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Increased c-kit and stem cell factor expression in the pulmonary vasculature of nitrofen-induced congenital diaphragmatic hernia☆

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ABSTRACT

Purpose: Persistent pulmonary hypertension(PPH) in congenital diaphragmatic hernia (CDH) is caused by increased vascular cell proliferation and endothelial cell (EC) dysfunction, thus leading to obstructive changes in the pulmonary vasculature. C-Kit and its ligand, stem cell factor(SCF), are expressed by ECs in the developing lung mesenchyme, suggesting an important role during lung vascular formation. Conversely, absence of c-Kit expression has been demonstrated in ECs of dysplastic alveolar capillaries. We hypothesized that c-Kit and SCF expression is increased in the pulmonary vasculature of nitrofen-induced CDH.
Methods: Timed-pregnant rats received nitrofen or vehicle on gestational day 9(D9). Fetuses were sacrificed on D15, D18, and D21, and divided into control and CDH group. Pulmonary gene expression levels of *c-Kit* and *SCF* were analyzed by qRT-PCR. Immunofluorescence double staining for c-Kit and SCF was combined with CD34 to evaluate protein expression in ECs of the pulmonary vasculature.
Results: Relative mRNA levels of *c-Kit* and *SCF* were significantly increased in lungs of CDH fetuses on D15, D18, and D21 compared to controls. Confocal laser scanning microscopy confirmed markedly increased vascular c-Kit and SCF expression in mesenchymal ECs of CDH lungs on D15, D18, and D21 compared to controls.
Conclusion: Increased expression of c-Kit and SCF in the pulmonary vasculature of nitrofen-induced CDH lungs suggest that increased c-Kit signaling during lung vascular formation may contribute to vascular remodeling and thus to PPH.

Congenital diaphragmatic hernia (CDH) is a relatively common malformation, currently affecting about 2.3 per 10,000 live births [1]. Although the surgical repair of the underlying diaphragmatic defect is relatively easy, the main problem is the associated disturbed lung development, resulting in severe pulmonary hypoplasia and persistent pulmonary hypertension (PPH) [2]. Apart from hypoplastic lungs, further changes are present in the pulmonary vascular components of CDH patients consisting of arterial media hyperplasia, peripheral muscularisation of smaller vessels and adventitial thickening [3–5]. Pulmonary hypoplasia and PPH both occur to a variable extent in newborn infants with CDH and because of the absence of sufficient lung-protective strategies, most of the newer treatment modalities have replaced mortality with a higher rate of long-term morbidity [6,7]. Thus, CDH remains one of the major therapeutic challenges in neonatal intensive care units [8].

Most of our current knowledge about the structural and molecular changes in CDH derives from experimental animal models [9]. Administration of the herbicide nitrofen (2,4-dichloro-phenyl-p-nitrophenyl ether) to pregnant rats on gestational day 9 (D9) has been shown to result in CDH in approximately 70% of the offsprings with associated pulmonary hypoplasia and abnormal pulmonary vascular remodeling, both remarkably similar to the human situation [10,11].

Recent studies have demonstrated that the distal pulmonary vasculature originates from endothelial cells (ECs) of the lung mesenchyme by means of vasculogenesis [12,13]. PPH in CDH is caused by increased vascular cell proliferation and endothelial cell dysfunction, which in turn leads to obstructive changes in the pulmonary vasculature and thus to elevated pulmonary artery resistance [14]. While the pathophysiology of PPH has been extensively studied, the molecular basis of the abnormal architectural changes in the pulmonary vasculature of CDH-associated PPH remains largely unknown.

C-Kit, a transmembrane tyrosine kinase receptor, and its ligand, stem cell factor (SCF) are both expressed by ECs in the developing fetal lung mesenchyme [12], indicating an important role in lung vascular formation. Conversely, absence of c-Kit expression has recently been found in ECs of dysplastic pulmonary capillaries [15], suggesting that PPH in CDH may result from increased c-Kit signaling in mesenchymal ECs during pulmonary vasculogenesis, leading to characteristic vascular thickening and remodeling. We therefore designed this study to investigate the hypothesis that c-Kit and SCF expression is increased in the pulmonary vasculature of rats with nitrofen-induced CDH.

☆ LEVEL OF EVIDENCE: 2b individual cohort study

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1. Material and methods

1.1. Animals, drugs and experimental design

After obtaining ethical approval (REC668b) from the local research ethics committee, pathogen-free adult Sprague–Dawley rats® (Harlan Laboratories, Shardlow, UK) were mated overnight, and females were checked daily for presence of a vaginal plug. The day of plugging was defined as embryonic day 0.5 (E0.5) and timed pregnant animals were randomly divided into two experimental groups: "Nitrofen" and "Control". On gestational day 9 (D9), dams received either intragastrically 100 mg of nitrofen (WAKO Chemicals GmbH, Neuss, Germany), dissolved in 1 ml of olive oil, or vehicle alone. On the selected time-points D15, D18 and D21, animals were anesthetized with 2% volatile isoflurane (Piramal Healthcare UK Ltd., Morpeth, United Kingdom) and fetuses were delivered *via* caesarean section. After decapitation, fetuses underwent laparotomy and diaphragms were inspected under a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) for CDH. Whole lungs of nitrofen-exposed fetuses with a diaphragmatic defect ($n = 12$ per time-point) and controls ($n = 12$ per time-point) were dissected *via* thoracotomy and stored either in a TRIzol® reagent (Invitrogen, Carlsbad, USA) for total RNA isolation or fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for immunofluorescence-double-staining. All animal procedures were performed following current guidelines for management and welfare of laboratory animals and were approved by the Department of Health and Children (Ref. B100/4378) under the Cruelty to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005).

1.2. Total RNA isolation and complementary DNA synthesis

Total RNA was isolated from whole lung specimens with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA quantification was performed spectrophotometrically (NanoDrop ND-1000 UV–Vis® Spectrophotometer, Wilmington, USA) and the RNA solution was stored at −20 °C. Synthesis of cDNA was performed using a Transcript High Fidelity cDNA Synthesis Kit® (Roche Diagnostics, Grenzach-Whylen, Germany) according to the manufacturer's protocol. All cDNA samples were stored at 4 °C until further use.

1.3. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Gene-specific primer pairs used in this study are listed in Table 1. After an initialization phase at 95 °C for 5 min, 55 amplification cycles were carried out. Each cycle included an initial denaturation step at 95 °C for 10 s, an annealing step at 60 °C for 15 s and an elongation step at 72 °C for 10 s. The final elongate temperature was 65 °C for 1 min. Relative mRNA expression levels of *c-Kit* and *SCF* were measured with a Light Cycler® 480 instrument (Roche Diagnostics, West Sussex, UK) and gene levels were normalized to the housekeeping gene *β-actin*. All experiments were carried out in duplicate for each sample and primer pair.

Table 1
Primer sequences for quantitative real-time polymerase chain reaction.

Gene	Sequence (5′–3′)	Product size (bp)
c-Kit		
Forward	GAC AGG CTC ATG AAT GGC AG	128
Reverse	ATC TGT ACG TCT ACT GGC GG	
SCF		
Forward	TCT GTC TTG GAG CTG CAT GA	84
Reverse	AAG AGC AGC CAC CAT GTA CT	
β-actin		
Forward	TTG CTG ACA GGA TGC AGA AG	108
Reverse	TAG AGC CAC CAA TCC ACA CA	

1.4. Immunofluorescence-double-staining and confocal laser scanning microscopy

Following overnight fixation in 10% PFA, fetal whole lungs from each time-point were paraffin embedded, transversely sectioned at a thickness of 5 μm and mounted on polylysine-coated slides. Tissue sections were deparaffinized with xylene and rehydrated through ethanol and distilled water. After washing in phosphate-buffered saline (PBS) + 0.05% Tween, sections were incubated with 1.0% Triton X-100 (Sigma Aldrich Ltd., Arklow, Ireland) for 20 min to improve cell permeabilization. Following another washing step, sections were blocked with 3% bovine serum albumin (Sigma Aldrich Ltd., Arklow, Ireland) for 30 min to avoid nonspecific absorption of immunoglobulin. The blocking solution was then rinsed off and sections were incubated with primary antibodies against c-Kit (rabbit polyclonal, sc-168, 1:100), SCF (mouse polyclonal, sc-13,126, 1:100) and CD34 (goat polyclonal, sc-7045, 1:100) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) at 4 °C overnight. On the next day, sections washed and incubated with corresponding secondary antibodies (donkey anti-rabbit Alexa 647-A150067, 1:250; donkey anti-mouse Alexa 488-A150109, 1:250 and donkey anti-goat Alexa 555-A21432, 1:250) (Abcam plc, Cambridge, UK) at room temperature for 1 h. After another washing step, sections were counterstained with a DAPI antibody (10,236,276,001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) at room temperature for 10 min and mounted with glass coverslips using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). Finally, all sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and evaluated independently by two investigators.

1.5. Statistical analysis

All numerical data are presented as means ± standard error of the mean. Differences between two groups were tested using an unpaired Student's *t* test when the data had normal distribution or a Mann–Whitney *U* test when the data deviated from normal distribution. Statistical significance was accepted at *P* values of less than 0.05.

2. Results

2.1. Relative mRNA expression levels of c-Kit and SCF in fetal rat lungs

Following qRT-PCR, the relative mRNA expression levels of *c-Kit* and *SCF* were significantly increased in nitrofen-exposed CDH lungs of D15 (0.05 ± 0.02 *vs.* 0.03 ± 0.01; $P < 0.05$ and 0.28 ± 0.13 *vs.* 0.16 ± 0.09; $P < 0.05$, respectively), D18 (0.13 ± 0.04 *vs.* 0.10 ± 0.01; $P < 0.05$ and 0.49 ± 0.22 *vs.* 0.25 ± 0.06; $P < 0.05$, respectively) and D21 (0.10 ± 0.03 *vs.* 0.04 ± 0.01; $P < 0.05$ and 0.58 ± 0.22 *vs.* 0.28 ± 0.18; $P < 0.05$, respectively) fetuses compared to controls (Table 2).

Table 2
Relative mRNA expression levels of *c-Kit* and *SCF* in fetal rat lungs.

	c-Kit		*SCF*	
	Control	Nitrofen	Control	Nitrofen
D15	0.03 ± 0.01	0.05 ± 0.02*	0.16 ± 0.09	0.28 ± 0.13*
D18	0.10 ± 0.01	0.13 ± 0.04*	0.25 ± 0.06	0.49 ± 0.22*
D21	0.04 ± 0.01	0.10 ± 0.03*	0.28 ± 0.18	0.58 ± 0.22*

* $p < 0.05$ *vs* control.