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A small molecule approach to engineering vascularized tissue

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ABSTRACT

The repertoire of growth factors determines the biological engagement of human mesenchymal stromal cells (hMSCs) in processes such as immunomodulation and tissue repair. Hypoxia is a strong modulator of the secretome and well known stimuli to increase the secretion of pro-angiogenic molecules. In this manuscript, we employed a high throughput screening assay on an hMSCs cell line in order to identify small molecules that mimic hypoxia. Importantly, we show that the effect of these small molecules was cell type/species dependent, but we identified phenanthroline as a robust hit in several cell types. We show that phenanthroline induces high expression of hypoxia-target genes in hMSCs when compared with desferoxamine (DFO) (a known hypoxia mimic) and hypoxia incubator (2% O_2). Interestingly, our microarray and proteomics analysis show that only phenanthroline induced high expression and secretion of another angiogenic cytokine, interleukin-8, suggesting that the mechanism of phenanthroline-induced hypoxia is distinct from DFO and hypoxia and involves the activation of other signaling pathways. We showed that phenanthroline alone was sufficient to induce blood vessel formation in a Matrigel plug assay *in vivo* paving the way to its application in ischeamic-related diseases.

1. Introduction

Human mesenchymal stromal cells (hMSCs) secrete a broad panel of factors that have trophic effects (e.g., anti-apoptotic, pro-angiogenic and anti-scarring) and immunomodulatory effects [1]. Several clinical trials have been completed or are currently on the way investigating the use of hMSCs for the treatment of, amongst others, autoimmune diseases [2–5], myocardial infarcts [reviewed in Ref. [6]], solid organ/graft transplantations [7,8] and ischemic wounds [9]. Debate exists on the mechanisms underlying the effects of infused MSCs. Although differentiation of MSCs into cells of the target tissue has been shown [10–12], low engraftment percentages, the short window in which effects are observed and the fact that conditioned medium alone also has effects, support a trophic effect [13–16].

Several studies have demonstrated that culture of hMSCs under hypoxic conditions, which closely resemble oxygen concentrations in natural bone marrow (1–7%) [17], results in enhanced secretion of pro-angiogenic trophic factors – of which most importantly vascular endothelial growth factor (VEGF) – but also improves survival, engraftment and differentiation of implanted cells [18–22]. Cellular responses to hypoxia are mainly regulated by hypoxia-inducible factors (HIFs)-heterodimers consisting of an α and β subunit [23,24]. The HIF-1α isoform is ubiquitously expressed in all cell types and, upon hydroxylation by prolyl hydroxylase domain proteins (PHDs), it is rapidly degraded by the proteasome [25]. PHD's inactivation leads to accumulation and stabilization of HIF-1α and subsequent translocation into the nucleus where it binds to hypoxia responsive elements (HREs) and initiates transcriptional activation of HIF-target genes [23,24]. Subsets of genes containing an HRE have been identified, including angiogenic, endothelial and metabolism-related genes.

Culture under hypoxia is expensive and, most importantly, restricted to an *in vitro* scenario. Alternatively, small molecules can be used that, similarly to hypoxia, activate HIF-1-target genes but are cheap and easy to use. In addition, controlled release of these molecules in an *in vivo* set-up is possible in a spatially and temporally controlled manner. Spatial control using antibodies

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specific for certain tissues or cell types coupled to liposomes was pioneered in the early 80's and has been broadly used ever since [26–28]. More recently, using phage display libraries, it was possible to identify small peptides possessing high-affinity for certain proteins and therefore use these instead of proteins [29]. Temporal control of drug release can be achieved using controlled hydrolysis of polymers, changes in polymer matrices leading to differences in diffusion rate, changes in cellular environment such as pH, temperature or enzymatic activity or even via external stimuli such as an electric field or ultrasounds and containing encapsulated drugs and therefore, tuning the release profile [29–35].

In the past, our lab has shown that by using a small molecule we can modulate the composition of trophic factors secreted by hMSCs and control their differentiation into the osteogenic lineage [36]. Nevertheless, the effects observed with hMSCs could not be extrapolated to different cell types or species, clearly demonstrating that the biological function attributed to some molecules in one species or cell type differ depending on the cellular context. For example, we have shown that the same molecule that induced osteogenesis in hMSCs (dibutyryl-cAMP) led to a distinct phenotype in MSCs from another species, i.e. adipogenesis in rat MSCs [37]. Based on this observation and on the fact that previous screens for activators of the HIF pathway have been performed mostly with cancer cells lines [21,38,39], we decided to develop a new screening strategy based on a more clinically relevant cell type. We generated a cell line using human immortalized MSCs containing an HRE-GFP reporter (HRE-GFP iMSCs) and used high-throughput screening (HTS) to identify compounds that activate the HIF-1 pathway.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in compliance with Dutch government guidelines and approved by the Institutional Committee for Animal Welfare of the Leiden University Medical Center (LUMC).

2.1.1. Cell culture

Bone marrow aspirates (5–15 mL) were obtained from patients with written informed consent and isolated as previously described [40]. Human mesenchymal stromal cells (hMSCs) were expanded in proliferation medium consisting of alpha minimal essential medium (α-MEM; Gibco, Carlsbad, CA), 10% fetal bovine serum (Lonza, Verviers, Belgium), 0.2 mM ascorbic acid (Sigma Aldrich, St. Louis, MO), 2 mM L-Glutamine (Gibco), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen, Carlsbad, CA) and 1 ng/mL of basic fibroblast growth factor (bFGF, Instruchemie, Delfzijl, The Netherlands). Basic medium consisting of proliferation medium without bFGF was used during the experiments. Human umbilical vein endothelial cells (HUVECs) were commercially obtained from Lonza and cultured in Endothelial Growth Medium-2 (EGM-2) with addition of the microvascular bullet kit (MV, all from Clonetics, Lonza), containing hEGF, hydrocortisone, gentamicin, 5% FBS, VEGF, hFGF-B, R3-IGF-1 and ascorbic acid. Cells were kept at 37 °C and 5% CO_2. Medium was refreshed three times per week and cells were trypsinised when a confluency of 70–80% was reached. MG-63 cells were cultured in basic medium and ACL cells were cultured in DMEM High Glucose (PAA) containing 10% FBS and 0.2 mM ascorbic acid.

2.1.2. LOPAC screen

To perform the screen, 4000 HRE-GFP iMSCs (see Supplementary information on preparation of the cell line) were seeded in black 96-well plates (BD Biosciences) and allowed to attach overnight. The next day, the LOPAC® library, a library consisting of 1280 pharmacologically active compounds, was added. As negative and positive controls, basic medium and 100 μM desferoxamine (DFO, Sigma Aldrich) in basic medium were added respectively. After one day of incubation with the test compounds, an Alamar Blue assay was performed to assess cell numbers/metabolic activity [41] and GFP intensity was measured as readout for the HRE activity. Briefly, medium containing 10% (v/v) Alamar Blue solution (Biosource, Camarillo, CA) was added and incubated at 37 °C for 4 h. Then, fluorescence was measured at 590 nm on a Victor plate reader (Perkin Elmer, Wellesley, MA). Upon removal of the Alamar Blue solution, cells were washed with phosphate buffered saline (PBS; Life Technologies) and GFP intensity was measured at 520 nm on a Victor plate reader.

2.1.3. Hit validation

Obtained hits were further tested. Briefly, four different concentrations of each hit were tested for their capacity to induce HRE activity. In addition, twelve compounds previously identified in an HRE screen using a cancer cell line [38] were tested along with our hits. These experiments were performed as described above for the primary screen.

Based on the HRE-GFP activity (high signal and low cytotoxicity) we selected a concentration for subsequent experiments with primary human mesenchymal stromal cells (hMSCs).

2.1.4. Gene expression analysis

hMSCs (or when mentioned MG-63 and ACL cells) were seeded in triplicate in 6-well plates at 5000 cells/cm^2 and allowed to attach for 10–15 h in basic medium. Upon reaching near confluency, cells were treated as described above. After 2 days of treatment and 2 subsequent days of incubation with fresh medium, cells were lysed immediately with TRIzol. RNA was isolated using a Bioke RNA II nucleospin RNA isolation kit (Machery Nagel) and RNA concentrations were measured using an ND100 spectrophotometer (Nanodrop technologies, USA). cDNA was synthesized from 100 ng of RNA, using iScript (BioRad) according to the manufacturer's protocol. For qualitative PCR, a master mix, containing distilled water, forward primer, reverse primer (Sigma Genosys), BSA, and SYBR green I mix (all from Invitrogen) was prepared. Real-time qPCR was performed in a Light-Cycler (Roche). Light-Cycler data was analyzed using the fit points method of Light-Cycler software. The baseline was set at the lower log-linear part above baseline noise and the crossing temperature (C_t value) was determined. C_t values were normalized to the 18S housekeeping gene and ΔC_t ($C_{t,\ control} - C_{t,\ sample}$) was used to calculate the upregulation in gene expression [42]. Primer sequences are listed in Table 1.

2.1.5. Protein expression analysis

hMSCs were seeded at 5000 cells/cm^2 in T25 flasks. Upon reaching near-confluence, medium was changed for basic medium, basic medium with 150 μM DFO or 200 μM Phenanthroline (Phen, Sigma Aldrich) or cells were added to a hypoxia chamber (2% O_2). After 2 days, cells were lysed with 250 μL RIPA buffer with addition of protease/phosphatase inhibitors (Roche). Total protein concentrations were determined using a BCA kit (Pierce) and 10 μg of total protein was used to determine concentrations of VEGF, IL-8, basic fibroblast growth factor (bFGF), growth-colony stimulating factor (G-CSF) and epidermal growth factor (EGF) using a Luminex assay (Invitrogen) according to the manufacturer's protocol. Briefly, cells and standards were incubated with fluorescent beads, followed by incubation with a biotinylated detection antibody. After incubation with streptavidin-R-Phycoerythrin and washing, both the fluorescence of the coupled beads and the R-phycoerythrin were measured using a Luminex® FlexMapTM (Luminex).

2.1.6. Whole genome expression analysis

hMSCs were seeded in T25 flasks at 5000 cells/cm^2 and allowed to attach overnight in proliferation medium. The next day, medium was added with the following conditions; basic medium, basic medium supplemented with 150 μM DFO or basic medium supplemented with 200 μM Phen. After 48 h, RNA was isolated as described above. From 500 ng of RNA, cRNA was synthesized using the Illumina TotalPrep RNA amplification Kit (Ambion), according to the manufacturer's protocol and the quality of RNA and cRNA was verified on a Bioanalyzer 2100 (Agilent). Microarrays were performed using Illumina HT-12 v4 expression Beadchips, according to the manufacturer's protocol.

2.1.7. Proliferation

hMSCs and HUVECs were seeded in triplicate in 6-well plates at 3000 cells/cm^2 and allowed to attach overnight in culture medium as described above (see cell culture). Then, cells were washed and conditioned medium (CM, see Supplementary information for details on preparation) was added. After 3 days, proliferation of hMSCs was determined by measuring the metabolic activity using a 10% (v/v) Alamar Blue (Invitrogen) and for HUVECs, nuclei were stained with DAPI (Sigma Aldrich) and counted.

2.1.8. Scratch wound healing assay

HUVECs were seeded in triplicate in 6-well plates at 10,000 cells/cm^2 and allowed to attach for 10–15 h in culture medium as described above (see cell culture). When the cells reached near confluency, a wound was created by scratching the surface with a pipette tip, and the medium was changed to different types of conditioned medium. After 12 and 20 h pictures were taken to examine migration of cells into the wound.

Table 1
Primer sequences.

Gene	Forward primer	Reverse primer
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
VEGF-A	Commercially obtained from SA biosciences	