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Hunterian Lecture

Challenging embryological theories on congenital diaphragmatic hernia: future therapeutic implications for paediatric surgery

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Lung hypoplasia is central to the poor prognosis of babies with congenital diaphragmatic hernia (CDH). Prolapse of abdominal organs through a diaphragmatic defect has traditionally been thought to impair lung growth by compression. The precise developmental biology of CDH remains unresolved. Refractory to fetal correction, lung hypoplasia in CDH may instead originate during embryogenesis and before visceral herniation. Resolving these conflicting hypotheses may lead to re-appraisal of current clinical strategies.

Genetic studies in murine models and the fruitfly, *Drosophila melanogaster* are elucidating the control of normal respiratory organogenesis. *Branchless* and *breathless* are *Drosophila* mutants lacking fibroblast growth factor (FGF) and its cognate receptor (FGFR), respectively. *Sugarless* and *sulphateless* mutants lack enzymes essential for heparan sulphate (HS) biosynthesis. Phenotypically, all these mutants share abrogated airway branching. Mammalian organ culture and transgenic models confirm the essential interaction of FGFs and HS during airway ramification. Embryonic airway development (branching morphogenesis) occurs in a defined spatiotemporal sequence. Unlike the surgically-created lamb model, the nitrofen rat model permits investigation of embryonic lung growth in CDH. Microdissecting embryonic lung primordia from the nitrofen CDH model and normal controls, we demonstrated that disruption of stereotyped airway branching correlates with and precedes subsequent CDH formation.

To examine disturbed branching morphogenesis longitudinally, we characterised a system that preserves lung hypoplasia in organ culture. We tested FGFs and heparin (an HS analogue) as potential therapies on normal and hypoplastic lungs. Observing striking differences in morphological response to FGFs between normal and hypoplastic lung primordia, we postulated abnormalities of FGF/HS signalling in the embryonic CDH lung. Evaluating this hypothesis further, we examined effects of an HS-independent growth factor (epidermal growth factor, EGF) on hypoplastic lung development. Visible differences in morphological response indicate an intrinsic abnormality of hypoplastic lung primordia that may involve shared targets of FGFs and EGF.

These studies indicate that lung hypoplasia precedes diaphragmatic hernia and may involve disturbances of mitogenic signalling pathways fundamental to embryonic lung development. What does this imply for human CDH? Fetal surgery may be 'too little, too late' to correct an

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established lung embryopathy. *In utero* growth factor therapy may permit antenatal lung rescue. Prevention of the birth defect by preconceptual prophylaxis may represent the ultimate solution.

Key words Congenital diaphragmatic hernia – Lung hypoplasia – Nitrofen – Branching morphogenesis – Epidermal growth factor – Fibroblast growth factor

Congenital diaphragmatic hernia (CDH) is an idiopathic human malformation comprising the Bochdalek diaphragmatic defect, intrathoracic herniation of abdominal viscera and hypoplastic lungs (Fig. 1). The incidence of 1:2500 births represents a new case every 24–36 h in the UK. Currently, 50% of these babies die largely due to respiratory failure resulting from hypoplastic lung development. Historically, newborns underwent emergency diaphragm repair and hernia reduction to improve lung expansion. Failure of immediate operation to enhance survival heralded innovations such as extracorporeal membrane oxygenation, inhaled nitric oxide and high frequency ventilation to stabilise the CDH baby pre-operatively. Despite such efforts, CDH mortality is little changed. The deadlock has driven surgeons to try to improve pulmonary growth in CDH antenatally.[1]

Traditional teaching maintains that pulmonary hypoplasia in CDH results from compression of the developing lung by prolapse of abdominal organs into the fetal chest. Based on this, paediatric surgeons modelled CDH by operating on fetal lambs to create a diaphragmatic defect and place abdominal viscera within the thorax. Following surgery, pregnancies were maintained by tocolytics. Apparently vindicating the 'compression' theory, newborn lambs with surgically-created CDH had hypoplastic lungs. Second stage fetal surgery to repair lamb CDH encouragingly improved lung growth by term and provided the bridge to pioneering human fetal surgery programmes. Disappointingly, fetal diaphragmatic repair and hernia reduction have not enhanced survival in human CDH.[2]

Prompted by the observation that congenital laryngeal atresia produces enlarged hyperplastic lungs, surgeons turned to augmenting lung growth of fetal CDH lambs by antenatal occlusion of the trachea. Based on the premise that ineffective diaphragmatic contraction in CDH causes pulmonary hypoplasia by allowing excessive tracheal loss of lung fluid, antenatal tracheal occlusion has been refined to the PLUG technique (plug the lung until it grows). During subsequent caesarean section, tracheal occlusion is removed and the newborn intubated prior to disconnection from placental support (EXIT procedure). Now performed fetoscopically, 'plugging' has yet to improve outcome reliably in human CDH.[3]

Appreciation that lung hypoplasia in CDH appears refractory to postnatal and fetal therapies has been accompanied by increased awareness that CDH is more than a 'diaphragmatic hole'. Associated anomalies identified in CDH newborns and affected fetuses imply that the cardinal features of CDH are part of a global embryopathy.[4] Accepting this thesis implies that lung hypoplasia arises during embryogenesis and before fetal diaphragmatic hernia. Evaluation of this hypothesis is

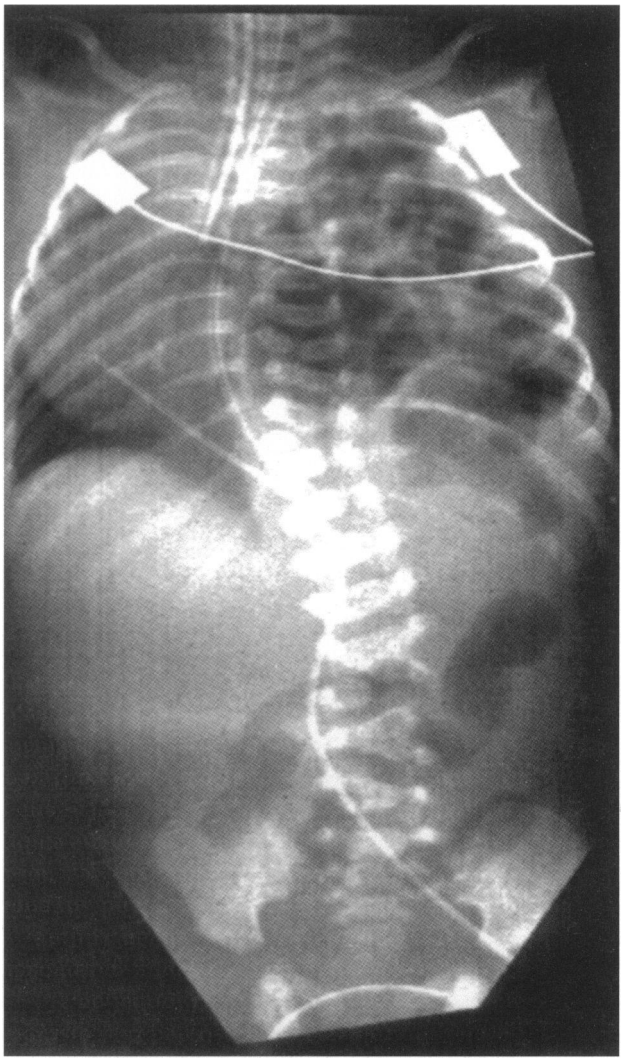

Figure 1 Plain radiograph of a human newborn with congenital diaphragmatic hernia. Gas-filled intestinal loops and stomach fill the left hemithorax displacing the heart, mediastinum and endotracheal tube. Highlighting the global embryopathy in CDH, vertebral anomalies are also apparent.

impeded by the fact that human embryogenesis, like normal diaphragmatic closure, is completed by 8 weeks of gestation. Embryological investigation of CDH, therefore, depends on animal models. However, 'manufacture' of CDH in normal fetal lambs cannot illuminate the embryogenesis of the human malformation. In contrast, the nitrofen model of CDH affords unparalleled opportunity to scrutinise the developmental biology of lung hypoplasia and CDH. Nitrofen, a herbicide with stereochemical resemblance to thyroid hormone, produces CDH and lung hypoplasia in 30–40% of newborn rats when administered to their mother during the embryonic stages of gestation. Whilst the mechanism is unknown, newborn rats with nitrofen CDH have lung hypoplasia and an incidence of associated anomalies with striking homology to the human phenotype.[5] Supporting the concept that lung hypoplasia forms part of a global embryopathy, contested findings in the nitrofen model suggested that lung hypoplasia arises prior to the diaphragmatic hernia.[6] If human lung hypoplasia is similarly determined during embryogenesis, strategies for treatment of CDH may require radical reappraisal.

Experimental work

Embryonic lung malformations may be specific precursors of CDH

The human lung arises at 4 weeks of gestation as a diverticulum of the foregut. Main, lobar and segmental bronchi are elaborated over the next 1.5 weeks by stereotypical airway branching. Normal diaphragmatic closure at 8 weeks is a substantially later event.[5] We hypothesised that equivalent stages of lung branching morphogenesis are disrupted prior to nitrofen-induced CDH.[7] Importantly, in the nitrofen model, CDH newborns have severe lung hypoplasia whilst litter-mates without CDH have minor pulmonary hypoplasia.[8] If the hernia alone induces severe pulmonary hypoplasia in CDH pups, then all nitrofen-exposed lungs should develop similarly until the hernia supervenes. We hypothesised that the severe lung hypoplasia phenotype accompanying CDH is discernible prior to (and hence not due to) visceral herniation.

Methods

Sprague-Dawley rats were fed 100 mg nitrofen in olive oil on day 9.5 of gestation in the manner described to induce lung hypoplasia and CDH in newborns (term = day 22). Control animals received olive oil. Embryonic lungs were microdissected on day 13.5 of pregnancy (equivalent to 4.5 weeks of human gestation), just after lung primordia bud from the primitive foregut and prior to herniation in the model.[5] Branching morphogenesis was measured by counting terminal buds on lung specimens.

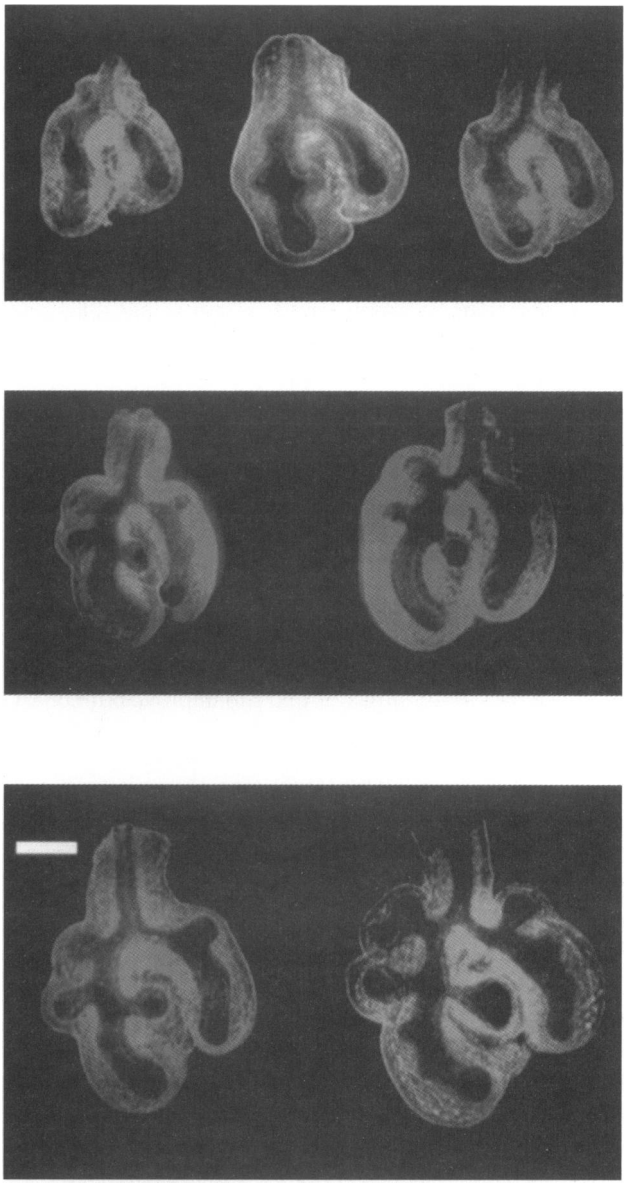

Figure 2 Photomicrographs of whole unsectioned rat lung primordia at day 13.5 *in vivo* and prior to established diaphragmatic hernia. All specimens are at the same magnification with the trachea superiorly: scale bar = 250 μm. Top, nitrofen-exposed lung rudiments with reduced lobar bronchi (36% of exposed embryos); middle, nitrofen-exposed lung rudiments with normal bronchial anatomy (64% of exposed embryos); bottom, normal control lung rudiments.

Results

At day 13.5 of gestation, prior to herniation in this CDH model, nitrofen-exposed lung primordia (n = 170) already had fewer terminal buds than normal lungs (n = 130) at the

same stage ($P < 0.001$). Normal lungs had a minimum and median 6 terminal buds at day 13.5 gestation. Though subnormal in size, 64% of nitrofen-exposed lungs obeyed this stereotype with at least 6 terminal buds. In contrast, 36% of lung primordia from day 13.5 nitrofen-exposed embryos were 'stunted' with diminished elaboration of the lobar bronchi (< 6 buds; Fig. 2).

Discussion

Lung branching morphogenesis is abnormal prior to herniation in this CDH model. Secondly, nitrofen-exposed lungs are dissimilar before the hernia supervenes: the majority retain normal lobar bronchi, whilst the remainder are 'stunted'. Therefore, the severe lung hypoplasia characteristic of nitrofen CDH appears before the hernia. Strikingly, the 36% incidence of 'stunted' nitrofen-exposed lungs matches the 30–40% incidence of left CDH subsequently seen at term in this model. These identical frequencies further support the thesis that the severe lung hypoplasia seen in pups with CDH is apparent before, and hence not entirely attributable to, the hernia. 'Stunting' of early airway branching appears to be a specific precursor of CDH.

Hypoplastic lung morphogenesis can be modelled in culture

Organ culture has yielded invaluable information on normal lung development. Roles of epithelium, mesenchyme, extracellular matrix and growth factors have all been clarified employing culture models. Furthermore, numerous findings from *in vitro* studies of lung development have recently been confirmed by transgenic experiments *in vivo*.[9] Therefore, we developed an organ culture model to further study embryonic lung hypoplasia in the nitrofen CDH model.

Methods

Nitrofen-exposed and normal control embryos were generated as described. Day 13.5 embryonic lungs were transferred to membrane culture-dish inserts (Millicell®, Millipore Corp., UK) on serum-free medium (DMEM/F12 1:1, GibcoBRL, Life Technologies, UK) incorporating penicillin (100 IU/ml) and streptomycin (100 µg/ml; GibcoBRL). Lungs were incubated at 37°C in 5% CO_2 for up to 78 h. Culture media were changed every 48 h. Lung growth *in vitro* was recorded by daily photography. Terminal bud counts were recorded daily for each lung specimen. Lung epithelial contour was traced on scanned photomicrographs using Lucida® software (Kinetic Imaging, Liverpool, UK) which calculated individual epithelial areas and perimeters.

The Mann-Whitney U test was used to compare normal and nitrofen-exposed lungs. A significance level of 0.05 was employed.

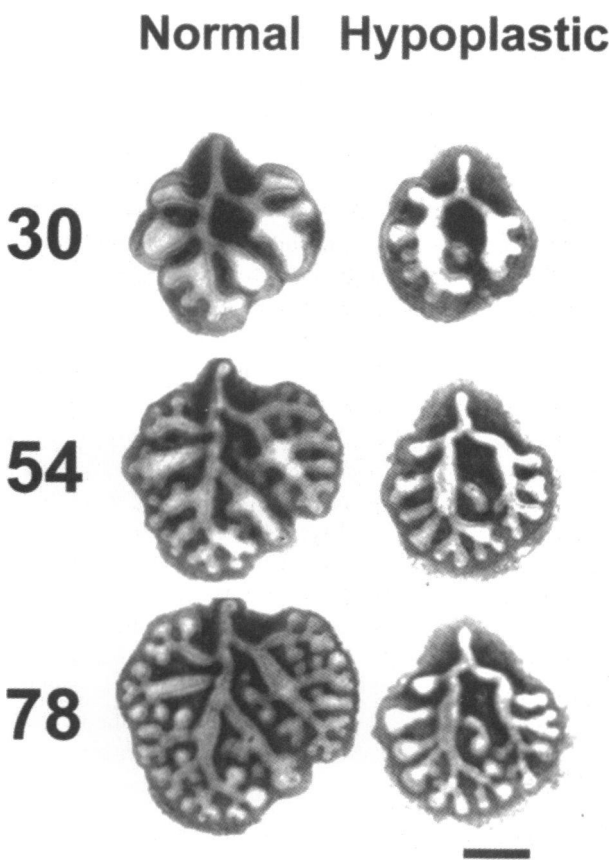

Figure 3 Photomicrographs of whole unsectioned lung primordia in organ culture. Shown on the left of the figure is the longitudinal development of a normal lung rudiment at 30, 54 and 78 h in culture. For comparison, the development of a nitrofen-exposed lung rudiment is shown on the right at the same time-points *in vitro*. All specimens are at the same magnification: scale bar = 400 µm. The trachea is seen superiorly in each case.

Results

Normal and nitrofen-exposed lungs increased in size and branching in organ culture. Cultured lungs underwent pseudo two-dimensional morphogenesis remaining smaller and less complex than *in vivo*.

In vitro studies (Fig. 3) revealed that nitrofen-exposed lungs had significant reductions in lung area at 6, 30 and 54 h in culture compared to normal controls ($P = 0.001$, $P < 0.001$ and $P = 0.001$, respectively). Lung perimeter and terminal lung bud count were reduced in the nitrofen group at 6 h and 30 h in culture ($P < 0.001$ and $P = 0.002$ versus normal perimeter; $P < 0.001$ and $P = 0.01$ versus normal terminal bud count). Significant morphological differences between nitrofen-exposed and normal control lungs were evident for up to 54 h in culture.

Discussion

Lung explants underwent branching morphogenesis throughout the culture period. Having established that lung embryogenesis is disrupted prior to nitrofen CDH, we have demonstrated that nitrofen-induced lung hypoplasia persists in organ culture. Compelling evidence from organ culture and gene targeting studies has emphasised the essential role of growth factors to lung branching morphogenesis.[9] Given these seminal findings, we sought to exploit the culture model of lung hypoplasia to establish whether targeted growth factors will rescue hypoplastic lung embryogenesis.

Fibroblast growth factor signalling may be disturbed in embryonic lung hypoplasia

Mammalian lung development requires interaction of the fibroblast growth factor family (FGFs), cognate receptors (FGFRs) and heparan sulphate (HS) proteoglycans.[10,11] This has been elucidated from studies in mice and the fruitfly, *Drosophila melanogaster*.[9] *Branchless* and *breathless* are *Drosophila* mutants lacking FGF and its receptor (FGFR), respectively.[9] *Sugarless* and *sulphateless* mutants lack heparan sulphate (HS). All these *Drosophila* mutants share abrogated airway branching.[12] Organ culture and transgenic models confirm the requirement of mammalian airway ramification for FGFs and HS.[13] Binding of the FGFR2IIIb receptor is essential for lung branching morphogenesis.[13] FGF1 binds FGFR2IIIb and is known to increase branching of normal lung explants. In contrast, FGF2 essentially does not bind FGFR2IIIb.[14] Heparin is an HS analogue that modulates FGF kinetics. We hypothesised that FGF1 (± heparin) would stimulate branching of hypoplastic lung primordia *in vitro*.

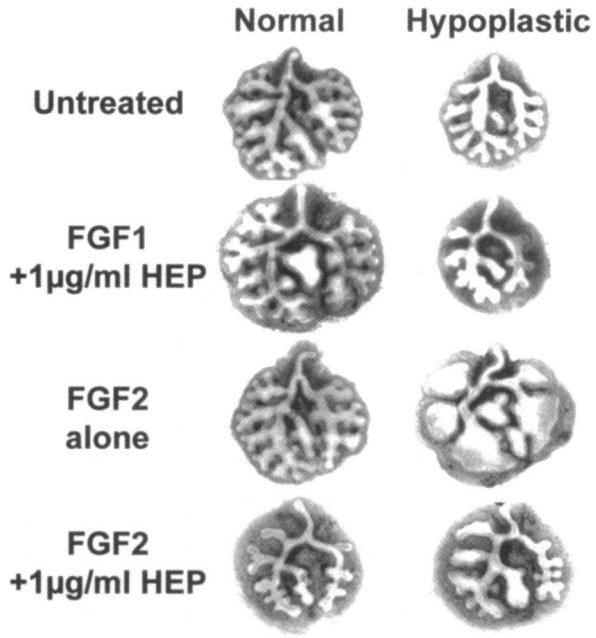

Figure 4 Photomicrographs of normal and nitrofen lung rudiments all taken after 54 h *in vitro*. Lung primordia are photographed at the same magnification (original x8). HEP = heparin. Normal lungs (left) and nitrofen-exposed lungs (right) were cultured in plain media or with added FGFs and heparin. Comparing each lung rudiment with normal and hypoplastic nitrofen lungs cultured in plain media (top): (1) FGF1 + HEP stimulates normal lung growth but inhibits the nitrofen-exposed lung; (2) FGF2 has minimal effect on normal lung, but produces marked cystic dilatation of nitrofen-exposed lungs; (3) FGF2 + HEP appears to strongly inhibit normal lung with limited effects on nitrofen-exposed lung. Clear differences are, therefore, apparent between the FGF responsiveness of normal lung and that of the hypoplastic nitrofen-exposed lung.

Methods

Normal and nitrofen-exposed embryonic lung primordia were retrieved at day 13.5 of gestation and cultured as described. Combinations of serum-free media ± heparin (100 ng/ml or 1 µg/ml) ± FGF1 or FGF2 (a 'control' FGF) were employed to give 9 experimental groups ($n \geq 4$ per group). After literature review and pilot studies, FGF1 and FGF2 were used at 600 ng/ml and 60 ng/ml, respectively. Fibroblast thymidine incorporation assays confirmed growth factor bioactivities before and after 24 h and 48 h in culture. Heparin (porcine intestinal mucosa derived, Sigma-Aldrich Company Ltd, UK) concentrations of 100 ng/ml and 1 µg/ml have been shown to chaperone optimally FGF2 and FGF1, respectively. Higher concentrations diminish branching morphogenesis and smooth muscle cell proliferation and were, therefore, avoided. Lung primordia were incubated at 37°C in 5% CO_2 for up to 78 h. Culture media were changed every 48 h. *In vitro* morphological development was measured as described.

The Mann-Whitney U test was used to compare growth factor treated lungs with specimens in plain media. For each heparin dose, specimens were compared with and without growth factor. The comparisons were conducted for nitrofen-exposed and normal control lung rudiments. A significance level of 0.05 was employed.

Results

Heparin alone produced only transient alterations of lung development. Normal lung growth was briefly and modestly enhanced by heparin early in the study period. In contrast, nitrofen-exposed lungs were minimally inhibited by heparin toward the end of the culture period.

FGF1 ± heparin significantly increased terminal bud counts, lung epithelial area and lung perimeter of normal

lungs, In contrast, nitrofen-exposed lungs cultured with FGF1 ± heparin had significantly reduced lung area, lung perimeter and terminal bud counts compared to nitrofen-exposed lungs in plain medium.

FGF2 alone had only minor effect on normal lungs, whilst FGF2 + heparin was actually inhibitory. Nitrofen-exposed lungs cultured with FGF2 alone demonstrated striking increases in lung area coupled with marked reductions in terminal bud count. FGF2 + heparin only transiently and modestly altered nitrofen lung morphogenesis (Fig. 4).

Discussion

FGF1 (± heparin) stimulated branching morphogenesis of normal lungs *in vitro*. In contrast, nitrofen-exposed lung primordia were unexpectedly inhibited by this morphogen and they also exhibited strikingly abnormal responses to FGF2 ± heparin. Most notably, FGF2 produced massive dilatation of the epithelial lumen of nitrofen lungs.

These findings indicate that FGF1 alone is unlikely to rescue hypoplastic lung growth *in vivo*. Secondly, the abnormal responsiveness of nitrofen-exposed lungs to FGFs further indicate that they are intrinsically abnormal prior to visceral herniation. We postulated that disturbance of the FGF/FGFR/HS network (or its downstream intracellular targets) may comprise at least part of such an intrinsic lesion. We now present a model to explain reduced airway branching in nitrofen-exposed lungs and their abnormal response to FGFs *in vitro*.

A model for a FGFR2IIIb lesion in embryonic lung hypoplasia

FGF10 and FGFR2IIIb are mammalian homologues of *Drosophila*'s *branchless* FGF and *breathless* FGFR. The FGFR2IIIb receptor is essential to airway morphogenesis.[13] FGF1 avidly binds the FGFR2IIIb receptor whilst FGF2 does not. Both ligands bind the FGFR2IIIc receptor.[14] Considered 'reciprocal' isotypes, segregation of epithelial FGFR2IIIb from mesenchymal FGFR2IIIc has suggested a key role in epithelial-mesenchymal interactions.[13]

We propose an imbalance of these receptors in hypoplastic lung primordia. The FGF1 stimulation of branching morphogenesis in normal lungs was anticipated from previous studies and on current evidence is mediated via FGFR2IIIb binding.[13] The failure of FGF1 to enhance nitrofen-exposed lungs may, therefore, result from reduced FGFR2IIIb expression and/or function. Diminished FGFR2IIIb expression in the nitrofen-exposed lungs may be accompanied by reciprocal increase in the elaboration and/or activity of FGFR2IIIc. Unbalanced up-regulation of FGFR2IIIc may explain the 'cystic' response of nitrofen-exposed lungs to FGF2.

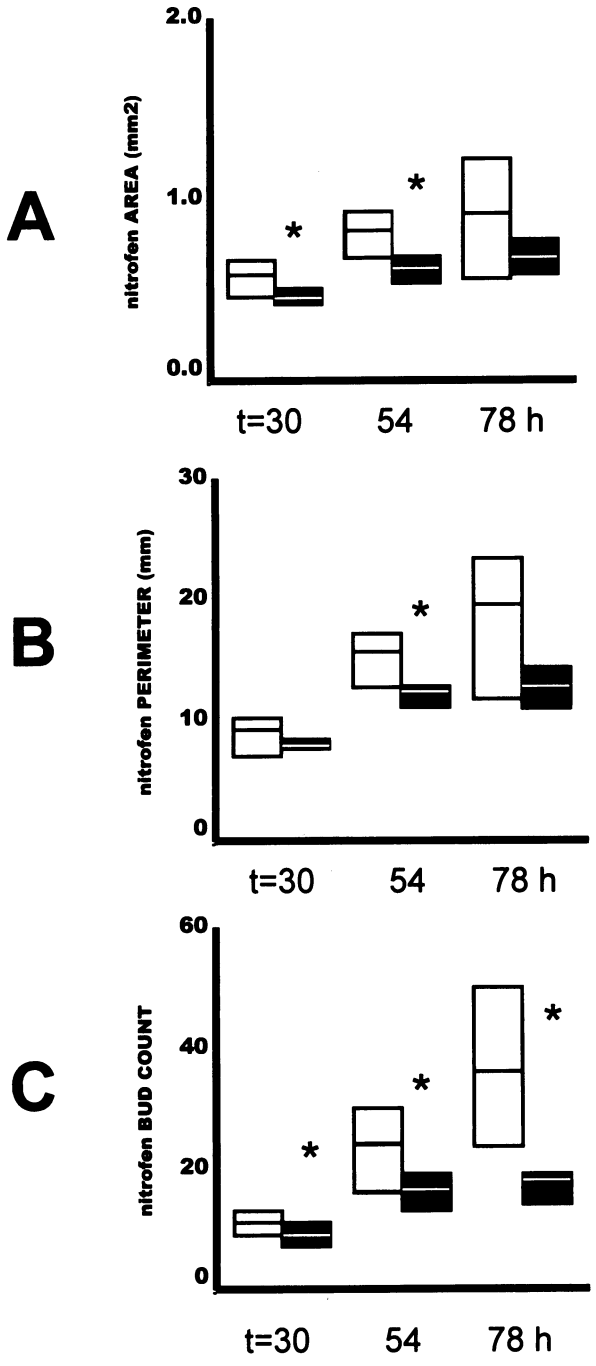

Figure 5 Morphological development of nitrofen-exposed lungs cultured with or without EGF (25 ng/ml). Graphs demonstrate lung area (A), lung perimeter (B) and terminal bud count (C) versus time (h). Data are expressed as medians (horizontal lines through each box) and interquartile ranges (boxes). Lungs were cultured without EGF (white) or with 25 ng/ml EGF (black); t, hours in organ culture; $^*P < 0.05$ for lungs cultured with EGF versus lungs in plain medium at the same time-point.

Epidermal growth factor (EGF) and in vitro *embryonic lung hypoplasia*

If FGF–FGFR–HS interactions alone are abnormal in the hypoplastic lung, nitrofen-exposed lungs might respond in a more normal fashion to HS-independent morphogens. Epidermal growth factor (EGF) signalling is ubiquitous in development and HS-independent.[15] We, therefore, selected EGF to test our hypothesis that FGF/FGFR/HS activity is **specifically** abnormal in cultured hypoplastic lungs.

Methods

Normal and nitrofen-exposed lung rudiments were harvested at day 13.5 of gestation and cultured as explained. Lungs were grown with or without EGF (gift from Dr J. Smith, University of Liverpool). EGF bioactivity was checked using cell proliferation assays. The EGF dose of 25 ng/ml was based upon pilot studies and literature review. Lungs were incubated at 37°C in 5% CO_2 for up to 78 h. Culture media were changed every 48 h. Morphological and statistical analyses were performed as reported above.

Results

In cultured normal lungs, EGF produced minor inhibitory changes in morphological development. The only significant alterations in EGF-treated lungs were decreased lung area at 30 h ($P = 0.04$) and lung perimeter at 78 h *in vitro* ($P = 0.002$).

In nitrofen-exposed lungs, EGF yielded moderate reductions in lung area at 30 h and 54 h *in vitro* ($P = 0.03$ and $P = 0.01$, respectively). Culture with EGF diminished nitrofen-exposed lung perimeter at 54 h ($P = 0.004$) and reduced terminal bud counts amongst nitrofen-exposed lungs at 30 h ($P = 0.03$), 54 h ($P = 0.02$) and 78 h ($P = 0.001$) *in vitro* (Figs 5 & 6).

Discussion

EGF did not enhance branching morphogenesis of our cultured normal lungs. Whilst in agreement with certain investigators,[16] this conflicts with the experience of others.[17] Conflicting reports of EGF's effect on lung explants may have a number of explanations. Choice of rodent may be significant because $EGFR^{-/-}$ phenotypes vary profoundly depending on mouse strain.[15] Secondly, EGF activity may be acutely sensitive to *in vitro* conditions. Thirdly, EGF may have a peripheral and hence unpredictable role in airway development.

EGF inhibited morphological development of nitrofen-exposed lung primordia. Whilst effects of EGF on *in vitro* lung hypoplasia may represent an indirect consequence of FGF/FGFR/HS dysfunction, these findings may instead indicate a lesion of nitrofen-exposed lungs that encompasses both EGF and FGF signalling.

For example, both FGF and EGF activate MAPK and are antagonised by *sprouty*.[18] *Sprouty* restrains FGF-driven airway branching[9] probably by inhibition of MAPK.[18] Several mammalian *sprouty* homologues have been described.[19] In light of the present study, we may need to extend our concept of a signalling lesion in lung hypoplasia to include *sprouty*–MAPK interactions as a common pathway for both FGF and EGF networks.

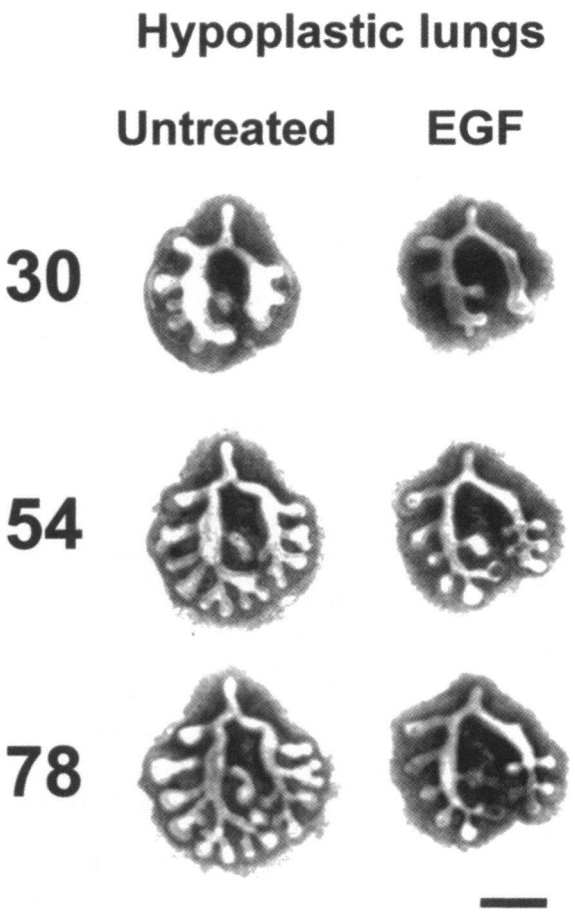

Figure 6 Photomicrographs of whole nitrofen-exposed lung primordia in organ culture. Shown on the left of the figure is the longitudinal development of a nitrofen-exposed lung rudiment after 30, 54 and 78 h cultured in plain media. On the right, a nitrofen-exposed lung rudiment cultured with 25 ng/ml EGF is shown at the same time-points *in vitro*. All specimens are at the same magnification: scale bar = 400 μm. The trachea is seen superiorly in each case.

Overview of experimental results

Lung hypoplasia begins during embryogenesis in CDH

Nitrofen-exposed lungs are abnormal prior to established CDH. Early lung malformations in the nitrofen model may in fact be specific precursors of CDH.[7]

Hypoplastic lung morphogenesis can be modelled in culture

Nitrofen-induced lung hypoplasia persisted *in vitro*. Hypoplastic lung development can, therefore, be modelled, measured and manipulated in culture.[7]

Abnormal growth factor signalling may underlie embryonic lung hypoplasia

Nitrofen-exposed lungs respond abnormally when cultured with heparin and/or selected FGFs. This supports the hypothesis that disturbed FGF–FGFR–HS signalling underpins embryonic lung hypoplasia.[10,11] Whilst not provoking the contrasting effects of the FGFs, EGF did yield greater inhibition of nitrofen-exposed lungs than normal ones. Perturbed growth factor signalling may, therefore, extend to *sprouty*–MAPK interactions in the hypoplastic lung.

Future therapeutic implications for paediatric surgery

Paediatric surgeons have the enviable opportunity to translate developmental biology into clinical benefit for patients with birth defects. If lung hypoplasia begins during embryogenesis in human CDH, refinements of postnatal treatment may remain 'too little, too late'.[7] Fetal surgery as currently configured would similarly have limited impact on embryonic lung defects in CDH. What new antenatal approaches can be envisaged?

Preconceptual prevention would circumvent lung hypoplasia by reducing the incidence of CDH.[7] Secondly, antenatal glucocorticoids improve lung compliance and several parameters of lung maturation in experimental CDH. They are subject to a multicentre trial in human CDH and may yet contribute to improved survival.[20] Thirdly, fetoscopic instillation of growth factors to enhance antenatal airway development remains a goal of the present investigations.[11] The startling effect of FGF2 on hypoplastic lungs raises the tantalising possibility of a pharmacological 'PLUG' to be used with antenatal steroids in order to augment lung size and lung maturation in tandem. Future co-administration of angiogenic factors may ameliorate the 'pruned' hypermuscularised pulmonary vasculature that contributes to respiratory failure in CDH. Finally, tissue engineering may eventually permit stem cell grafts to repair the hypoplastic lung. Clarifying the precise embryology of lung hypoplasia in CDH remains crucial to transforming these concepts into effective clinical treatments for this lethal human anomaly.

Acknowledgements

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