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Routine isolation and expansion late mid trimester amniotic fluid derived mesenchymal stem cells in a cohort of fetuses with congenital diaphragmatic hernia

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ABSTRACT

Objective: To assess the feasibility of routine isolation and expansion of amniotic fluid derived mesenchymal stem cells (AF-MSC) in fetuses diagnosed with isolated congenital diaphragmatic hernia (CDH).
Study design: Redundant AF samples of fetuses with CDH and normal fetuses were obtained. Cell colonies were mechanically selected for each sample. Proliferation capacity was expressed as population doubling time (PDT). Cell lines were further characterized with flow cytometry, differentiation assays and qRT-PCR (OCT4 and NANOG). After cell labeling with LacZ *in vivo* tracking was performed after fetal tracheal injection in rabbits.
Results: Fourteen consecutive CDH samples (median gestational age (GA) of 32.9 weeks; IQR: 27.8–34.3 weeks) and seven control samples (30 weeks; IQR: 28.9–34.4 weeks) were obtained. PDT was similar in both groups (45.4 h ± 1.9 vs. 52.3 h ± 3.4;NS). AF-MSCs expressed a typical mesenchymal CD marker profile. Clones could be differentiated in osteogenic, adipogenic and chrondrogenic lineages. Expression of multipotency markers was low in all cell lines. We confirmed the presence of injected cells inside the fetal lung three days after intratracheal injection.
Conclusion: Routine isolation and expansion of AF-MSCs in CDH is feasible and cell lines generated were comparable to those of control samples. AF-MSCs from affected fetuses could potentially be used in future stem cell therapy.

Introduction

Mesenchymal stem cells (MSC) are cells with a self-renewal capacity and the ability to differentiate into mesodermal cell types, such as adipogenic, osteogenic and chondrogenic cells. MSCs were initially isolated from adult bone marrow [1], but they have been isolated from numerous sources of adult tissues, as well as from fetal and extra-embryonic tissues [2–4]. Considering the ethical constraints for using embryonic stem cells (ESC), MSCs are an interesting alternative for cell therapy and tissue engineering. Furthermore MSCs, unlike ESCs, do not appear to form teratoma when injected *in vivo* and their immunomodulatory capacities reduce an immunologic response of the host after transplantation [5].

The amniotic fluid is a rich source of various differentiated and stem cell populations originating from different organ systems of the developing fetus [6–9]. More recently, a distinct population of cKit (CD117) positive cells was described, possessing the ability to differentiate into cells outside the mesodermal lineage [10]. These broad multipotent cells are well-characterized, differentiate to various cell lineages belonging to the 3 germ layers and their therapeutic potential has been explored both using *in vitro* as well as *in vivo* experiments [11]. However, in order to obtain mesenchymal progenitors, simpler methodologies, which do not

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require processes based on immunoisolation, have been proposed [6]. Likewise, we recently reported on the routine isolation of AF-MSCs following mechanical selection in early mid trimester amniotic fluid samples of structurally normal fetuses [12].

Cell therapy has been suggested in the management of congenital malformations, including congenital diaphragmatic hernia (CDH). CDH is a rare birth defect with an incomplete closure of the diaphragm resulting in the herniation of abdominal organs into the thorax, thereby interfering with pulmonary development. The resulting pulmonary hypoplasia of both lungs leads to potentially fatal ventilatory insufficiency and pulmonary hypertension after birth. Despite advances in both neonatal care, as well as the potential benefit of antenatal therapy a substantial part of fetuses with severe CDH will not survive after birth. There is early evidence of a beneficial effect of AF-MSCs on the developing lung as demonstrated in the nitrofen rat model [11]. If this would be confirmed in higher species and clinical applications are contemplated, one would need to routinely isolate and expand AF-MSCs at the moment of diagnosis, which typically would be in the late second or early third trimester. Also, the isolation of lung progenitors could provide new insights regarding the etiology of CDH, since this is still largely unknown [13,14]. There is an increasing evidence that certain candidate genes (such as, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), Wilms tumor 1 (WT1) and GATA binding protein 4 5GATA4) or signaling pathways (retinoid acid pathway) could play an important role [13]. Cytogenetic analysis of amniotic fluid stem cells could be useful in order to further unravel the underlying causes of this condition.

Theoretically, the developmental aberrations and a decreased surface area of conducting airways could influence the amount of mesenchymal cells that are shed from the lungs into the amniotic fluid. This might alter the MSC population in the amniotic fluid and could potentially influence the isolation capability.

Therefore we set up a study to investigate the feasibility of routine isolation and expansion of AF-MSC. Our second objective was to evaluate if such cell lines could be used *in vivo*, e.g., by administration into the developing fetal airways.

Materials and methods

Clinical sample collection

Redundant amniotic fluid (around 5–10 ml) from clinical AF samples, e.g., taken at the time of assessment or at fetal surgery from fetuses with an isolated CDH was used. Typical time points were either late diagnostic amniocentesis or fetoscopic endoluminal tracheal occlusion (FETO) for balloon insertion or balloon removal [15]. Controls were redundant fluid samples from structurally normal fetuses that underwent amniotic fluid sampling at the time of other diagnostic or fetal surgery procedures in the second or third trimester, such as fetoscopic laser photocoagulation for twin-to-twin transfusion syndrome (TTTS) or intrauterine transfusion for fetal anemia.

Cell culture

After filtration (40 μm strainer) and centrifugation (240 RCF, 5 min) the cells were seeded in a tissue culture treated petridish and allowed to attach. Incubation was done at 37 °C in a 5% humidified CO_2 chamber for expansion. Cell culture medium consisted of α-MEM (GIBCO® Invitrogen, Ghent, Belgium), 15% fetal bovine serum (FBS) (GIBCO® Invitrogen), 1% L-glutamine (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 18% Chang B and 2% Chang C (Irvine Scientific, Brussels, Belgium). After a few days in culture, separate cell colonies (4 colonies/sample) were mechanically picked up under inverted microscope using fine-tipped pipettes. The aspirated cells of each colony were re-seeded into an individual well of a 96-well plate. Cells were allowed to reach 70% confluence and were expanded for further experiments. At each passage, cells were detached by exposing the cell plate to trypsin/EDTA solution (Sigma–Aldrich, St. Louis, MO, USA). Cells were counted manually using a cell counter chamber and with each passage, 25×10^3 cells were reseeded in a single well of a six-well plate. Proliferation capacity was evaluated with growth curves expressing population doubling time (PDT = $(t_1 - t_0) \times \log 2/\log$(number of cells at t_1/number of cells at t_0))

Surface marker analysis with flow cytometry

After expansion, early passage (4–6) AF-MSCs were used for characterization with fluorescent activated cell sorting (FACS) to evaluate the surface marker expression profile. Antibodies (CD45, CD34, CD73, CD90, CD105, CD44, CD117, HLA-DR, HLA-ABC (BD Biosciences, Erembodegem, Belgium) and CD29 (Acris, Herford, Germany)) were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). At least 10^5 cells were incubated for 30 min at 4 °C for both the antibody as well as the appropriate isotype control. Unstained cells were used to determine the background autofluorescence. Cell analysis was performed with a FACSort cytometer (BD Biosciences) using Cell Quest software; offline data interpretation was done using FlowJo software (Tree Star, Ashland, OR, USA).

In vitro differentiation assays

Early passage (5–8) AF-MSCs were evaluated for differentiation capacities into osteogenic, adipogenic and chondrogenic cell lineages. Osteogenic differentiation was induced in 70% confluent cultures by the addition of a commercially available differentiation medium (GIBCO® Invitrogen) for 21 days. Alizarin staining of the calcified extracellular matrix deposition was used to confirm the formation of osteoblasts. Adipogenic differentiation was induced in a 100% confluent culture into eight-chamber slides (Nunc). The cells were submitted to cyclic changes (every 3 days) with induction and maintenance medium. Induction medium consisted of DMEM low glucose (Invitrogen), dexamethasone (1 mM), indomethacin (100 μM), 3-isobutyl-L-methyl-xanthine (0.5 mM), human recombinant insulin (10 μg/ml), FBS (10%), penicillin and streptomycin (1%) for 14 and 21 days. Maintenance medium consisted of DMEM with the addition of only FBS, antibiotics and human recombinant insulin (10 μg/ml). Oil Red O staining was used for determination of adipogenic differentiation. Chondrogenic differentiation was induced in high-density pellet mass cultures for 14 days. These cultures were initiated in a 24-well plate by seeding a 20 μl droplet of cell suspension containing 400,000 cells in PBS. The cells in the droplet were allowed to attach without medium for three hours, thereafter the cells were cultured in the normal medium described above for 24 h. The next day the medium was replaced by commercially available differentiation medium (GIBCO® Invitrogen) specific for chrondrogenic differentiation. Chondrogenesis was confirmed with Alcian Blue staining.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The gene expression of *OCT4* and *NANOG*, which are considered multipotency markers were analyzed using qRT-PCR. Total RNA was obtained using TriPure isolation reagent (Roche Diagnostics, Vilvoorde, Belgium) according to the manufacturer's guidelines