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Isolation and characterization of chicken lung mesenchymal stromal cells and their susceptibility to avian influenza virus

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ABSTRACT

In this study, we isolated and characterized mesenchymal stromal cells (MSCs) from the lungs of 1- to 2-week-old chickens. Microscopically, the cultured cells showed fibroblast-like morphology. Phenotypically these cells expressed CD44, CD90, CD105 and the transcription factor PouV, which has been shown to be critical for stem cell self-renewal and pluripotency. The multipotency of chicken MSCs was demonstrated by their ability to undergo adipogenic and osteogenic differentiation. Like chicken bone marrow MSCs and mammalian MSCs, chicken lung MSCs had immunoregulatory activity and profoundly suppressed the proliferative capacity of T cells in response to a mitogenic stimulus. Next, we examined the susceptibility of these cells to H1N1 and H9N5 avian influenza (AI) viruses. The lung MSCs were shown to express known influenza virus alpha-2,3 and alpha-2,6 sialic acid receptors and to support replication of both the avian H1N1 and avian H9N5 influenza strains. Viral infection of MSCs resulted in cell lysis and cytokine and chemokine production. Further characterization of lung MSCs in chicken and other mammalian species may help in understanding the pathogenesis of infectious and non-infectious lung diseases and the mechanisms of lung injury repair.

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1. Introduction

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells which have the ability for self-renewal and a multilineage differentiation capacity for the repair of damaged cells and tissues. Friedenstein et al. [1] initially isolated bone marrow (BM)-derived MSCs by their adherence to plastic dishes. MSCs have been isolated from different tissues including adipose tissue, skeletal muscle, synovium, spleen, thymus, blood, lung, fetal blood and amniotic fluid [2]. Several studies using a variety of animal models have shown that MSCs may have a role in the repair and regeneration of injured lung, infarcted myocardium, and damaged bone, tendon and cartilage [3–6]. Following infusion of in vitro expanded MSCs in the lung, MSCs have been detected as type I and type II alveolar epithelial cells, endothelial cells, fibroblasts, and bronchial epithelial cells [7]. Although not as tested yet, logically lung MSCs would be more efficient in treating lung diseases as compared to BM-MSCs.

Another important characteristic of MSCs is their immunoregulatory function; they inhibit inflammation and immunological responses both in vitro and in vivo [2]. MSCs exert pleiotropic immunosuppressive effect by inhibiting lymphocyte proliferation, downregulating cytokine production by both antigen-presenting cells and T cells by producing several soluble factors such as transforming growth factor-β, hepatocyte growth factor, indoleamine 2,3-dioxygenase, nitric oxide and prostaglandin E2 [8–12]. In addition, MSCs-induced immunosuppression can also be produced by regulatory T cells (T regs) generated upon interaction with T cells [13].

Influenza viruses belong to the family Orthomyxoviridae and cause highly contagious respiratory infection in humans and animals. These viruses cause seasonal epidemics and infrequent pandemics in humans. Avian influenza (AI) viruses caused three human pandemics during the last century. The 1918 pandemic was caused by H1N1 virus that was derived from an AI virus adapted in a mammal host [14,15]. The 1957 Asian H2N2 and 1968 Hong Kong H3N2 pandemics were caused by avian and human influenza virus reassortants [16]. The currently circulating pandemic H1N1 influenza virus is a triple reassortant influenza virus whose genome contains segments from avian, swine and human influenza viruses [17].

Excessive virus replication and enhanced production of proinflammatory cytokines and chemokines (cytokine storm) have been detected in humans, chickens and mice infected with...
highly pathogenic AI (HPAI) H5N1 and with recreated 1918 pandemic influenza virus [18–22]. The mechanisms responsible for deterioration of lung function and the loss of capacity for lung repair after AI virus infection are not well understood. In addition to the HPAI H5N1 viruses, human infections with other subtypes of AI virus including H9N2, H5N1, H7N7, H7N3, and H10N7 have also been reported [23]. Thus, AI viruses pose a potential pandemic threat to human population. Understanding the pathogenesis of AI virus is very important to devise strategies for the control and prevention of AI in avian species and subsequent pandemics in humans.

In the present study we have isolated and characterized MSCs from chicken lungs. The chicken represents an important animal model for studies of evolution, embryology, immunology, oncology, cell biology, virology, and gene regulation, and was the first farm animal with a completely sequenced genome. Rous sarcoma virus, the first tumor virus, and the oncogene (src) were identified in the chicken thus, the chicken serves as a model organism for the study of viruses and cancer. The chicken immune system provided the first indications of the distinctions between T- and B-cells, with the B-cell nomenclature based on the avian bursa of Fabricius [24]. MSCs derived from lungs could be a very valuable source for use in cell therapy applications on the lung. In addition, we have also provided evidence that chicken lung MSCs are susceptible to infection with AI virus and infected MSCs produce proinflammatory cytokines/chemokines, and therefore, may play an important role in the pathogenesis of influenza infection. We also discuss the potential implications of these observations for infection with highly pathogenic influenza viruses.

2. Materials and methods

2.1. Isolation and digestion of lung tissue

Lungs were obtained from 1- to 2-week-old specific pathogen free chickens (HyVac, Ames, IA). Lung tissue was washed extensively with phosphate buffered saline (PBS) and minced with scissors in a 50 ml conical tube. The lung tissue was digested by incubating it in sterile filtered 0.5 mg/ml collagenase type IV (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) for 30 min at 37°C. The tissue debris was removed by filtering single cell suspensions through sterile 70 μm filters (BD, Falcon, USA) and mononuclear cells were obtained by density gradient centrifugation over Ficoll-Hypaque (density gradient 1.079). Cells were resuspended at a concentration of 1 × 10^6 cells/ml in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and 10 mM HEPES. After 3–4 days, non-adherent cells were removed and fresh medium was added. Subsequently, the medium was refreshed every 3–4 days and cells trypsinized at confluency. The cells between passages 2 and 5 were used.

2.2. MSCs differentiation

Lung MSCs were examined for differentiation into adipocytes and osteocytes [10,25]. Adipogenic differentiation was induced by culturing confluent MSCs cultures in DMEM supplemented with 10% FBS, 1 μM dexamethasone, 10 μg/ml insulin, and 0.2 mM indomethacin for 21 days. The medium was replaced every 3–4 days. Adipogenic potential was assessed by Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in a medium consisting of 100 nM dexamethasone, 10 mM β-glycerophosphate (Sigma), 0.05 mM l-ascorbic acid-2-phosphate (Sigma), and 10% FBS in DMEM. The medium was refreshed every 3–4 days and cells maintained in these conditions for 21 days. Osteogenesis as indicated by calcium deposition was evaluated by Von Kossa staining.

2.3. RNA extraction and RT-PCR

RT-PCR was used to examine the gene expression of PouV, CD44, CD90 and CD105 [10]. RNA was isolated using TRIzol total RNA isolation reagent (Life Technologies, Rockville, MD). To detect gene expression RT-PCR was used with the following primers: PouV primers 5′-AAAT GTGTTGACGCGAGAT-3′ and 5′-TTGTTGGAGTTGCGATGTA-3′; CD44 primers 5′-GGTCTACATGTGCGAGCTGA-3′ and 5′-AAAGCTAGGTTGGAGGA-3′; CD90 primers 5′-ACGGATGACACGATGAA-3′ and 5′-ATGAGAGGCTCCAAAGGT-3′; and for the β-actin 5′-CATCACCATTGGCAATGAGAG-3′ and 5′-GCAACGAGGTA CGTGAATC-3′. After PCR, 10 μl of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining.

2.4. T-cell proliferation assay

Splenocytes were prepared by centrifugation over Ficoll-Hypaque gradients [10,26]. The cells were suspended in triplicate in 100 μl RPMI 1640 medium supplemented with 5% FBS, 2 mM l-glutamine (Invitrogen), 0.1 mg/ml streptomycin, and 100 U/ml penicillin G (Invitrogen) in 96-well plate in the presence or absence of MSCs. The cultures were stimulated or unstimulated with mitogen (ConA or Phorbol 12-myristate 13-acetate (PMA) and ionomycin) and incubated for 48 h at 37°C. The cells were pulsed with [3H] thymidine during the last 5 h of incubation. Lymphoproliferation was measured as counts per minute by a Matrix 9600 beta counter (Packard Instrument Co., Meriden, CT). All experiments were performed in triplicate. ConA purchased from Sigma (St. Louis, MO) was used at 5 μg/ml. PMA and ionomycin were from Sigma and were used at concentrations of 50 ng/ml and 1 μg/ml, respectively.

2.5. Detection of sialic acid receptors on MSCs

Sialic acid receptors on MSCs were detected by immunohistochemical staining [27]. MSCs grown on coverslips were fixed with 3.7% formaldehyde in PBS for 30 min. After blocking the cells with 2% bovine serum albumin (BSA) in PBS for 30 min, the cells were incubated with biotinylated Maackia amurensis lectin II (MAA) (20 μg/ml) or Sambucus nigra agglutinin (SNA) (Vector Laboratories, Burlingame, CA) for 1 h, followed by the addition of ABC reagent (Vector Laboratories, Burlingame, CA) and developed with 2,3’ diaminobenzidine tetrahydrochloride.

2.6. Viruses and infection of MSCs

Two AI virus strains (GWT/0H/91 H1N1 and G/MD/95 H9N5; referred to as H1N1 and H9N5 in the text) were propagated in 10-day-old embryonated chicken eggs. MSCs were infected with H1N1 and H9N5 virus at a multiplicity of infection (MOI) of 1. Following washing with PBS, the cells were incubated with viruses diluted in DMEM supplemented with 0.02 μg/ml TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin and 0.2% bovine serum albumin. After adsorption for 1 h, the cells were washed and fresh medium was added to the cells. At indicated intervals, the supernatants containing viruses were titrated in MDCK cells and viral proteins in infected cells were detected by immunofluorescence assay (IFA) using fluorescent antibodies specific for nucleoprotein of influenza A virus (Chemicon, CA) [28].

2.7. Detection of apoptosis

Cells were stained with acridine orange to determine nuclear morphology and with ethidium bromide to distinguish cell...
viability. Both dyes were used at final concentrations of 1 μg/ml for each [28,29]. The MSCs were analyzed for evidence of DNA fragmentation using in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay. TUNEL assay was performed with an ApopTag Plus Peroxidase apoptosis detection Kit (Chemicon, CA) according to the manufacturer’s instructions.

2.8. Quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was used to examine the transcription of mRNA encoding IL-8 and IL-6. Expression of 28S rRNA was used as internal housekeeping gene to normalize data collection [26,30]. Fold increase of IL-8 and IL-6 transcript expression over uninfected controls was calculated with the $2^{ΔΔCT}$ method [26,31].

3. Results

3.1. Isolation and differentiation of lung MSCs

MSCs isolated from chicken lungs showed a fibroblast-like morphology. The initial adherent spindle-shaped cells appeared as individual cells on the third day. In 4–8 days, the culture became more confluent and reached 65–70% confluency within 2 weeks. Cell population appeared more homogeneous consisting mainly of fibroblastic cells (Fig. 1A).

First passage cells from the lungs were readily differentiated into adipocytes and osteocytes upon culturing in appropriate induction media. MSCs treated for 21 days with adipogenic medium showed differentiation into adipocytes. In adipogenic cultures, intracellular accumulation of lipid droplets were stained with Oil Red O, indicating adipogenesis (Fig. 1B). In osteogenic cultures mineralized nodule-like structures showing calcium deposition were observed, which stained black with Von Kossa staining (Fig. 1C).

Chicken lung MSCs expressed CD44, CD90 (Thy-1) and CD105 (Endoglin) genes indicating that these cells had mesenchymal lineage (Fig. 1D). Additionally, transcription factor PouV was also detected in lung MSCs. PouV is a chicken homologue of mammalian Oct4 and like Oct4 has been shown to regulate the pluripotency and self-renewal of chicken embryonic stem cells [32].

3.2. Suppression of T-cell proliferation by MSCs

Next we assessed the ability of MSCs to inhibit T-cell proliferation in response to mitogens. The addition of MSCs to splenocytes stimulated with ConA or PMA/ionomycin inhibited their proliferation (Fig. 2). We have shown previously that MSCs isolated from BM inhibit T-cell proliferation; therefore, these cells were included as positive controls in this study [10]. This indicates that, like mammalian MSCs, chicken BM and lung-derived MSCs also exert immunosuppressive effects on T cells.

3.3. Detection of α-2,3 and α-2,6-linked sialic acid receptors on MSCs

Influenza virus infects cells through binding to cell surface sialic acid receptors. To examine the susceptibility of MSCs to influenza virus, we first evaluated by immunohistochemistry these cells for the presence of sialic acid receptors. The α-2,3 and α-2,6-linked sialic acid receptors were detected on the surface of MSCs by lectin staining. A majority of the lung MSCs expressed both α-2,3 and α-2,6 linked sialic acid receptors, indicating that viruses of both avian and mammalian lineages might replicate in these cells (Fig. 3A).

![Fig. 1. Characterization of MSCs isolated from chicken lung. (A) Morphology of MSCs derived from lung. (B) Oil Red O staining for lipid-filled vesicles (red) after 21 days of adipogenic differentiation of MSCs derived from lung. (C) von Kossa staining for calcified mineral nodules (black) after 21 days of osteogenic differentiation of MSCs derived from lung. (D) Phenotype of chicken lung MSCs. Reverse transcription-polymerase chain reaction was performed using total RNA extracted from lung MSCs with specific primers. The center column shows the results with total RNA from lung MSCs. Total RNA prepared from normal chicken spleen cells was used as a positive control for CD44, CD90 and CD105 expression and for PouV, total RNA isolated from bone marrow MSCs was used as positive control (left column). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](image-url)
3.4. MSCs as targets for AI virus replication and apoptosis

To assess the susceptibility of MSCs to AI virus, MSCs were infected with H1N1 or H9N5 virus. Cells were incubated with viruses at an MOI of 1 for 1 h, washed extensively, and cultured for various time periods, after which infectious virus in culture supernatant was measured by titration in MDCK cells. As shown in Fig. 3B, infectious viruses were observed in culture supernatants 24 h post-infection (hpi) and viral titers further increased at 36 hpi.

To assess the relationship between virus replication and cell death, we examined cell viability in MSCs exposed and non-exposed to AI virus at 24 and 36 h after virus exposure. The replication of these viruses in lung MSCs was accompanied by cell lysis. Both viruses induced cell lysis, however, H1N1 virus was more cytolytic than H9N5 virus (Fig. 3C). The production of cytopathic effects required live, infectious virus because cell death was not observed when heat-inactivated virus was used (data not shown).

Additionally we detected AI viral proteins in in vitro infected lung MSCs. MSCs monolayers were exposed to H1NI or H9N5 for 24 h, fixed and incubated with fluorescent antibodies specific for nucleoprotein of influenza A virus. Viral nucleoprotein was detectable in virus-infected cells but not in mock-infected cells by immunofluorescence staining, indicating AI virus infection of MSCs in vitro (Fig. 3D).

3.5. AIV-induced expression of IL-6 and IL-8 in MSCs

To determine the proinflammatory cytokine and chemokine production following AI virus infection, MSCs were infected with H1N1 or H9N5. After 6 and 12 hpi total RNA was isolated. The expression levels of IL-6 and IL-8 were analyzed by qRT-PCR. The levels of IL-6 and IL-8 were substantially greater in H1N1 and H9N5 infected MSCs than in uninfected controls (Fig. 5). However, no significant differences (P > 0.05) were observed in the expression of both IL-6 and IL-8 between the two viruses tested (Fig. 5).
representative of two separate experiments.

uninfected controls was calculated with the 2^(-ΔΔCT) method. RNA isolated from uninfected control cultures was used as the reference sample (calibrator) at each time point. The relative change in mRNA concentration (ΔCT) for each virus-infected sample was then determined from the difference between the calibrator CT and the CT of each virus-infected sample. Fold increase of IL-6 and IL-8 transcript expression over uninfected controls was calculated with the 2^ΔΔCT method. The data are representative of two separate experiments.

4. Discussion

We have isolated and characterized for the first time MSCs from the lungs of hatched chickens. These cells exhibited fibroblastic morphology, expressed a pluripotency marker and differentiated into adipocytes and osteocytes suggesting that they are multipotent. As noted previously with MSCs isolated from chicken BM and MSCs of other species, lung MSCs also inhibited the T-cell proliferation in vitro in response to T-cell mitogens. Most importantly, lung MSCs were susceptible to infection with AI virus and infected cells had upregulated expression of proinflammatory cytokine and chemokine genes.

Similar to our study, lung multipotent MSCs have been identified in mouse and humans [33–35]. Like human lung MSCs, chicken lung MSCs also differentiated into adipocytes and osteocytes [33,34]. Additionally, we detected the expression of PouV in MSCs isolated from chicken lung. PouV is a chicken homologue of mammalian Oct4 [32]. In mammals, Oct4 is expressed in pluripotent stem cells such as embryonic stem cells and induced pluripotent stem cells, and MSCs where it regulates self-renewal and pluripotency [36]. Expression of PouV in chicken BM and lung MSCs may, therefore, also be associated with pluripotency and self-renewal of these cells.

MSCs are known to inhibit inflammation and immunological responses both in vitro and in vivo [2]. They exert pleiotropic immunosuppressive effects by inhibiting lymphocyte proliferation, and by downregulating cytokine production by both antigen-presenting cells and T cells by production of several soluble factors. As shown previously with chicken BM-MSCs and human lung MSCs, chicken lung MSCs also had immunoregulatory properties such that they inhibited the proliferation of T cells [10,37].

Previously, we and other groups have shown the susceptibility of BM-MSCs to variety of viruses [38–40]. However, this is the first report showing the infection of lung MSCs with influenza virus. Additionally, we detected the expression of IL-6 and IL-8 in AI virus-infected lung MSCs. Production of IL-6 and IL-8 was also detected in human BM-MSCs stimulated with poly(I:C) [41,42]. IL-6 regulates inflammatory responses and hematopoiesis [43], and its overproduction relates to the pathology of autoimmune diseases and tissue remodeling. Conversely, IL-8 is present in the inflammatory milieu during tissue repair [44]. The production of IL-6 and IL-8 thus suggests that infection of lung MSCs with AI virus might affect their differentiation potential and potentiate the inflammatory response as observed in avian and mammalian species following infection with influenza virus [18,21,22]. The mechanism responsible for this hyperimmune activation is not known. Based on our findings and other reports on mechanisms of immune regulation by MSCs, we speculate that infection and lysis of MSCs by AI virus might result in local immune dysregulation allowing infiltration and proliferation of inflammatory cells in the infected lung with excessive production of proinflammatory cytokines. Secondly, as MSCs have been shown to induce the production of Tregs [13], lysis and/or apoptosis of MSCs by AI virus and excessive proinflammatory cytokine production by immune cells and MSCs may mediate the depletion of Tregs, again contributing to the dysregulated immune response in the lung. Future studies will be directed to examine the proportions of Tregs in influenza virus-infected MSCs-T-cell co-cultures and the mediators responsible for inducing alterations in the proportions of Tregs.

In our preliminary studies we observed the infection of Oct4 expressing cells at the bronchoalveolar junction of the lung of mice infected with HPAI H5N1. Oct4, the octamer-binding transcription factor 4, is an important binding transcription factor that regulates the development and differentiation and pluripotency of MSCs [45]. Due to non-availability of cross-reactive Oct4 antibody, we were not able to directly assess infection of lung MSCs with AI virus in virus-infected chicken lung sections. Interestingly, earlier studies [46,47] showed the susceptibility of Oct4 expressing putative lung stem cells to SARS-corona virus infection in mouse lungs. Additional experiments are needed in chickens and mammalian species to confirm the infection of lung MSCs in vivo and further delineate their roles in regulating the local immune response following infection with Al/Influenza virus.

In conclusion, we have isolated MSCs from chicken lung and have presented evidence that the lung MSCs serve as targets for AI virus infection. Our findings suggest that in addition to respiratory epithelial cells, AI virus infects lung MSCs resulting in cell lysis and the production of proinflammatory cytokines. The death of the lung MSCs and the resulting local immune dysregulation may contribute to lung damage, loss of repair capacity, and respiratory failure. Further characterization of these MSCs in chicken and other...
mammalian species may help in understanding the pathogenesis of infectious and non-infectious lung diseases and the mechanisms of lung injury repair.

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