Amniotic fluid derived mesenchymal stromal cells augment fetal lung growth in a nitrofen explant model

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A B S T R A C T

Purpose: Recent experimental work suggests the therapeutic role of mesenchymal stromal cells (MSCs) during lung morphogenesis. The purpose of this study was to investigate the potential paracrine effects of amniotic fluid-derived MSCs (AF-MSCs) on fetal lung growth in a nitrofen explant model.

Methods: Pregnant Sprague–Dawley dams were gavage fed nitrofen on gestational day 9.5 (E9.5). E14.5 lung explants were subsequently harvested and cultured ex vivo for three days on filter membranes in conditioned media from rat AF-MSCs isolated from control (AF-Ctr) or nitrofen-exposed (AF-Nitro) dams. The lungs were analyzed morphometrically and by quantitative gene expression.

Results: Although there were no significant differences in total lung surface area among hypoplastic lungs, there were significant increases in terminal budding among E14.5 + 3 nitrofen explants exposed to AF-Ctr compared to explants exposed to medium alone (58.8±8.4 vs. 39.0±10.0 terminal buds, respectively; p<0.05). In contrast, lungs cultured in AF-Nitro medium failed to augment terminal budding. Nitrofen explants exposed to AF-Ctr showed significant upregulation of surfactant protein C to levels observed in normal fetal lungs.

Conclusions: AF-MSCs can augment branching morphogenesis and lung epithelial maturation in a fetal explant model of pulmonary hypoplasia. Cell therapy using donor-derived AF-MSCs may represent a novel strategy for the treatment of fetal congenital diaphragmatic hernia.

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Pulmonary hypoplasia and pulmonary hypertension continue to be major problems in neonates with congenital diaphragmatic hernia (CDH). Despite ongoing advances in state-of-the-art care, including fetal tracheal occlusion, extracorporeal membrane oxygenation, and permissive hypercapnia, mortality rates for infants with CDH remain in excess of 30% [1–4]. Among survivors, the pulmonary morbidity associated with this disease can be quite significant, as validated by the spawning of multidisciplinary clinics involving surgeons, pulmonologists, and other specialists at major children’s hospitals nationwide [5]. Novel treatments, aimed at better facilitating fetal and/or postnatal lung growth, are needed if further inroads are to be made in the management of this debilitating disease.

Over the past decade, stem cell-based approaches have been explored as an innovative strategy towards understanding lung regeneration in a variety of pediatric lung disorders [6–8]. In particular, recent experimental work suggests that an underlying disruption of stem cells within the lung mesenchyme, as opposed to lung epithelia itself, may be a major initiator of lung hypoplasia in CDH and other diseases such as bronchopulmonary dysplasia (BPD) [9]. In parallel with these findings, several investigators have demonstrated the potential therapeutic role of exogenously delivered mesenchymal stromal cells (MSCs) derived from either bone marrow or umbilical cord on lung regeneration in pediatric disease models [10,11]. The mechanisms involved in the observed lung repair by MSCs have not been well elucidated but may involve paracrine modulation of inflammatory responses by growth factors, possibly with activation of adjacent lung epithelial progenitors and endothelial cells [12].

Our laboratory, among others, has focused on the potential role of cells normally present within the amniotic fluid during organogenesis [13–15]. The amniotic fluid is known to contain a heterogeneous population of MSCs originally derived from placenta, lung, skin, and other organs [16–20]. Although these amniotic fluid-derived MSCs (AF-MSCs) are largely CD117-negative and do not have three germ layer differentiation potential [21], they express selected markers of pluripotency, are easy to isolate across a wide range of gestational ages, and have been shown to exert paracrine effects including the acceleration of fetal wound healing [21–24]. Moreover, compared to postnatal bone marrow MSCs, fetal derived MSCs express higher levels of stimulatory pulmonary morphogens, including hepatocyte growth factor (HGF), a heterodimeric heparin-binding growth factor regarded as a major initiator of normal lung organogenesis [19,25]. To date, little is known about the possible salutary effects of AF-MSCs on lung development in both normal and pathological states. In this study, we explored the novel theoretical concept of fetal MSC-
mediated lung growth by investigating the paracrine action of AF-MSCs on branching morphogenesis in a nitrofen lung explant model.

1. Methods

1.1. Nitrofen-induced lung explant model

This study was approved by the University of Michigan Unit for Laboratory Animal Medicine under protocol 10495-1 in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. To induce fetal lung hypoplasia, timed-pregnant Sprague–Dawley rats (Harlan Laboratories) were gavage fed 100 mg of nitrofen (2,4-dichlorophenyl p-nitrophenyl-ether; Sigma-Aldrich) dissolved in 1 ml of olive oil on gestational day 9.5 (E9.5) as previously described elsewhere [26]. Control dams were gavage fed 1 ml of olive oil only. On day 14.5 (E14.5), dams were deeply anesthetized, and all fetuses were harvested by cesarean section. Fetal lungs were dissected out intact in Hank’s buffered saline under a dissecting microscope.

1.2. Amniotic fluid mesenchymal stromal cells

Amniotic fluid samples were collected from pregnant control and nitrofen-exposed Sprague–Dawley dams (n=5 each) on E14.5. Rat AF-MSCs were isolated and cultured based on established protocols in the laboratory [13]. Plastic adherent, spindle-shaped cells between passage 3 and 5 were evaluated using criteria as described elsewhere [27,28]. For flow cytometry analyses, 5 × 10^5 AF-MSCs were incubated with 5% fetal bovine serum, and primary antibody at 4 °C for 30 min. Antibodies against CD11 (BD Biosciences), CD34 (Santa Cruz), CD45, CD73 (BD Biosciences), CD79 (Novus Biologicals), CD90 (BD Biosciences), and CD105 (Novus Biologicals) were used as well as same isotype antibodies as negative controls. Evaluation of staining was performed using the LSRII flow cytometer (BD Biosciences), and data were analyzed using Flojo software (Tree Star).

For adipocyte differentiation of AF-MSCs, confluent cells were placed in adipogenic differentiation medium [0.5 mM isobutyl-methylxanthine (EMD Millipore), 200 μM indomethacin (MP Biomedical), 10^{-6} M dexamethasone (MP Biomedical), and 10 μg/ml of insulin (Sigma-Aldrich) in αMEM (Gibco)] supplemented with 10% fetal bovine serum. For osteoblast differentiation, confluent cells were cultured in osteogenic differentiation medium [10^{-7}M dexamethasone (MP Biomedicals), 10 mM β-glycerophosphate (Alfa Aesar) in DMEM (Gibco)] supplemented with 10% fetal bovine serum.

Rat dermal fibroblasts were isolated from adult rat skin as described elsewhere [29]. One day prior to fetal lung dissection, cells were plated at a density of 20,000 cells/cm² in 12-well plates and grown for 24 h in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM l-glutamine at 37 °C in a 5% CO2 atmosphere.

1.3. Explant culture system

E14.5 lung explants were placed into polyethylene terephthalate hanging insert filters (0.4 μm, EMD Millipore) and divided into four groups based on cell type: medium alone (medium, n=6), AF-MSCs from control rats (AF-Ctr, n=6), AF-MSCs from nitrofen-exposed rats (AF-Nitro, n=6), and rat dermal fibroblasts (dermis, n=3). Normal lungs harvested from E14.5 control dams were grown in medium alone. Explants were cultured under serum-free hypoxic conditions (3% O₂) for three days to mimic the low-oxygen tension of the fetal environment.

Fig. 1. Characteristics of rat amniotic fluid-derived mesenchymal stromal cells (AF-MSCs) harvested on gestation day 14.5. Phase contrast photomicrographs of AF-MSCs from control (A) and nitrofen-exposed (B) fetuses (20× magnification). Flow cytometry analyses of AF-MSCs from control (C) and nitrofen-exposed (D) fetuses, demonstrating a predominant population of CD45⁻, CD73⁺, CD90⁺, and CD105⁺ cells.
1.4. Morphometry

Each explant was photographed daily under an inverted phase-contrast microscope (4× magnification). Digital images from day 0 (E14.5), day 1 (E14.5+1), and day 3 (E14.5+3) were blindly analyzed for total lung surface area and terminal lung budding using Leica Application Suite V3 software. For lung surface area calculations, the outline of the lung explant was manually traced and integrated by the imaging software. For branching analysis, a terminal bud was defined as a single acinus separated by distinct septae at the periphery of the explant.

1.5. RNA extraction and quantitative gene expression

Relative gene expression of several lung maturity markers, namely surfactant protein B (SP-B), surfactant protein C (SP-C), and club cell secretory protein (CCSP), was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from E14.5+3 explants (n=6) using TRIzol (Invitrogen). First-strand cDNA was prepared from 2 μg of total RNA with a Taqman Reverse Transcription Reagent kit (Applied Biosciences) and oligo dT primers. Samples without reverse transcriptase were processed in parallel as negative controls. Quantitative RT-PCR was carried out on a Mastercycler ep realplex (Eppendorf) using Fast SYBR Green Master Mix (Applied Biosystems) under the following thermal cycling conditions: enzyme activation at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Actin beta (ACTB) was used as a reference gene for the normalization of target gene expression using the $2^{-\Delta\Delta Ct}$ method. PCR primers were designed with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences: SP-B forw: 5’CCAGTGAACAGGCTATGCCA3’, SP-B rev: 5’CTGCTCA-CACTTTTGTGTC3’, SP-C forw: 5’CCCACCGGATTACTCGACAG3’, SP-C rev: 5’CCACCAAAACGAGATGA3’, CCSP forw: 5’AGCT-CCGCCCTTCGGAAC3’, CCSP rev: 5’GGTCTGAGGCAGGTGAAA3’, ACTB forw: 5’TGTCTGACAGGATGCAAG3’, ACTB rev: 5’TAGACCCCAAATCCACAC3’.

1.6. Statistical analyses

Quantitative data were presented as the mean±SEM. Data were analyzed by the Student’s t-test or one-way analysis of variance with post hoc testing by Dunnett or Bonferroni correction for multiple comparisons, as appropriate, using Prism 6 (GraphPad). Results were considered to be statistically significant if p<0.05.

2. Results

Rat E14.5 amniotic fluid-derived cells from control and nitrofen-exposed animals were isolated by plastic adherence from all specimens. The cells were heterogenous in morphology and

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Fig. 2. Representative photomicrographs of nitrofen fetal rat lung explants between E14.5 to E14.5+3 (after three days in culture). Each column depicts a different treatment group, namely lung explants exposed to medium alone (medium), amniotic fluid-derived mesenchymal stromal cells from control fetuses (AF Ctr), amniotic fluid-derived mesenchymal stromal cells from nitrofen-exposed fetuses (AF Nitro), and rat dermal fibroblast (dermis).
predominantly CD90+, CD73+, CD105+, CD11−, CD34−, CD45−, and CD79−, consistent with a mesenchymal stromal phenotype (Fig. 1). AF-MSCs could be selectively differentiated into adipogenic and osteogenic lineages (data not shown).

E14.5 fetuses exposed to nitrofen consistently had lung hypoplasia compared to lungs from control E14.5 fetuses, as demonstrated by a significant reduction in total surface area (1.77±0.04 vs. 2.53±0.07 mm², respectively; p<0.05) and branching (15.03±0.48 vs. 20.65±0.94 terminal buds, respectively; p<0.001).

To determine the paracrine effects of AF-MSCs among hypoplastic lungs, E14.5 explants from nitrofen-exposed fetuses were cultured for three days in the presence of AF-Ctr (Fig. 2). At E14.5+1, no significant changes were observed in terms of surface area and lung branching based on culture conditions (Fig. 3A). At E14.5+3, lung surface areas were still comparable among the groups. However, exposure to AF-Ctr caused a significant increase in airway branching (AF-Ctr 58.8±8.4 vs medium 39.0±10.0; p<0.05; Fig. 3B). In contrast, exposure of fetal lungs to AF-Nitro failed to augment lung branching (35.3±7.6 terminal buds). Explants cultured with dermis also had a stimulatory effect on branching (64.7±12.4 terminal buds; p<0.05), comparable to lungs exposed to medium but was not significantly different compared to AF-Ctr.

In order to evaluate developmental maturation of E14.5+3 hypoplastic explants, gene expression of lung epithelial markers was measured by qRT-PCR (Fig. 4). AF-MSCs were associated with a marked increase in SP-C expression in AF-Ctr and AF-Nitro explants. Expression of SP-C among AF-Ctr lungs was similar to levels achieved in normal control lungs and reached statistical significance compared to hypoplastic lungs in medium alone (N+medium). Although there was a trend towards increased upregulation of SP-B among explants cultured with AF-Ctr and AF-Nitro, no significant differences in gene expression were observed. There were no differences in CCSP expression.

3. Discussion

The impact of amniotic fluid on lung development has been appreciated for decades [20,30,31]. Neurogenic conditions and mechanical factors that prevent fetal breathing of amniotic fluid are
associated with pulmonary hypoplasia [32–34]. More recently, it has been speculated that the severe and potentially lethal pulmonary hypoplasia that occurs in fetuses with severe oligohydramnios may not be solely from volume-mediated distention of the fetal lungs, but rather from inadequate exposure of the lung to growth factors present within the amniotic fluid [35,36]. Taken together, it appears that the cellular milieu of amniotic fluid, in addition to the contribution of volume and pressure, may play a critical and active role during the process of lung morphogenesis. However, significant gaps in knowledge remain with regards to the potential mechanistic pathways and the relative importance of amniocytes during the various stages of fetal lung development.

Unlike MSCs that are typically derived from bone marrow and other tissues, fetal-derivedstromal cells, such as AF-MSCs, have several unique properties that suggest their role in the development of fetal lungs and other organs [37]. First, in addition to the typical MSC characteristics as outlined elsewhere [38], AF-MSCs have immunomodulatory properties and a distinctive phenotype consistent with more primitive cell origins, expressing selected pluripotency markers including OCT4, SOX2 and SSEA4 [39,40]. Based on hierarchical clustering and differential expression analyses of the entire amniocyte transcriptome, these cells express canonical regulators associated with pluripotency and stem cell repression, and are distinct from human embryonic stem cells, induced pluripotent stem cells, and neonatal dermal fibroblasts [41]. Second, fetal stromal cells express high levels of several pulmonary morphogens, including HGF, keratinocyte growth factor, and fibroblast growth factor-2, [19,42] but low levels of transforming growth factor-β1 (TGF-β1) which has been shown to inhibit fetal lung branching and leads to aberrant differentiation of perivascular cells [43]. TGF-β1 has also been associated with hypoalveolarization consistent with a BPD-like phenotype [44,45].

In this report, we utilized an established ex vivo fetal nitrofen lung model to study the effects of AF-MSCs during the pseudoglandular stage of pulmonary development. Developmental biologists have suggested that the pseudoglandular stage, a period marked by progressive branching of the airways and vascular networks, is the primary period associated with aberrant lung development in CDH [34]. Evidence to support this notion includes autopsy data in CDH neonates, demonstrating a reduction in airway generations to half the normal amount with relatively normal numbers of alveoli when related to the number of terminal bronchioles [46].

In our model, we identified a significant stimulatory effect of AF-Ctr, but not AF-Nitro, on terminal budding of explanted E14.5 hypoplastic lungs. The exposure of E14.5 nitrofen-exposed lungs to AF-Ctr augmented terminal branching to levels exceeding those of normal lungs. At the same time, we did not observe a significant enhancement in overall lung size, as measured by total lung surface area, thereby suggesting that the predominant effect of AF-Ctr on lung hypoplasia was on the induction of branching morphogenesis. The observed acceleration in pulmonary terminal bud formation occurred in a paracrine fashion since cell-to-cell contact between AF-MSCs and cells within the explant itself was not required.

Airway branching morphogenesis, a process completed in humans by the sixteenth week of gestation, is a complex developmental process that is likely regulated through multiple signaling pathways involving fibroblast growth factor-10, among others [47]. The mechanism for the observed branching acceleration by AF-Ctr in this study has yet to be defined. Previous investigators have largely focused on the cytoprotective and anti-inflammatory effects of MSCs [48]. At this point, we can only speculate that secretion of pulmonary morphogens and other growth signaling molecules within the microenvironment by a subpopulation of AF-MSCs may activate resident stem cells to stimulate branching [48,49]. However, the paracrine effects mediated by AF-MSCs do not seem to be entirely specific to amniocytes since dermal fibroblasts also produced the same positive effects on lung branching in our study [50]. During fetal lung development, distal lung epithelial cells are not fully committed to a specific phenotype and still have the plasticity to be responsive to mesenchymal signaling factors [51]. Further research is clearly needed to better characterize the mechanistic pathways and potential mediators in the process of AF-MSC-induced branching morphogenesis [52].

Another intriguing finding of the present study was the observation that in contrast to AF-Ctr, AF-Nitro did not accelerate branching morphogenesis of E14.5 nitrofen-exposed lungs. Compared to hypoplastic lungs in medium alone, AF-Nitro exerted no significant effect on either total lung surface area or terminal bud formation. Therefore, in the nitrofen model, the observed fetal lung hypoplasia could, in theory, be secondary to a primary process within the lung parenchyma that is further exacerbated by the poor lung growth milieu of dysfunctional AF-MSCs surrounding the fetus. The AF-MSCs harvested from nitrofen-exposed fetuses may be functionally unique and therefore may represent a novel prenatal biomarker useful for further characterizing and predicting the degree of hypoplastic lung disease before birth. Recently, increased levels of human MSCs isolated from tracheal aspirates in neonates soon after birth have been shown to be predictive of the likelihood of developing BPD [9,53]. We are currently looking at the therapeutic effect of human AF-MSCs harvested from normal and CDH fetuses in this model since our rat amniotic fluid data would suggest that the autologous delivery of AF-MSCs in CDH patients might not enhance lung growth to the same degree as donor AF-MSCs obtained from fetuses with normal lungs.

This study also found evidence for enhancement of distal type II epithelial cell differentiation within hypoplastic lungs exposed to AF-MSCs, as shown by significantly increased expression of SP-C among AF-Ctr explants. Expression of SP-C, a specific marker for distal lung type II epithelial maturation, begins during the pseudoglandular phase. After only a three-day exposure of hypoplastic lungs to AF-Ctr, levels of SP-C expression within E14.5+3 nitrofen-exposed lungs were comparable to those seen in normal E14.5+3 lungs in medium alone. Although no change was observed in the expression of SP-B and CCSP, the lack of any downregulation of these lung maturity markers is encouraging. Surfactant deficiency is known to be persistent both in late term nitrofen-exposed fetal rats [54] and in some neonates with CDH. The observation of upregulation in SP-C by AF-MSCs in our study also stands in contrast to reports on sustained tracheal occlusion, an experimental fetal intervention that has also been shown to stimulate branching morphogenesis in hypoplastic lungs but has been associated with massive loss of type II epithelial cells and a reduction in overall surfactant production [55].

AF-MSCs are readily accessible via amniocentesis throughout the entire gestation, do not require tedious cell sorting techniques to identify rare subpopulations, and can be rapidly expanded in the laboratory under clinical-grade conditions from a 5 mL specimen [22]. However, despite the aforementioned paracrine effects of AF-MSCs on branching morphogenesis, this study has some caveats and limitations that warrant further inquiry before considering its application as an approach to therapeutic lung rescue in severe cases of CDH. As with most animal models produced using teratogenic compounds, nitrofen lungs may not fully recapitulate the features of the developing human CDH lung [56]. To date, we have also studied a relatively small number of hypoplastic fetal lungs, and there are problems inherent with interpreting data from an ex vivo fetal lung model since the concomitant effect of ipsilateral compression on the lung by the hernia cannot be taken into account. Given these issues as well as the desire to translate this therapeutic concept into the clinical arena, we are also working with in vivo models to assess the impact of donor-derived AF-MSC delivered during the canalicular stage of lung development.

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Discussion

Discussant: Dr. Harold Lovorn, Nashville, TN. Thank you, Dr. Kunisaki for that very clever study. So the difference in the condition media from the control amniotic fluid stem cells versus the nitrogen how
are you going to discriminate those, what technology/techniques will you use?

Response: Dr. Kunisaki: We have done some preliminary work looking at various cell surface markers as well as the relative plasticity of the MSCs derived from control and nitrofen animals, and we have not been able to detect any differences between the two groups. Morphologically the nitrofen-exposed MSCs do appear differently, but they seem to proliferate at the same rate in culture. Since then we have performed some microarray studies starting first with human actually amniotic fluid MSCs to look at differences and those analyses remain ongoing, but I think a microarray approach looking at genes that are associated with pulmonary growth for example PDGF and TGF beta I think may be fruitful in terms of sorting out the actual mechanism that is going on here.