Angiogenic factors produced by fetal lung mesenchymal stromal cells

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Abstract

Background
The multi-potency and immune suppressive properties of mesenchymal stromal cells (MSC) have made them a promising candidate for cell-based therapies. For adult MSC the observed and potential therapeutic effects are largely mediated by paracrine factors, capable of inducing angiogenesis. Data on the potential pro-angiogenic effect of fetal MSC is limited and contradictory.

Objectives
We investigated whether human fetal lung MSC (FL-MSC) produce factors that can activate endothelial cells towards a pro-angiogenic state.

Methods/Results
Gene expression of FL-MSC was studied using a dedicated angiogenesis PCR Array. We identified increased transcript levels of CXCL1, FGF-2, HGF, IL-6, MDK, u-PA, PGE₂, TGF-β1, and VEGF in FL-MSC, which were verified on protein level in conditioned medium (FL-MSC-CM) by ELISA. Protein concentrations of FGF-2 and IL-8 were relatively low in FL-MSC-CM (< 1 ng/ml), whereas high levels of CXCL1, HGF, IL-6, TGF-β1, VEGF, and SDF-1α were measured (>1 ng/ml). Proliferation, an important component of angiogenesis, was measured by ³H-thymidine incorporation. Incubation of hMVEC with FL-MSC-CM resulted in a significant increase in proliferation, which was decreased (50-60%) by addition of neutralizing antibodies for VEGF and HGF. Incubation of endothelial cells with FL-MSC-CM supplemented with TNF-α significantly stimulated tube formation in a 3D fibrin matrix. Addition of neutralizing antibodies for VEGF and HGF completely abrogated the induction of tube formation by FL-MSC-CM.

Conclusions
Human FL-MSC can stimulate endothelial proliferation and capillary-like tube formation by endothelial cells, two major components of the angiogenesis process. HGF and VEGF secreted by FL-MSC are the major contributors to this stimulation.
Introduction

Mesenchymal stromal cells (MSC) are multipotent progenitor cells with a high degree of plasticity, capable of giving rise to a number of unique, differentiated mesenchymal and non-mesenchymal cell types\textsuperscript{1-10}. MSC are easily isolated from bone marrow and expanded\textsuperscript{11}, although they most likely exist in virtually all organs\textsuperscript{12-14}. MSC have become a promising candidate for cell-based therapy in various clinical settings, because of their multi-potency and immune tolerant features\textsuperscript{15, 16}. In most acute clinical conditions, for which the use of MSC might be considered, allogeneic MSC would be the only option, as the limited time frame for clonal expansion would not allow for the time-costly in vitro proliferation of autologous MSC\textsuperscript{17}. However, use of MSC for cell therapy is feasible, as they display multi-site engraftment when systematically injected in vivo\textsuperscript{18} and exhibit an intrinsic ability to home to injured sites\textsuperscript{19-23}. MSC have shown promising results in preclinical and clinical studies for a number of conditions, such as bone or cartilage defects\textsuperscript{24-26}, cardiovascular disorders\textsuperscript{27-32}, central nervous system or spinal cord injury\textsuperscript{21, 33-39}, auto-immune\textsuperscript{40}, and lung diseases (for reviewed see\textsuperscript{41-44}). Their immunosuppressive effect has been utilized in the treatment of graft-versus-host disease in haematological stem cell transplantation, and in preclinical studies on solid organ transplantation and autoimmune diseases. Furthermore, MSC also have been encountered in the perivascular niche\textsuperscript{11, 45-55}, which suggests a potential pericyte role on the blood vessel and its endothelium, in particular in conditions of tissue repair and neovascularization\textsuperscript{56, 57}.

The therapeutic effect of MSC was originally thought to be attributed to their ability to participate physically in a supportive microenvironment or by differentiation into different cell types needed to repair or replace damaged tissue. Indeed an MSC cell line has been used to stabilize new vascular structures derived from endothelial progenitor cells\textsuperscript{58}. However, in other conditions, such as after acute myocardial infarction, it is controversial whether MSC infusion resulted in engraftment, differentiation and functional integration into the myocardium\textsuperscript{59, 60}. Recent reports show that the observed and potential therapeutic effects of MSC are (partly) mediated by paracrine factors secreted by these cells\textsuperscript{37, 61-70}. The factors released by MSC could well play a role in inhibiting fibrosis (scar formation), apoptosis, and in enhancing angiogenesis\textsuperscript{69, 71, 72}. These for tissue (re)generation very important roles are summarized as “the trophic effect” of MSC\textsuperscript{73, 74}.

A positive role of paracrine factors from MSC on neovascularization has been described by a number of groups. It was shown that adult stromal cells from adipose tissue or bone marrow of mouse as well as human origin can produce factors, such as VEGF and HGF, that induce pro-angiogenic and anti-apoptotic effects on endothelial cells (EC) in vitro\textsuperscript{20, 61-63, 66, 75-80}. 
Literature on the angiogenesis-promoting properties and potential therapeutic use of fetal MSC is limited and contradictory. Iop et al.\textsuperscript{81} compared bone marrow-derived MSC to fetal tissue (amnionic fluid)-derived MSC in a rat model of heart cryo-injury. Fetal MSC displayed a higher proliferative rate than BM-MSC and differences were found in the differentiation of these two types of MSC into endothelial- and cardiomyocyte-like cells. However, in a different study by Ishikane et al.\textsuperscript{82}, no differences were reported between bone marrow- and fetal membrane-derived MSC with respect to growth factor production, immunosuppressive properties and stimulation of blood perfusion and capillary density in a rat model of hind limb ischemia. Roelen et al.\textsuperscript{83} provided compelling evidence that human fetal MSC and adult MSC are in fact different. Fetal MSC possessed a stronger immunosuppressive capacity and produced significantly more IL-10 and VEGF, as compared to adult MSC. We have previously shown that human fetal lung MSC (FL-MSC) promote engraftment of CD34\textsuperscript{+} cells in NOD/SCID mice\textsuperscript{84}.

The aim of the present study was to investigate whether human MSC of fetal lung origin (FL-MSC) secrete products that can activate endothelial cells toward a pro-angiogenic state, and to identify factors secreted by FL-MSC that mediate this pro-angiogenic effect. To that end, we characterized gene expression and secretion of pro-angiogenic factors secreted by FL-MSC and investigated by using neutralizing antibodies their contribution to the MSC-induced proliferation and capillary-like tube formation by human microvascular endothelial cells.

Materials and methods

Isolation and culture of human fetal lung mesenchymal stromal cells (FL-MSC)

FL-MSC were isolated and cultured as described before\textsuperscript{84}. Briefly, after informed consent fetal lung tissue (gestational age: 15-22 weeks) was obtained according to a protocol, approved by the Medical Ethical Review Board of the Leiden University Medical Center. Cells were cultured in M199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 200 μg/ml penicillin-streptomycin (Invitrogen), 20μg/ml Endothelial Cell Growth Factor (ECGF) (Roche Diagnostics GmbH, Mannheim, Germany), and heparin (8 U/ml), which will be further indicated as M199c. Flasks were kept in a humidified atmosphere at 37°C (5 % vol/vol CO\textsubscript{2}). Medium was refreshed every other day, and cells were replated every two weeks.
**FL-MSC-conditioned medium (FL-MSC-CM)**
Conditioned medium of FL-MSC was harvested during a period of 5-7 days in the second week of culture when cells reached confluence. FL-MSC-CM was collected from cells in passage 4-7.

**Isolation and culturing of microvascular endothelial cells**
Human foreskin microvascular endothelial cells (hMVEC) were isolated from neonatal foreskin dermis as described previously. Briefly, endothelial cells were isolated and purified by fluorescence-activated cell sorting (FACS) using anti-CD31-PE (BD Biosciences, San Diego, CA, USA) (purity 97.8 ± 1.7%). After purification, hMVEC were cultured in M199 medium, supplemented with 10% human serum (PAA Laboratories, Pasching, Austria) 10% new born calf serum (Invitrogen), supplemented with 150 μg/ml crude endothelial growth factor from bovine brain. hMVEC were used from passage 8 to 10 for experiments. In each experimental set-up, three different hMVEC donors were used to exclude donor dependency of the results.

**In vitro angiogenesis assay after stimulation with FL-MSC-CM**
In vitro angiogenesis assays were performed in human fibrin matrices as described previously. In short, highly confluent hMVEC (0.7x10^5 cells/cm²) were seeded in a 1.25:1 split ratio on fibrin matrices and cultured in M199 medium supplemented with 10% HS, 10% NBCS and penicillin-streptomycin. After 4 hours, the medium was replaced with 90% FL-MSC-CM/10% NBCS to which TNF-α (Sigma-Aldrich, St. Louis, MO, USA) was added. This was repeated every other day. As a positive control for tube formation, 25 ng/ml VEGF (PeproTech, Rocky Hill, NJ, USA) was used in combination with 30 ng/ml TNF-α. Invading cells and tubular structures of hMVEC in the 3D fibrin matrix were analyzed by phase-contrast microscopy. The length and amount of the tube-like structures was analyzed with Optimas image analysis software version 6.0 (MediaCybernetics, Bethesda, MD, USA). Four fixed microscopic fields per well were analyzed and used to quantify the total pixel length of the tube-like structures expressed as arbitrary units (a.u.).

**Angiogenesis PCR array**
A RT² Profiler PCR Array (SABiosciences, Frederick, MD, USA) was used to detect expression of genes related to angiogenesis. The expression of 84 genes, five housekeeping genes (HKG: β₂-microglobulin, β-actin, hypoxanthine-guanine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde 3-phosphate dehydrogenase) and three RNA quality controls was profiled simultaneously in a 96 wells plate.
RNA was isolated from cultured FL-MSC as well as from hMVEC cultured in control medium or FL-MSC-CM for 48 hours, both supplemented with 10% human serum. RNAzol B (Campro Scientific GmbH, Berlin, Germany) was used to isolate RNA according to the manufacturers’ instructions. Concentration and quality of RNA was determined by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Subsequently, cDNA was made and the PCR Array performed according to the manufacturers’ instructions. The normalized gene expression of the genes was calculated as: \( \Delta Ct \) sample = (Ct sample GENE) – (Ct of HKG (average of 5 HKG)).

**Enzyme-Linked ImmunoSorbent Assay (ELISA)**

The concentration of FGF-2, CXCL1 (GROα), HGF, PGE2, TGF-β1, VEGF was determined using Quantikine sandwich ELISA (R&D Systems, Minneapolis, MN, USA). IL-6 and IL-8 were determined by PeliKine ELISA (Sanquin Reagents, Amsterdam, Netherlands), and Midkine by MDK ELISA (Peprotech). Urokinase-type plasminogen activator (u-PA) was measured as described before. All factors were determined in different batches of FL-MSC-CM and culture media.

**hMVEC proliferation assay**

Proliferation of hMVEC after stimulation with FL-MSC-CM was determined by \(^3\)H-thymidine incorporation. hMVEC (6000 cells/cm\(^2\)) were seeded in 96 wells plates. Twenty-four hours after plating, cells werestimulated for 48 hours with FL-MSC-CM or with FL-MSC-CM supplemented with blocking antibodies against VEGF (100 μg/mL, Avastin (Hoffmann-La Roche, Basel, Switzerland)), FGF-2 (10 μg/ml), HGF (1 μg/ml), CXCL1 (10 μg/ml) or Midkine (10 μg/ml) (all from R&D Systems) Anti-SDF-1α (40ng/ml), anti-IL-6 (5μg/ml) and anti-IL-8 (5ug/ml) were a kind gift from Dr. L.A. Aarden of Sanquin Research, Amsterdam. In blocking experiments, the neutralizing antibody (or combination of antibodies) was added to conditioned medium before it was used for stimulation. To test whether the proliferation was indeed blocked by the antibody, controls were included of purified protein FGF-2 (10 ng/ml), HGF (30 ng/ml), and VEGF (10 ng/ml) with or without the addition of its specific neutralizing antibodies. Twenty-four hours after stimulation, tracer amounts of \(^3\)H-thymidine were added (1 μCi/well) and the cells were incubated for an additional 18 hours. Afterwards, the cells were washed with PBS, 100% methanol, and 5% trichloroacetic acid. Cells were lysed with 0.3 M NaOH, collected and counted in a liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA).
**Statistical analysis**
Statistical significance was evaluated using the students T-test. Significance was assumed at a p-value < 0.05. Results are expressed as mean ± standard deviation (sd), unless mentioned otherwise.

**Results**

*Enhanced tube formation in fibrin matrix after stimulation with FL-MSC-CM and TNF-α*
We previously demonstrated that hMVEC growing on fibrin matrices are able to form tube-like structures after simultaneous stimulation by TNF-α and VEGF. Here we demonstrate that conditioned medium of FL-MSC (FL-MSC-CM) also stimulated tube formation by hMVEC on a 3D fibrin matrix (Fig. 1A,B), which occurred in a TNF-α concentration dependent way (Fig. 1C). Addition of FL-MSC-CM in combination with 30 ng/ml TNF-α induced a significant tube formation as compared to control medium (153 ± 24 vs. 27 ± 8; p<0.05), while it was comparable with tube formation following stimulation by medium supplemented with 25 ng/ml VEGF and TNF-α (153 ± 24 vs. 133 ± 24).

*FL-MSC express angiogenesis related genes*
mRNA samples of FL-MSC were analyzed by an angiogenesis-specific RT-PCR array for transcripts of soluble proteins involved in angiogenesis. Table 1 summarizes the ΔCt from several genes encoding soluble growth factors. From this list we selected a group of soluble mediators on the basis of two criteria. First, we selected genes encoding factors that had relevant transcript levels (determined by a ΔCt below 9). Additionally, we compared the expression of genes in FL-MSC and in endothelial cells (data not shown), and selected those factors that were higher or equally expressed in FL-MSC (Table 1, genes marked with *). Subsequent studies were performed to elucidate the role of CXCL1, FGF-2, HGF, IL-6, MDK, u-PA, PGE₂, TGF-β1, and VEGF.

*Protein concentration of angiogenic factors/cytokines in FL-MSC-CM*
To confirm the presence of the protein products of the genes mentioned above in FL-MSC-CM, their concentrations were determined in different batches of FL-MSC-CM by ELISA (Table 2). For FGF-2 and IL-8 relatively low levels were found, while high levels were measured for CXCL1, HGF, IL-6, TGF-β1, and VEGF. Since IL-8 is known to be produced in considerable amounts by activated endothelial cells, it is unlikely that the low levels of IL-8 are responsible for the stimulatory effect of FL-MSC-CM. In contrast, despite its low levels, FGF-2 may be of potential interest,
Figure 1. FL-MSC-CM added to hMVEC cultured on a fibrin matrix enhanced tube formation. Conditioned medium of FL-MSC was added to hMVEC cultures on a fibrin gel (B). After an additional 4 days of culture in the presence of TNF-α (30 ng/ml), tube-like structures appeared, as compared to control medium supplemented with TNF-α (B vs A; control). Quantification of tube length showed that tubular structures are significantly induced after stimulation with FL-MSC-CM in combination with high doses of TNF-α (10 and 30ng/ml)(C). Stimulation with FL-MSC-CM was found to be even more sensitive for TNF-α concentrations (1ng/ml, open bars; 10ng/ml, grey bars) than stimulation with VEGF. (* indicates that p < 0.05 between different media)(# indicates that p < 0.05 as compared to the same stimulation medium with 1 ng/ml TNF-α)($ indicates that p < 0.05 as compared to same stimulation medium with 10 ng/ml TNF-α).
given its potency to stimulate endothelial cells. In addition, high levels of SDF-1α were found, which, being not part of the array, was assayed separately because of its known contribution to angiogenesis\textsuperscript{90-92}. In the non-conditioned culture medium most cytokines/angiogenic factors were at undetectable levels. Due to technical reasons, we were not able to measure reliable MDK levels. To rule out a possible contribution of MDK, we included MDK in the experiments with protein specific neutralizing antibodies. Thus, we further evaluated the contributions of CXCL1, HGF, IL-6, MDK, u-PA, SDF-1α, FGF-2 and VEGF in the stimulation of proliferation and endothelial tube formation, two important parameters of angiogenesis, by FL-MSC-CM.

**FL-MSC-CM enhances proliferation of hMVEC**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Δ Ct FL-MSC</th>
<th>Gene</th>
<th>Δ Ct FL-MSC</th>
</tr>
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<tr>
<td>ANGPT1</td>
<td>11.59</td>
<td>IL-8</td>
<td>6.81</td>
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<td>ANGPT2</td>
<td>15.64</td>
<td>LEP</td>
<td>16.44</td>
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<td>ANGPTL3</td>
<td>16.88</td>
<td>MDK</td>
<td>5.91*</td>
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<td>ANGPTL4</td>
<td>14.22</td>
<td>MMP-2</td>
<td>3.06</td>
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<td>CCL2</td>
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<td>PDGFA</td>
<td>13.78</td>
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<td>CXCL1</td>
<td>5.79*</td>
<td>PF4</td>
<td>22.7</td>
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<td>CXCL10</td>
<td>14.01</td>
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<td>13.37</td>
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<tr>
<td>EGF</td>
<td>16.45</td>
<td>u-PA</td>
<td>2.36*</td>
</tr>
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<td>aFGF</td>
<td>10.79</td>
<td>PTGS1</td>
<td>7.06</td>
</tr>
<tr>
<td>FGF-2</td>
<td>6.93*</td>
<td>SERPINE1</td>
<td>9.15</td>
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<tr>
<td>HGF</td>
<td>8.32*</td>
<td>TGF-α</td>
<td>14.24</td>
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<td>IFNA1</td>
<td>14.57</td>
<td>TGF-β1</td>
<td>5.18</td>
</tr>
<tr>
<td>IFNB1</td>
<td>17.59</td>
<td>TGF-β2</td>
<td>10.19</td>
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<tr>
<td>IGF1</td>
<td>15.45</td>
<td>TNF-α</td>
<td>16.17</td>
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<tr>
<td>IL-1β</td>
<td>9.98</td>
<td>VEGF</td>
<td>8.03*</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.84*</td>
<td>VEGF-C</td>
<td>6.50*</td>
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</table>

**Table 1: Δ Ct of genes expressed by FL-MSC**

The gene expression as determined by PCR array, represents soluble proteins that are involved in angiogenesis. Δ Ct values in bold represent relevant transcript levels (<9). Δ Ct values marked with * represent transcript levels comparable or smaller (i.e. higher level of expression) than those of endothelial cells.
Proliferation of hMVEC was used as part of a read-out for the effect of angiogenic factors. Incubation of hMVEC with FL-MSC-CM (Fig. 2A) resulted in a significant increase in $^3$H-thymidine incorporation after 48 hours (14-fold increase as compared to control (M199c)). This proliferation was significantly higher than the proliferation induced by high concentrations of VEGF (10 ng/ml), FGF-2 (10 ng/ml) or HGF (30 ng/ml) (resp 3.8-fold, 6.1-fold and 3.4-fold as compared to control) (Fig. 2A). Such high concentrations of VEGF or FGF-2, however, were never measured in FL-MSC-CM, except for HGF (see table 2). Therefore, a combination of these cytokines and/or other factor(s) produced by FL-MSC must be responsible for the substantial induction of proliferation of hMVEC by FL-MSC-CM.

**VEGF and HGF contribute to FL-MSC-CM enhanced proliferation of hMVEC**

Next, we determined which of the angiogenic factors as measured in FL-MSC-CM (see table 2) might contribute to proliferation by using neutralizing antibodies. The neutralization dose used was able to completely block the proliferation induced by that particular protein as shown for FGF-2, VEGF and HGF in figure 2A. Neutralizing FGF-2, CXCL-1, IL-6, IL-8, MDK or SDF-1α did not decrease the FL-MSC-CM-induced proliferation of hMVEC (Fig. 2B). However, as shown in figure 2B, the antibodies neutralizing VEGF or HGF were able to reduce the FL-MSC-CM-induced proliferation of hMVEC by 26 ± 9.7% (p<0.01) and by 23 ± 9.5% (p<0.01), respectively. Combining these two antibodies resulted in an additive inhibition of proliferation (49 ± 13.9% inhibition, p<0.01). A combination of neutralizing antibodies against VEGF, HGF, CXCL1 and MDK did not further reduce hMVEC proliferation (Fig. 2B).

<table>
<thead>
<tr>
<th>Protein (pg/ml)</th>
<th>FL-MSC-CM</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>&gt;45,000</td>
<td>7</td>
</tr>
<tr>
<td>FGF-2</td>
<td>162 ± 61</td>
<td>7</td>
</tr>
<tr>
<td>HGF</td>
<td>23,283 ± 15,505</td>
<td>7</td>
</tr>
<tr>
<td>IL-6</td>
<td>2,403 ± 229</td>
<td>5</td>
</tr>
<tr>
<td>IL-8</td>
<td>827 ± 234</td>
<td>5</td>
</tr>
<tr>
<td>PGE2</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>6,217 ± 848</td>
<td>8</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>2,040 ± 611</td>
<td>7</td>
</tr>
<tr>
<td>VEGF</td>
<td>1,758 ± 1,388</td>
<td>6</td>
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</table>

Table 2: Concentrations of angiogenic factors/cytokines

Concentrations of different angiogenic factors/cytokines were measured in FL-MSC-CM and unconditioned medium (in pg/ml, mean ± sem) by ELISA as indicated in Materials and Methods. ND: no increment as compared to non-incubated culture medium.
Formation of tubular structures is completely inhibited by neutralizing VEGF and HGF in FL-MSC-CM

To determine which growth factors in FL-MSC-CM were essential for tube formation of hMVEC in the fibrin matrix; neutralizing antibodies were also applied in the in vitro tube formation assay. Since, VEGF and HGF were found to be in part responsible for the induction of hMVEC proliferation by FL-MSC-CM, we initially tested these growth factors for their involvement in FL-MSC-CM-induced endothelial tube formation. FL-MSC-CM in combination with 30 ng/ml TNF-α potently induced tube formation of hMVEC (Fig. 3A). Neutralizing HGF or VEGF in FL-MSC-CM, resulted in a significant inhibition in tube formation of 30% and 90%, respectively (p< 0.05)(Fig. 3B and C). Blocking both VEGF and HGF in FL-MSC-CM inhibited in vitro angiogenesis completely (Fig. 3D).

Figure 2. FL-MSC-CM significantly enhanced proliferation of hMVEC, which is for 50% mediated by VEGF in combination with HGF.

FL-MSC-CM induced a significant increase in proliferation of hMVEC as compared to control medium, but also as compared to potent angiogenic growth factors, such as VEGF and FGF-2. The several antibodies used for blocking angiogenic factors were able to completely block the proliferation induced by that particular protein (A). Neutralizing antibodies against VEGF, FGF-2, HGF, MDK, CXCL-1, IL-6, IL-8, SDF-1α, or combinations of antibodies were added to FL-MSC-CM and tested for their ability to block the effect of FL-MSC-CM. The antibodies neutralizing VEGF or HGF were able to reduce proliferation of hMVEC for 26 ± 9.7% (p < 0.01) and for 23 ± 9.5 % (p < 0.01), respectively. Combining these two antibodies resulted in an additive inhibiting effect on proliferation; 49 ± 13.9% inhibition of proliferation was reached (p < 0.01) (B).
Figure 3. Neutralizing angiogenic factors in FL-MSC-CM reduced tube formation of hMVEC in fibrin matrix.

HGF (B), VEGF (C) or both factors (D) were neutralized in FL-MSC-CM, and this resulted subsequently in an inhibition of 30%, 90% (p < 0.05) or an impressive and complete inhibition (p < 0.05) of tube formation by hMVEC in the fibrin matrix.
Regulation of genes involved in angiogenesis upon stimulation of hMVEC with FL-MSC-CM

The effect of FL-MSC-CM on the expression of several angiogenesis related genes by hMVEC (n=2) and HUVEC (n=3) was also analysed by RT-PCR array. FL-MSC-CM induced in both type of endothelial cells a down regulation of CXCL10 and IGFB1 (both ~10-fold in hMVEC), EFNB2, ITGAV MMP-2 and TGF-β2 (all three ~2-fold in hMVEC). VEGF was found to be slightly (~2-fold) upregulated in both hMVEC and HUVEC. For all other genes no pronounced effects were observed in hMVEC (data not shown).

Discussion

At present no data are available on the synthesis and secretion of angiogenic factors by fetal lung mesenchymal stromal cells (FL-MSC). Here we showed that FL-MSC produced and secreted considerable amounts of VEGF, HGF, CXCL1, SDF-1α, IL-6, TGF-β1 and u-PA, and moderate quantities of FGF-2 and IL-8. VEGF and HGF were completely responsible for FL-MSC-CM-stimulated outgrowth of capillary-like tubes by endothelial cells that were simultaneously stimulated by TNF-α. In addition, these two growth factors explained 50-60% of the the FL-MSC-CM stimulated proliferation of hMVEC, while the other growth factors and chemokines had no effect.

Studies on bone marrow and adipose tissue-derived MSC have identified the expression and secretion of various angiogenic factors. Transcription studies demonstrated that these MSC upregulate VEGF, FGF-2, HGF, SDF-1α, MCP-1, PIGF, IL-6 MMP-9 and TGF-β, while the transcripts of VEGF, FGF-2, FGF-7, PIGF, IL-1, IL-6, TGF-β and TNF-α were up-regulated after exposure of these MSC to hypoxia66, 93. The production and secretion by bone marrow MSC was demonstrated for VEGF66, 82, 94-97, HGF82, 95, 98, FGF-262, PIGF66, MCP-166 and IGF199, IGF2, EGF99, keratinocyte GF99, Ang199, SDF-1α99, MIP1α99, and EPO99. Indeed, a number of paracrine factors produced by adult MSC were capable of inducing endothelial cell proliferation66, 99-102. Our data identified fetal lung MSC-derived VEGF and HGF as major regulators of the invasive process of tube formation and in endothelial proliferation in vitro, as was previously demonstrated for the stimulation of endothelial cell proliferation by adipose tissue-derived MSC conditioned medium101. Neutralization of the activities of CXCL1 (also called GRO1α), FGF-2, IL-6, IL-8 or midkine (MDK) did not affect endothelial proliferation.

TGF-β1 was also produced by FL-MSC. TGF-β is an angiogenic factor with both pro- and anti-angiogenic properties103. It can inhibit angiogenesis by stimulating PAI-1 production104, 105. Because hMVEC were preincubated in heat-inactivated human serum, the cells had been desensitized to additional TGF-β treatment in our experi-
mental conditions. Therefore, it is unlikely that TGF-β caused the additional proliferation by FL-MCS-CM. PlGF transcription was found enhanced in marrow-derived MSC\textsuperscript{62}, but we found only a limited expression in FL-MSC as compared to endothelial cells themselves. Furthermore, PlGF interacts with VEGFR1, while VEGFR2 is responsible for the endothelial proliferation\textsuperscript{106}. IL-1 and TNF-α inhibit endothelial proliferation rather than stimulating it\textsuperscript{107, 108}.

Two other growth factors have been indicated to be transcriptionally enhanced in bone marrow MSC after exposure to hypoxia, namely FGF-7\textsuperscript{76} and NGF\textsuperscript{78}. While FGF-7 is usually considered as an epithelial growth factor, a recent study by Padela et al.\textsuperscript{109} suggested a proliferation stimulating effect of FGF-7 in epithelial and endothelial cells in intact lung alveoli. However, it is uncertain whether this stimulatory effect occurred by a direct effect of FGF-7 on endothelial cells or whether this stimulation was indirectly (e.g. via VEGF induction) as is currently thought. With respect to NGF, Park et al.\textsuperscript{110} showed that NGF stimulated endothelial cell invasion and cord formation on Matrigel in vitro, but had marginal effect on proliferation and migration of these cells. However, other authors reported a direct effect of NGF on endothelial proliferation\textsuperscript{111, 112}. NGF production was not included in our study on FL-MSC, and therefore NGF cannot be excluded yet as a possible factor contributing to FL-MSC-CM-stimulated endothelial proliferation.

During the course of our studies we observed that MSC secrete large amounts of u-PA. Previously it was found that u-PA is involved in endothelial tube in a fibrin matrix\textsuperscript{87, 104}. Furthermore, in the presence of TNF-α, exogenous u-PA is capable of enhancing tube formation. As our evaluations of FL-MSC-CM-induced tube formation occurred in the presence of TNF-α, it may be possible that u-PA secreted by MSC also contributed to the outgrowth of tubular structures. Similarly, two reports documented the stimulatory effects of adult MSC on endothelial tube formation in 3D fibrin gels due, in part, to degradation of the matrix by proteases (mainly MMP) produced by the MSC\textsuperscript{65, 102}. Indeed we found a 23% reduction of tube formation when blocking u-PA. However, these experiments cannot discriminate between the contribution of endogenous endothelial u-PA and u-PA derived from MSC.

In summary our data demonstrate that human FL-MSC can stimulate endothelial proliferation and endothelial growth as tubular structures into a 3D fibrin matrix, two major components of the angiogenesis process. HGF and VEGF secreted by FL-MSC are the major contributors to this stimulation. This provides perspective on using these cells in conditions that requires angiogenesis stimulation, including applications in tissue engineering.
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