 13-kDa heparin-binding growth factor, is a RA-responsive growth factor involved in numerous processes including cell migration, inflammation, and angiogenesis [15]. During fetal development, MK expression is widespread early in gestation and becomes restricted to specific site [16]. In the normal lung development, MK mRNA expression is observed in the fetal developing lung from embryonic day (E) 13.5 to adult [17]. High levels of MK mRNA are observed at E13.5–E16.5 and decreased E18.5 to postnatal day (PN) 1, and increased thereafter from PN5 to PN12 [17]. These findings have suggested that MK gene may play a key role especially in the early lung development and in the postnatal alveolarization. In vitro studies have reported that MK expression is regulated by RA during alveolarization in the fetal rat lung cell culture [15, 18]. However, little is known about the expression of MK gene and the effect of RA on MK gene expression in vivo.

We designed this study to investigate the hypothesis that the pulmonary MK gene expression is downregulated in the early lung morphogenesis in the nitrofen-induced hypoplastic lung, and to evaluate the effect of prenatal RA treatment on pulmonary MK gene expression in the nitrofen-induced CDH model.

Materials and methods

Animals and treatment

Adult Sprague–Dawley rats were mated and the presence of spermatozoids in the vaginal smear was verified and was considered as gestational day (D) 0. Pregnant female rats were then randomly divided into experimental group and control group. Animals in the experimental group received intragastrically 100 mg of nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 ml of olive oil on D9, whereas those in the control group received only vehicle. On D18 the rats were randomly injected intraperitoneally with all *trans*-RA 5 mg/kg in cottonseed oil (Sigma, St Louis, MO, USA) with 100% ethanol as diluents, and the injections were repeated on D19 and D20. Pregnant rats were sedated with isofluorane and killed by cervical dislocation on D15, D18, and D21, and then fetal left lungs were harvested and divided into five groups: control, nitrofen without CDH [CDH(−)], nitrofen with CDH [CDH(+)], control + RA and nitrofen + RA ($n = 8$ for each group). In order to obtain representative numbers, the fetuses in each group came from at least three different dams. The Department of Health and Children approved all the animal experiments (ref. B100/4022) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002 and 2005.

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

The peripheral region of left lungs dissected from the thoracic cavity were immediately suspended in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and stored at −20°C. After thawing frozen sample in TRIzol® reagent, the total RNA of each lung derived from fetuses was isolated according to recommended protocol. As reverse transcription (RT), first strand cDNA was synthesized from RNA by using RETROscript® (Ambion/Applied Biosystems, TX, USA) according to manufacturer's instruction. Following RT at 44°C for 60 min, PCR was performed using a LightCycler® 480 SYBR Green I Master (Roche Diagnostics, West Sussex, UK) according to the manufacturer's protocol. Gene-specific primers used in this study are listed (Table 1). After initial denaturation step of 5 min at 95°C, 45 cycles of amplification for each primer pair was carried out. Each cycle included a denaturation step; 10 s at 95°C, an annealing step; 15 s at 60°C and an elongation step; 10 s at 72°C. Final elongation temperature was 65°C for 1 min. Relative levels of gene expression were measured using a LightCycler® 480 (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instructions. The relative changes in the expression levels of MK genes were normalized against the level of β-actin gene expression in each sample. Experiments were carried out at least in duplicate for each data point.

Immunohistochemical staining

The paraffin-embedded fetal lungs were sectioned at a thickness of 5 μm, and the sections were deparaffined with

Table 1 Primer sequences for real-time RT-PCR

Gene	Sequence (5′-3′)	Product size (bp)
β-actin		
Forward	TTG GAT GCC TGT GGT CTG TC	108
Reverse	TAG AGC CAC CAA TCC ACA CA	
MK		
Forward	CCT GCA ACT GGA AGA AGG AG	189
Reverse	CTT TGG CCT TTG ACT TGG TC	

xylene and then rehydrated through ethanol and distilled water. Tissue sections were immersed in target retrieval solution (DAKO, Cambridgeshire, UK) in a microwave oven (at 750 W) for 10 min followed by 10 min incubation in 0.3% H_2O_2 to block endogenous peroxidase activity. Sections were incubated at 4°C overnight with each of the primary antibodies including a 1:100 dilution of rabbit polyclonal antibodies against MK (Lot sc-20715; Santa Cruz Biotechnology, CA, USA) and treated in horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodies (Lot: K4003; DAKO Ltd, Cambridgeshire, UK). Sections were then developed with a diaminobenzidine (DAB)-H_2O_2 substrate complex, and counterstained with hematoxylin.

Statistical analysis

All numerical data in the results of RT-PCR are presented as means ± standard deviation. Differences between five groups were tested by Tukey–Kramer post-hoc test following one-way ANOVA. Statistical significance was accepted at P values <0.05.

Results

Relative mRNA expression levels of Midkine in fetal rat lungs

The pulmonary gene expression levels of MK were significantly decreased in nitrofen group (4.24 ± 1.38) compared to controls (8.89 ± 3.62) ($p < 0.01$). However, there were no significant differences in the expression levels of MK gene among all groups on D18 and D21 (Table 2). Pulmonary mRNA expression levels of MK were significantly upregulated in nitrofen + RA group (7.15 ± 1.70) compared to controls (0.35 ± 0.16) ($p < 0.01$), CDH(−) (0.24 ± 0.15) ($p < 0.01$), CDH(+) (0.39 ± 0.19) ($p < 0.01$) and control + RA group (4.70 ± 1.28) ($p < 0.05$) (Fig. 1).

Table 2 Pulmonary mRNA expression levels of MK in each developmental stage

	D15	D18	D21
Control	8.89 ± 3.62	2.54 ± 0.31	0.35 ± 0.16
Nitrofen			
CDH(−)	4.24 ± 1.38[§]	2.16 ± 1.56	0.24 ± 0.15
CDH(+)		3.09 ± 0.52	0.39 ± 0.19

[§] $p < 0.01$ versus control

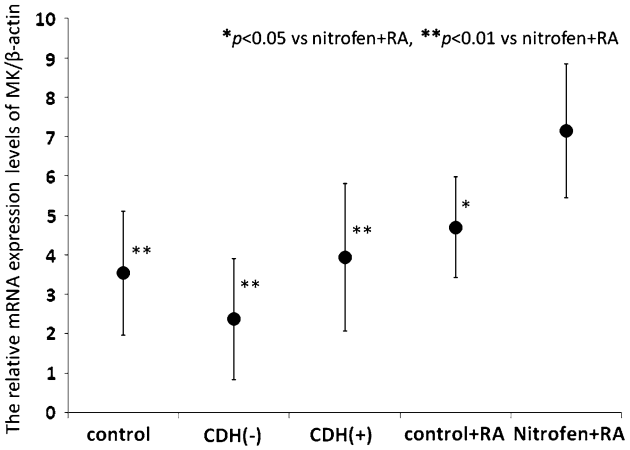

Fig. 1 The relative mRNA expression levels of MK on D21. Pulmonary mRNA expression levels of MK were significantly upregulated in nitrofen + RA group (7.15 ± 1.70) compared to controls (0.35 ± 0.16) ($p < 0.01$), CDH(−) (0.24 ± 0.15) ($p < 0.01$), CDH(+) (0.39 ± 0.19) ($p < 0.01$) and control + RA group (4.70 ± 1.28) ($p < 0.05$)

Pulmonary gene expression levels of Midkine at D21 after RA treatment

Immunohistochemical study of Midkine at D21 in fetal rat lungs

To determine whether the altered amounts of MK transcripts in the nitrofen-induced hypoplastic lung were reflected the protein expression, immunohistochemical study using polyclonal antibody against MK was performed. Immunoreactivity of MK was markedly decreased in nitrofen-induced hypoplastic lung compared to controls on D15 (Fig. 2). There were no remarkable differences in immunoreactivity of MK between control lung and nitrofen-induced hypoplastic lung on D18 and D21 (data not shown). On D21, markedly increased expression of MK was observed in nitrofen + RA lung compared to the lungs from the other group (Fig. 3). After prenatal treatment with RA, lung morphological immaturity observed in nitrofen group was also markedly improved with thinner alveolar septae and larger air spaces.

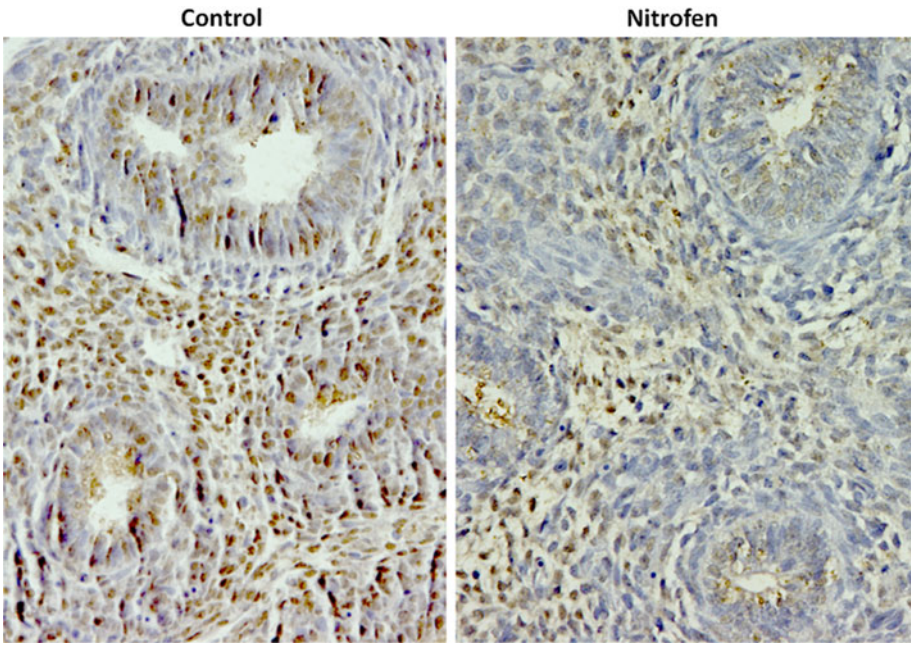

Fig. 2 Immunohistochemical study using polyclonal antibody against MK on D15. Immunoreactivity of MK was markedly decreased in nitrofen-induced hypoplastic lung on D15 compared to control lung

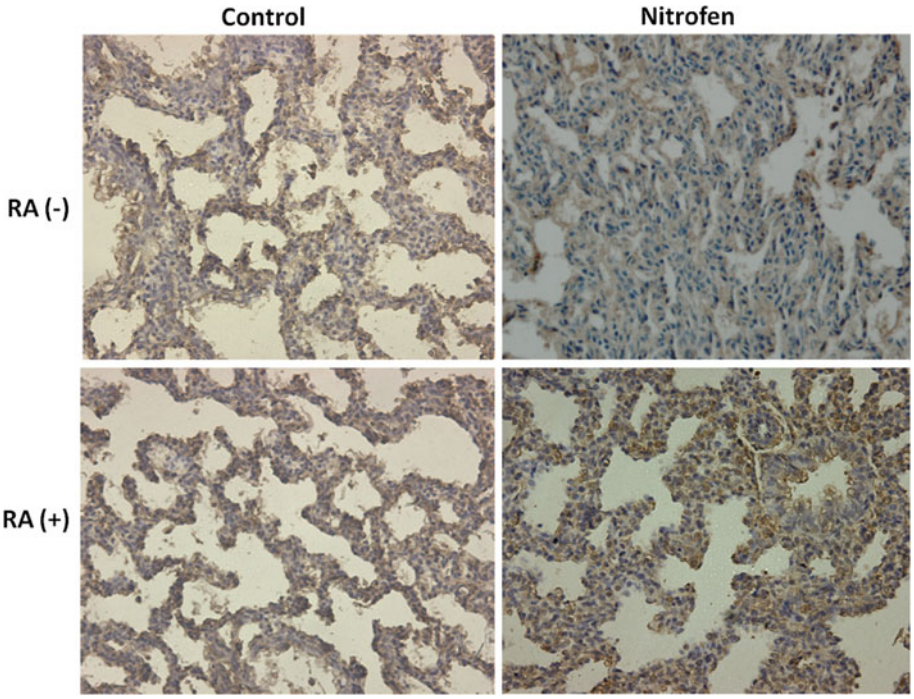

Fig. 3 Immunohistochemical study for MK on D21. MK immunoreactivity on D21 was markedly increased in nitrofen + RA lung, whereas there were no differences in immunoreactivity of MK in the other groups

Discussion

RA, active metabolite of retinoids, is necessary for the lung development in each developmental stage [8]. It has been reported that there is a reduction in plasma retinol and retinal-binding protein levels in a group of CDH newborn compared to matching controls [19]. Postnatal treatment with RA has been shown to increase the number of pulmonary alveoli in rats [20]. It has also been demonstrated that RA can induce alveolar regeneration in the adult mice [21]. In the nitrofen model of CDH, studies from our laboratory have previously demonstrated that prenatal treatment with RA during later gestational days alters expression of genes involved in lung morphogenesis, and rescues pulmonary hypoplasia by stimulating alveologenesis [11–14]. Although these extensive findings support the concept of a therapeutic potential of RA in reverting lung hypoplasia in CDH, the precise molecular mechanisms by

which RA acts in reverting hypoplastic lung are still not clearly understood.

MK is a RA-responsive heparin-binding growth factor that is implicated in mesenchymal–epithelial interactions in fetal organogenesis [22] and is thought to mediate multiple developmental processes including cell migration, proliferation and vasculogenesis in various organs, including lung [17]. Exposure of D21 fetal rat lung distal airway epithelial cells and adjacent fibroblasts in primary culture to exogenous RA has been shown to enhance MK expression [18]. During neonatal alveolarization in rats, it has been further reported that RA treatment counteracts dexamethasone-induced inhibition of alveolar formation and increases the number of alveoli [23].

It has been reported that MK is also able to be regulated by thyroid transcription factor (TTF)-1 [17]. TTF-1 is a member of the Nkx2 transcription factor family regulating formation and gene expression in the lung parenchyma [17]. TTF-1 is expressed in the developing organs including fetal lung, and TTF-1 mRNA has been detected predominantly in the peripheral lung during fetal development [24]. TTF-1 gene activates the expression of various genes critical to lung development and function [25]. It has been reported that inactivation of TTF-1 causes pulmonary hypoplasia [26], and the mRNA expression of TTF-1 is decreased in the nitrofen-induced hypoplastic lung [27]. MK expression has been shown to be absent in the lung tubules of the TTF-1 null mice [17]. In this study, we showed downregulation of MK gene in the nitrofen-induced hypoplastic lung. Our findings are consistent with the idea that TTF-1 regulates the expression of MK during lung morphogenesis, and thus suggest that downregulation of MK and TTF-1 genes during critical period of lung morphogenesis in the nitrofen model may contribute to pulmonary hypoplasia associated with CDH.

In the present study, we clearly showed that gene expression levels of MK were significantly downregulated in the nitrofen-induced hypoplastic lung. We further demonstrated that prenatal treatment with RA upregulated the pulmonary gene expression levels of MK in the nitrofen model of CDH. In addition, we observed that the immunoreactivity of MK was also markedly decreased on D15 and markedly increased after prenatal treatment with RA in the nitrofen-induced hypoplastic lung, validating altered amount of transcripts in its pulmonary protein expression. In the light of recent evidence that MK, a RA-responsive growth factor, is a critical regulator of alveologenesis, it is tempting to speculate that upregulation of MK genes during the alveolar period of lung morphogenesis rescues lung hypoplasia and enhances lung growth in the nitrofen CDH model, by stimulating alveolarization via RA-mediated MK signaling.

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