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Research paper

Impact of low oxygen on the secretome of human adipose-derived stromal/stem cell primary cultures

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ABSTRACT

Tissue fibrosis can lead to organ dysfunction, patient morbidity, and mortality. Adipose-derived Stromal/stem Cells (ASCs) represent a potential therapeutic. Immediately following grafting, ASCs would reside in a lower O_2 environment. ASC secretome was examined under 5% O_2 ("low O_2") and 21% O_2 ("ambient O_2") culture conditions. ASCs from five female donors were cultured in low or ambient O_2 conditions for 3 days and pooled conditioned medium was compared by two-dimensional liquid chromatography and tandem mass spectrometry (2D-LC–MS/MS). Of 71 proteins identified, five proteins involved in extracellular matrix (ECM) remodeling exhibited ≥2-fold decrease under low O_2 culture and were confirmed by Western immunoblot and qRT-PCR: fibronectin 1, TGF-β1-induced protein (βig-h3), osteonectin, and collagens type 1α_1 and α_2. ELISAs performed using 10 donors also confirmed significant decreases during low O_2 culture in 4–6 ASC donors. For low abundant proteins, a 36 cytokine/chemokine array was performed. Fifteen cytokines/chemokines including Type 2 cytokines IL-13, MCP-1, and CD40 ligand were detected in ambient O_2 ASC medium. IL-6 was detected in low O_2 but not ambient O_2 ASC medium. These findings demonstrate that low O_2 ASC exposure resulted in reduced ECM protein and Type 2 cytokine secretions that are significant with regard to inflammation in fibrosis.

1. Introduction

Tissue fibrosis is a complication and/or end point in a number of diseases that can lead to significant organ dysfunction and patient morbidity and mortality [1–6]. Fibrosis is characterized by excessive extracellular matrix (ECM) deposition which interferes with normal tissue architecture and function. The current therapeutic regimens, including anti-inflammatory/immunosuppressive agents such as colchicine, interferon-gamma (IFN-γ), corticosteroids and cyclophosphamide [7,8], are limited, nonspecific, and largely ineffective in a number of diseases [6,9–11].

Recent studies demonstrated the efficacy of adipose tissue-derived stromal/stem cells (ASCs) in animal models and human therapies of multiple fibrotic diseases [12–17]. Additional studies have demonstrated that both bone-marrow mesenchymal stem cell (BMSC) and ASC therapy ameliorates damage induced by ischemia and reduced both acute and chronic tissue fibrosis in varying models of disease progression, including pulmonary, renal, and hepatic fibroses [12–15,17,18]. Although the mechanisms of action in these models are not fully understood, growing evidence suggests that even small changes in ASC secretions of soluble ECM remodeling proteins and inflammatory mediators can result in improved tissue structure and function [12,14,19,20].

In a severe model of renal ablation, Semedo, et al. [21] demonstrated that the administration of BMSCs attenuated chronic low O_2 and oxidative stress and reduced the production of inflammatory cytokines, such as IL-1β and TNF-α, that are present during chronic kidney disease. Donizetti-Oliveira et al. [22] demonstrated that

Abbreviations: 2-D, two-dimensional; ASCs, adipose tissue-derived stromal/stem cells; CD40 L, CD40 ligand; COL1α1, collagen type 1α1; COL1α2, collagen type α2; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; FDR, false discovery rate; HIF-1α, hypoxia-inducible factor-1α; IFN-γ, interferon-gamma; IL-1β, interleukin-1β; IL-4,6,10,13, interleukins-4, -6, -10, and -13; LC–MS/MS, liquid chromatography and tandem mass spectrometry; MCP-1, monocyte chemotactic protein-1; PAI-1, plasminogen activator inhibitor-type 1; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; ROC curve, receiver operating characteristic curve; serpins, serine protease inhibitors; SPARC/osteonectin, secreted protein acidic, cysteine-rich; SVF, stromal vascular fraction; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue; βig-h3, TGF-β1-induced protein.

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ASCs can halt renal fibrosis at different times of disease progression by the modulation of inflammatory mediators such as IL-6, TNF-α, IL-4, IL-10, and ECM proteins including collagen type I and vimentin under low O_2. Several other studies have described the beneficial effects of ASC administration for the accelerated healing processes of skin wounds [23], cardiac wall regeneration [24], repair after myocardial infarction [25], and improvement of cellular function following stroke [26]. Taken together, these data suggest that in a low O_2 environment, ASCs may contribute to ECM remodeling by altering inflammatory mediators of the immune response. However only limited studies of the secretome of ASCs cultured under low O_2 conditions have been reported [27]. The purpose of this study was to compare the secretomes of ASCs cultured under ambient and low O_2 conditions and to assess donor effect on the secretomes.

2. Materials and methods

2.1. Isolation, collection, and culture of human ASCs

The procedures used are modifications of published methods [28]. Human adipose tissue was acquired from elective plastic surgical procedures, with the patient's informed consent as approved by the Pennington Biomedical Research Center Institution Review Board (Protocol PBRC #23040). ASCs were obtained from the subcutaneous abdominal adipose tissue region of female patients of European descent ($n = 5$) 28–61 years of age with a mean ± SD of 41.3 ± 8.9 years. The patients displayed a mean body mass index (kg/m^2) (±SD) of 25.8 ± 4.6 (summarized in Table 1). Primary cultures were prepared as described in Dubois et al., 2008. Briefly, liposuction tissues were transported to Pennington Biomedical in saline solution within 2 h post surgery. The tissue was washed at least three times with 2 volumes of Phosphate Buffered Saline (PBS) to remove blood. The tissue was then digested with one volume of PBS supplemented with 0.1% collagenase type I (Worthington Biochemicals, Brunswich NJ), 1% bovine serum albumin, and 2 mM $CaCl_2$ for 60 min at 37 °C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction (SVF) by centrifugation (300× g) for 5 min at room temperature. The supernatant containing mature adipocytes was aspirated and discarded and the remaining pellet was identified as the SVF. The SVF cells were suspended and plated immediately in T175 flasks in Stromal Medium (DMEM/F-12 Ham's, 10% FBS [Hyclone, Logan, UT, http://www.hyclone.com], 100 U penicillin/100 g streptomycin/0.25 g fungizone) at a density of 0.156 ml of tissue digest/cm^2 of surface area for expansion and culture. This initial passage of the primary cell culture was referred to as passage 0 (P0). For cryopreservation, the ASCs were resuspended in cryopreservation medium (10% dimethylsulfoxide, 10% Dulbecco's modified Eagle's medium [DMEM]/F-12 Ham's, 80% fetal bovine serum [FBS]), frozen at −80 °C in an ethanol-jacketed closed container, and subsequently stored in liquid nitrogen prior to thawing for individual assays. The cells were then replated and expanded in cell factories in the Stromal Medium (DMEM/F-12 Ham's, 10% FBS [Hyclone, Logan, UT, http://www.hyclone.com], 100 U penicillin/100 g streptomycin/0.25 g fungizone) in the Adult Stem Cell Core Facility at the Louisiana Cancer Research Consortium.

Table 1
ASCs donors. ASCs were isolated from 11 different donors between the ages of 28 and 61. All ASC cultures were derived from subcutaneous abdominal aspirates of female Caucasian patients. All ASCs were used at passage 2.

Donor	Age	BMI
1	47	19.99
2	40	21.18
3	33	21.63
4	42	21.97
5	48	23.65
6	44	24.98
7	37	30.65
8	33	29.4
9	61	26.49
10	28	31.58
11	41	32.75
Mean ± SD	41.3 ± 8.9	25.8 ± 4.6

2.2. Low O_2 cell culture

Five 150 cm^2 plates of ASC were maintained in Stromal Medium and the medium was replaced every 3rd day. *In vitro* low O_2 experiments were performed with a Single Set point O_2 & CO_2 controller inserted in a standard incubator (Biospherix C-Chamber Low O_2 Chamber; Bio-Spherix, Lacona, NY, http://www.biospherix.com) that continuously infused a calibrated gas mixture (95% N_2, 5% CO_2). Experiments were performed at O_2 concentrations of 21% (ambient air) or 5% (low O_2). After three days, the media were removed from both the low O_2 and ambient O_2 plates and replaced with Serum Free Medium (DMEM/F-12, 1% antibiotic/antimycotic). After 1 h, the medium was removed and discarded. Fresh Serum Free Medium was added and each dish was incubated overnight (16 h) after which time all low O_2 and ambient O_2 cell conditioned medium for each donor lot was collected, pooled, adjusted to a final concentration of 2 mM PMSF by addition of a 100× stock solution, snap frozen in liquid nitrogen, and stored at −80 °C for future analysis. The complete culture medium was replaced with basal medium for 18 h prior to collecting the conditioned medium, so as to omit interference from nutritional proteins and false reporting of large abundant proteins that are present within fetal bovine serum of complete culture medium [29]. The process was repeated independently for statistic analysis (a total of 4 samples: two low O_2 samples and two ambient O_2 samples of pooled conditioned medium from each donor). The cells from the low O_2 and ambient O_2 conditions were harvested for total protein and RNA using the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen Inc. Valencia, CA, http://www.qiagen.com) according to the manufacturer's instructions. Protein and RNA samples were stored at −80 °C for future analysis.

2.3. Cell proliferation assay

Cell growth was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay. 100 μg MTT (50 μL of 2 mg/ml) solution was added to each well and the plates incubated for 4 h at 37 °C. MTT formazan crystals were then resolubilized by adding 150 μl of 100% dimethylsulfoxide (DMSO) to each well. Plates were then agitated on a plate shaker for 5 min and then analyzed for the spectrophotometric absorbance at 540 nm using a scanning multiwell spectrophotometer (Fluostar Optima, BMG Labtech; Durham, NC). Three independent sets of experiments were performed for each treatment. The mean value and standard deviation for each treatment were determined and a one-way ANOVA was performed followed by Bonferroni's post-test.

2.4. Cell viability assay

After culturing ASCs in Stromal Medium under low O_2 and ambient O_2 conditions for 3 days, effects on viability were observed by trypan blue dye exclusion using the countess automated cell counter (Life Technologies, Grand Island, NY). Data obtained from

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