RESCUE OF MURINE SILICA-INDUCED LUNG INJURY AND FIBROSIS BY HUMAN EMBRYONIC STEM CELLS

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ABSTRACT: Type II pneumocytes (ATII Cells) are considered as putative alveolar stem cells. Since no treatment is available to repair damaged epithelium and preventing lung fibrosis, novel approaches to induce regeneration of injured alveolar epithelium are desired.

The objective of this study was to assess both the capacity of human embryonic stem cells (HUES-3) to differentiate in vitro into ATII Cells and the ability of committed HUES-3 cells (HUES-3-ATII Cells) to recover in vivo a pulmonary fibrosis model obtained by silica-induced damage.

In vitro differentiated HUES-3-ATII Cells displayed alveolar phenotype characterized by multilamellar body and tight junction formation, by the expression of specific markers such as SP-B, SP-C and ZO-1 and the activity of CFTR-mediated chloride ion transport. After transplantation of HUES-3-ATII Cells into silica-damaged mice, histological and biomolecular analyses revealed a significant reduction of inflammation and fibrosis markers along with lung function improvement, weight recovery and increased survival. The persistence of human SP-C, human nuclear antigen and human DNA in the engrafted lungs indicates that differentiated cells remained engrafted up to ten weeks.

In conclusion, cell therapy using HUES-3 cells may be considered a promising approach to lung injury repair.

KEYWORDS: cell therapy, human embryonic stem cells, lung fibrosis, silica damage.
INTRODUCTION

The pulmonary alveolar epithelium is an endoderm-derived tissue comprised of two main cell populations: the alveolar type I (ATI) and type II (ATII) pneumocytes [1]. In addition, resident lung multipotent stem cells have been recently identified in the distal airways [4]. Beside characterizing their phenotype and functional properties in vitro and in vivo, the Authors described the capacity of these cells to give rise to different populations of endodermal epithelial cells and to pulmonary vessels. However their capacity to promote tissue restoration in patients with lung disease has not been demonstrated yet. As the lung is constantly exposed to environmental toxicants and pathogens capable of injuring and destroying ATI Cells on the alveolar surface, possibly leading to tissue destruction, lung structure distortion and respiratory dysfunction, ATII Cells play a key role in lung homeostasis by producing surfactant proteins and by their ability of undergoing proliferation and differentiation to replace ATI Cells [2]. Thus, the in vitro generation of ATII Cells, capable of repairing damaged alveolar units [3], is likely to be instrumental to the treatment of lung diseases characterized by aberrant tissue remodeling, such as pulmonary fibrosis, where no effective drug is available and lung transplantation is the only treatment option. Exposure to crystalline silica may cause lung tissue reactions leading to both acute and chronic pulmonary disease manifestations ranging from nodular to massive forms of pulmonary fibrosis [5,6]. As silica induced tissue reactions can be reproduced in the mouse, where inhalation of silica causes lung inflammation and fibrosis [7], this study was designed to assess the ability of human embryonic stem cells (HUES-3) to differentiate into functioning alveolar ATII Cells and to engraft into the lungs of mice with silica-induced pulmonary fibrosis in order to repair lung damage, mitigate pulmonary fibrosis and reduce mortality.

METHODS

Cell culture

HUES-3 cells (46,XY) were propagated in embryonic stem cell medium (unESCM), according to the manufacturer’s protocol [8]. For in vitro differentiation, cells were cultured in suspension in the embryoid body medium (HUES-3 medium and Activin A). Embryoid bodies (EBs) were cultured in adhesion in KO-DMEM 20% FBS for 10 days, and after in Small Airway Growth Medium (SAGM; Clonetics) for 3, 5, and 8 days. Cells cultured without SAGM were used as controls (Ctrl) [3]. This study on human embryonic stem cells was approved by the Bioethical Committee of the Tor Vergata University Hospital.

Immunostaining
Cells were fixed in 4% paraformaldehyde and stained with antibodies against human pro-
surfactant protein SP-C (1:2000; Chemicon) [3], human aquaporin5 AQP5 protein (1:50; 
Calbiochem) [9], human tight junction ZO-1 FITC conjugated (1:50; Zymed Laboratories) [10], 
human Cystic Fibrosis Transmembrane Conductance Regulator CFTR (1:500; Abcam) [11] and 
human Nuclear antigen HuNu (1:100; Chemicon) [12].

**Real-Time RT-PCR**
Quantitative Real-Time RT-PCR was performed according to the manufacturer’s protocol for 
surfactant protein B (SP-B) and C (SP-C117/134) [3]; for stem cell markers: Octamer-binding 
transcription factor 4 (isoform OCT-4A and OCT-4B), NANOG, CXC chemokine Receptor 4 
(CXCR4), Collagen Type 1 alpha 1 (Col1-α1) and the inflammatory markers Tumor Necrosis 
Factor (TNF-alpha), Monocyte Inflammatory Protein 2 (MIP-2), Interleukin 6 (IL-6), and 
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene, as it is 
stable in differentiating ES cell cultures. Oligonucleotide sequences are shown in table 1.

**Flow Cytometry Analysis**
HUES-3-ATII Cells after 3, 5 and 8 days of culture with SAGM, were analyzed after 
disaggregation by flow cytometry shortly after collection. Antibody directed specifically against 
human stage-specific embryonic antigen-4 (SSEA-4) (2µg/ml; Chemicon), Thyroid transcription 
factor 1 (TTF1) (1:30; GeneTex, Inc), CD105 (Endoglin) (1:20; BD Pharmigen), CD34 (1:20; BD 
Pharmigen) were used according to manufacturer’s protocols.

Cytofluorimetric analyses were performed using a FACSCalibur flow cytometer (Becton-
Dickinson,) and resulting data analyzed using CellQuest software.

**Fluorescence measurements of apical chloride efflux**
Chloride efflux was measured using the Cl⁻ sensitive dye MQAE in cell grown at confluence [13]. 
Fluorescence was recorded with a. CFTR-dependent chloride secretion was calculated as the 
difference of fluorescence stimulated by forskolin (FSK) +IBMX with or without the specific CFTR 
inhibitor, CFTRinh-172 [14].

**Measurement of transepithelial electrical resistance (TER)**
TER values were measured by Millicell-ERS Volt Ohm Meter (Millipore). All measurements were 
done in triplicate, and TER values were normalized for the area of the filter and obtained after 
background subtraction.
**Animals and experimental groups**

A total of 160 female Nude mice, 8-9 weeks old (Charles River) were used and splitted in a group of 50 mice treated with saline solution and a group of 110 mice treated with silica. Experiments were conducted in three consecutive times on different animals. Animal body weight was recorded daily. Ten more mice were used for teratoma formation.

Animal care was carried out in accordance with European Economic Community Council Directive 86/109, OJL 358, Dec 1 1987 and with NIH Guide for the Care and Use of Laboratory Animals.

**Silica treatment**

Crystalline Silica oxide powder (99.9% Alfa Aesar) was administered intranasally at a concentration of 5mg/mL (50 µl/mice/day) for 15 days. Control animals were sham treated with saline (50 µl/mice/day) for 15 days.

**HUES-3-ATIIICs transplantation into Nude Mice**

Silica-exposed mice were further subdivided in two groups, each comprised of 50 animals: *silica+cells*, receiving 2.5x10^6 cells by intratracheal instillation after 15 days of silica treatment and *silica*, receiving silica treatment for 15 days, but no cells. HUES-3-ATII Cells were injected after three days of SAGM treatment. A group of 10 silica-damaged animals inoculated intratracheally with 2.5x10^6 human fibroblasts.

A group of 10 animals were injected subcutaneously with 2.5x10^6 HUES-3-ATII Cells and sacrificed after 3 months to evaluate teratogenicity.

**Real-Time RT-PCR Detection of Murine/Human Chimerism**

Human DNA was detected in the lung mice using probes for the hTERT locus (Quantifiler kit; Applied Biosystem) for the quantification of human nuclear DNA [15] and/or probes for human Cytochrome B [16]. Data analysis was performed using the SDS software.

**Histological examination**

Both silica-exposed and silica-exposed cell-injected mice were sacrificed for histological evaluation five, 10 and 15 days after cell administration. Serial sections from formalin-fixed multiple slices of constant thickness [17] were stained with Hematoxylin&Eosin and Masson's trichrome to evaluate the extent of lung inflammation and the severity of fibrosis according to the Ashcroft method [18], respectively.

**Transmission Electron microscopy (TEM)**
Cells were post-fixed in 1% OsO4 for 2h and dehydrated through an alcohol series and propylene oxide before embedding in EPON 812. Thin sections were stained with toluidine blue and ultrathin sections were cut by an 8800 ultramicrotome III (LKB), counterstained with uranyl acetate and lead citrate and analyzed by a Philips Morgagni electron microscope.

**Protein analysis**

Proteins were extracted from mouse lung using RIPA buffer according to the manufacturer’s instructions, and analyzed by Bio-Rad dye-binding protein assay (Bio-Rad).

Transforming growth factor beta (TGF-β) (1:200; Santa Cruz Biotechnology), Collagen Type1 (Col1α1) (1:10000; AbCam), and β-actin (1:5000; Santa Cruz Biotechnology) was used as reported [19,20]. ECL Advanced detection kit (Amersham Pharmacia Biotech Ltd) was used, according to the manufacturer’s instructions.

**Oxygen saturation**

Blood arterial oxygen saturation was recorded using a small rodent oximeter sensor mounted on the thigh of each experimental animal (MouseOX; STARR Life Sciences, Oakmont, PA). Three groups of animals were recorded, each containing 5 mice. Data were collected for a minimum of 10 seconds, without any error code and the measurement was repeated six times within a 3-minute period.

**Statistical Analysis**

Data were expressed as mean values ±SEM for multiple independent experiments, as indicated by "n". For statistical analysis the unpaired Student’s t-test was used. A p value lower than 0.05 was regarded as significant.

**RESULTS**

**In vitro differentiation of HUES-3 into ATII Cells**

When HUES-3 cell colonies were cultured on a murine fibroblast layer (fig. 1A), and were detached from the feeder layer, differentiated in suspension to form fairly regular embryoid bodies (EBs) (fig. 1B), which could in turn form an endodermal layer when treated with Activin A. EBs, when transferred into adherent culture conditions, displayed an epithelial-like phenotype (fig. 1C) and differentiated into a alveolar type II epithelial cells after 8 days in SAGM medium culture (fig. 1D) [3]. This was shown after 8 days of culture in SAGM medium by immunocytochemistry with
confocal microscopy using anti-SP-C (an ATII Cell phenotype marker) and anti-AQP5 (an ATI Cell phenotype marker) antibodies, as differentiated HUES-3 cells expressed SP-C (fig. 2A) but not AQP5 (fig. 2E). In addition, they expressed the ZO-1 protein, a tight junction marker characteristic of epithelial structures (fig. 2C).

As expected, real time RT-PCR of stem cell markers revealed a decrease of OCT-4 (isoforms A and B), and NANOG from day 5 of differentiation (fig. 3A). Also flow cytometric analysis of the SSEA-4 protein reported a gradual reduction from day 3 to day 8 of differentiation (from 14% ± 0.03 to 6.7% ± 0.01) (fig. 3B). Differentiated cells revealed a cell population positive to TTF-1 cells (94.8% ± 0.02; fig.3E), a marker selectively expressed in type II alveolar epithelial cells, and only 2% of cells positive to CD105 (a mesenchimal cell marker; fig.3C) and CD34 (an endothelial cell marker; fig.3D), respectively. Furthermore, the expression of transcripts from SP-C isoforms and of SP-B progressively increased during differentiation in SAGM medium, respect to the control cells (Ctrl), not receiving SAGM medium (fig. 3F). Also chemokine receptor CXCR4 [21] expression markedly increased as soon as the cells started their differentiation treatment with SAGM medium (fig. 3G).

Similarly, two typical epithelial markers, the ATII Cells intracytoplasmic lamellar bodies in which SP-C is stored (fig. 4A; asterisk, and B at higher magnification), and the epithelial intercellular junctions, (fig. 4C; arrows), could be shown by transmission electron microscopy in HUES-3-ATII Cells after 5 days of SAGM culture.

**CFTR expression and activity in HUES-3-ATII Cells**

Confocal analysis of CFTR expression in HUES-3 cells grown with or without adding SAGM in cell medium showed heightened expression of the CFTR protein [22] on the apical membrane (ap) of HUES-3 cells after 5 days in SAGM medium (fig. 5C), compared to low and random expression on untreated cells. A detail of CFTR immunohistochemical analysis is reported in figure 3A where a tridimensional view of a single cell was cropped from a confocal Z-stack acquisition. Consistent with the above observations, HUES-3-ATII Cells, grown to confluence on permeable supports, displayed significantly increased trans-epithelial electrical resistance (TER), indicating differentiation toward the ATII Cells phenotype (fig. 5B). Similarly, HUES-3-ATII Cells after 3 and 5 days in SAGM culture showed significantly increased CFTR-dependent chloride efflux (black trace) when compared to control untreated (grey trace) cells (fig. 5D), as measured by fluorescence analysis with the chloride-sensitive dye MQAE after chloride/nitrate substitution in the apical perfusion medium (fig. 5E) [11].
Microscopic and molecular analyses of lung parenchymal damage before and after HUES-3-ATII Cells injection

After crystalline silica instillation, inflammatory infiltrates comprised of foamy cells, lymphocytes and neutrophils became progressively more widespread (fig. 6A,B; silica columns), leading to progressive multifocal fibrosing alveolitis with a nodular peribronchiolar organization [23]. Treatment with HUES-3-ATII Cells significantly reduced inflammation and fibrosis in silica-instilled mice after 10 and 15 days from cells injection (fig. 6A, B; sil + cells columns), in comparison to control mice as shown by Ashcroft score analysis (p<0.01) (fig. 6C). The engraftment of human ATII Cells into the mice lungs was demonstrated in the lungs of HUES-3-ATII Cells treated mice 4 weeks after cell administration. Expression of the human nuclear antigen (HuNu) (fig. 7A, arrows) and of human SP-C was shown to colocalize in ATII Cells (fig. 7B, arrows) by immunohistochemistry. Molecular analysis revealed the presence of human DNA in the same tissue samples (fig. 7D). The engraftment of human alveolar cells within murine lungs was confirmed by confocal microscopy on ATII Cells treated mice up to 10 weeks after cell transplantation (fig. 7C). Furthermore, teratogenicity of transplanted HUES-3-ATII Cells was ruled out by histological examination one year after HUES-3-ATII Cells injection in Nude mice (data not shown).

Assessment of recovery of lung damage and fibrosis in HUES-3-ATII Cells transplanted mice

Silica-induced lung damage and fibrosis were also evaluated by monitoring the expression of TGF-β protein during silica exposure (Silica 5d, 10d and 20d) as well as 20 days after HUES-3-ATII Cells administration (Sil+cells, 20d; fig. 8A). As reported by densitometric analysis, an increase of TGF-β, indicative of a fibrosis onset, was detected during silica exposure. On the other hand, administration of HUES-3-ATII Cells (Sil+cells 20d) resulted in a marked reduction of TGF-β expression in vivo compared to mice not receiving cells (Silica 20d; fig. 8A). No reduction was evidenced when fibroblast cells were injected instead of HUES-3-ATII Cells. A similar pattern was obtained for Col1-α1, indicative of fibrosis onset. Collagen expression was markedly reduced only when HUES-3-ATII Cells were injected (Sil+ cells 20d) and not when fibroblast cells were used (fig. 8B). In addition, the expression of the pro-inflammatory mediators IL-6, TNF-α and MIP-2 was clearly reduced after HUES-3-ATII Cells injection (silica+cells bars, grey colored) if compared to silica-injured (silica bars, white colored) at each time points (5,10,15 days) (fig. 8D). No reduction was found in silica-injured mice injected with fibroblast cells (silica+fib bars, black-colored). Consistent with pro-inflammatory and profibrotic cytokine expression, the level of the collagen deposition marker Col1-α1, showed a five-fold increase in silica exposed mice (white bars
at 15 days), with a significant reduction after HUES-3-ATII Cells administration (Silica+cells, grey bars). Quantitative values were normalized respect to those obtained in Ctrl mice injected with saline solution (Ctrl, striped bars) (fig. 8D).

Consistent with the observed reduction of inflammation and fibrosis, HUES-3-ATII Cells treatment also resulted in a marked improvement of lung function. Importantly, HUES-3-ATII Cells-treated mice (silica+cells; fig. 8C) showed, in comparison with silica-damaged mice (silica; fig. 8C) a marked improvement of blood oxygenation, as their saturation values remained in the range of silica-unexposed Ctrl mice (Ctrl; fig. 8C).

Finally, HUES-3-ATII Cells administration to silica-exposed mice resulted in an evident improved survival (fig. 8E). While weight loss progressed from the first day of damage in silica instilled mice not receiving HUES-3-ATII Cells (fig. 8E), a striking 95% recovery of pre-treatment weight was observed in mice 20 days after HUES-3-ATII Cells treatment (fig. 8E). Mice not receiving cells, or receiving fibroblasts, progressively died with an observed 100% mortality.

Confirming the specific effect of human embryonic stem cell treatment in ameliorating silica induced lung injury and fibrosis, silica injured mice, sham treated with human fibroblasts, showed no change in either weight loss progression or death rates (data not shown).

DISCUSSION

Pulmonary fibrotic disorders, either idiopathic, or associated with collagen vascular diseases or caused by environmental exposures may lead to lung structure distortion and dysfunction with ensuing respiratory failure. For the most severe fibrotic disorder, idiopathic pulmonary fibrosis/usual interstitial pneumonia, no medical treatment has been shown so far to be capable of arresting or reversing lung damage and fibrosis [24].

Bleomycin induced lung inflammation and fibrosis in mice have widely used as the most reliably model of pulmonary fibrosis, IPF in particular [25], and a number of studies have been carried out to attempt at restoring lung structure and function using stem cell transplant in bleomycin-treated mice. These studies showed that stem cell treatment can reduce inflammation and fibrosis while restoring alveolar structures in bleomycin induced pulmonary fibrosis [26-29]. Other strategies have been explored for the repair and regeneration of the injured lung. Intratracheal instillation of Palifermin (DN23-KGF) a recombinant keratinocyte growth factor has been shown to be capable of stimulating emphysematous lung regeneration in experimental animals, likely through enhanced production of cytokines such as VEGF and TGF-β [30]. Interestingly, transplantation of lentivirus-transduced bone marrow hemopoietic stem cells expressing KGF has been shown to
attenuate lung damage in a bleomycin murine model of pulmonary fibrosis [31], possibly via complex paracrine interaction. Another epithelial cell growth factor, the hepatocyte growth factor (HGF), has also been successfully used to treat bleomycin induced pulmonary fibrosis by toracothomic lung administration of an HGF plasmid (pCikhHGF) using electroporation [32]. Other studies have been carried out using stem cell transplantation (either mesenchymal cells or cells derived from fetal membrane of human term placenta) in animal models in which lung fibrosis has been induced by bleomycin and also by silica [26,33-36]. These cells were found to express alveolar epithelium markers after injection into mice and to exert a significant anti-inflammatory and anti-fibrotic effects in the bleomycin as well as in the silica models of fibrosis. However, only one group [27] reported evidence that long term engrafted cells could functionally differentiate into type II pneumocytes, by demonstrating secretion of SP-D expression following stimulation with corticosteroids. Bone marrow-derived cells, once instilled into silica injured mice, allowed a transient beneficial effect until two months after cell administration. Similar paracrine effects were described using bone marrow-derived mononuclear cells, for which no engraftment was reported despite an evident improvement of lung function [35].

With regard to human embryonic stem cells (hESCs), their ability of differentiating into functional ATII Cells has been shown in two studies [3,28]. Samadikuchaksaraei and Coll. reported in vitro differentiation of hESCs into a mixed population of cells containing a small percentage of ATII Cells, not suitable for transplantation because of the risk of producing teratomas after transplantation [3]. Wang and Coll. generated a stable transfected hESC line homogeneously expressing an ATII Cell phenotype that, once transplanted in a mouse model of bleomycin-induced lung injury, was able to functionally repair the acutely injured alveolar epithelium, without causing teratoma formation [28].

To date, no study using hESCs has been described in silica-induced lung injury in an in vivo mouse model.

The present study is, to our knowledge, the first one reporting the use of in vitro differentiated embryonic stem cells for the treatment of silica-induced pulmonary fibrosis in the mouse model, as it shows that HUES-3 cells were capable of differentiating into alveolar type II cells, graft into the mouse lung, repair lung injury and ameliorate disease course, rescuing mice from death.

Firstly, after differentiation in culture, HUES-3 cells expressed the alveolar epithelial cell markers SP-C, ZO-1, CXCR4 and active CFTR, while down-regulating the expression of the stem cell markers OCT-4, NANOG and SSEA-4, thus demonstrating that a type II alveolar cell phenotype could be obtained [37,38], through in vitro differentiation of HUES-3 cells.
Secondly, as it has been shown that the turnover of the adult mammalian alveolar epithelium is of about 3 to 4 weeks [39], the observation that transplanted HUES-3-ATII Cells could be identified in the lungs of silica injured mice up to 10 weeks after cell administration suggests the engraftment of HUES-3-ATII Cells after intrapulmonary injection.

Thirdly, the efficacy of intrapulmonary injection of HUES-3-ATII Cells in the treatment of lung inflammation and fibrosis in silica-injured mice, an experimental condition known to causes 100% mortality within three weeks [40], was proven both in pathological and clinical terms. It is well known that the expression of inflammatory cytokines, in particular of IL-6, is significantly elevated in silica-induced experimental lung injury, where it may act in synergy with TNF-α and TGF-β to perpetuate inflammation and fibrosis [41-47] resulting in increased type I collagen deposition. In our study, the reduction of inflammatory changes at the tissue level was associated with a marked reduction of the expression of IL-6, TNF-α and MIP-2 evidenced only in mice receiving HUES-3-ATII Cells, but not in those receiving equivalent volume of saline solution. Accordingly, pulmonary function was preserved and survival of silica-exposed mice was significantly improved after HUES-3-ATII Cells treatment.

The mechanism by which HUES-3-ATII Cells repair lung damage and reverse fibrosis remains to be elucidated. It has been shown that type-II alveolar cells function as epithelial cell precursors in the alveoli during fetal development and early life and that they are capable of regaining their migratory and proliferative potential after lung injury and alveolar damage, such in hyperoxic oxidative stress [48]. Alveolar type II cells are known to play a key role in the response to the exposure to toxic silica. Type II cell hyperplasia is seen in animal models as an early event following type I cell epithelial damage [39] and it is a common finding in the lower respiratory tract of individuals with pulmonary silicosis [49]. In this context, experimental studies demonstrating that type II alveolar epithelial cell disruption may lead to exaggerated fibrotic lung responses [50], are consistent with the hypothesis that failure of type II alveolar cells to express their regenerative potential may play a role in the relentless progression of idiopathic pulmonary fibrosis [51].

The data presented here strongly support the possibility that differentiated stem cells such as the HUES-3-ATII Cells, as they express CXCR4, may be capable of homing to the alveoli [52] where they can significantly reduce lung damage, type 1 collagen deposition and fibrotic tissue formation, represent a potentially applicable tool for innovative anti-fibrotic, cell-mediated treatment [28,53].

The results obtained in this silica-induced lung fibrosis model does bring further support to the potential usefulness of this differentiated stem cell therapy, as the administration of HUES-3-ATII Cells resulted both effective, reducing mortality of silica-injured mice, and safe, being devoid of teratogenic risks.
In conclusion, our results show that hESCs transplantation effectively reduce silica-induced lung fibrosis in mice after intratracheal injection, indicating the cell therapy approach as a potentially useful strategy for the treatment of fibrotic lung diseases.
REFERENCES


41 Davis GS. The pathogenesis of silicosis: state of the art. Chest 1986; 89:166S–169S.


### TABLE 1  Oligonucleotides used in Real-Time PCR assays

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Figure 5

**A**

**B**

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**C**

**D**

**E**

Chloride influx CFTR-dependent

\[ \Delta F_{E,\text{min}} \]

3 days

5 days
Figure 6

A

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B

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C

![Graph showing Ashcroft score over time](image)
Figure 7