Supporting cells in neovascularization: study on candidates for cellular therapy
van Beem, R.T.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Mesenchymal stem cells activate endothelial cells towards a proangiogenic state

Willy A Noort
Robert E Verloop
Rachel T van Beem
Marion Kleijer
Pieter Koolwijk
Jaap Jan Zwaginga
Victor W M van Hinsbergh
C Ellen van der Schoot

Manuscript in preparation
Abstract

Mesenchymal stem cells (MSC) secrete multiple paracrine factors, which might have therapeutic potential by enhancing angiogenesis and inhibiting fibrosis and apoptosis. We examined whether foetal human MSC, previously demonstrated to be able to enhance hematopoietic engraftment in a mouse model, were able to activate endothelial cells towards a pro-angiogenic state by paracrine factors.

The diversity and concentration of angiogenic factors produced by foetal lung (FL) derived-MSC were examined by an angiogenic specific PCR array and determination of multiple growth factors was performed by ELISA. *In vitro* angiogenesis of TNF activated MVEC stimulated with MSC-conditioned medium (CM) was studied in 3D fibrin matrices. Proliferation of stimulated MVEC, as determined by $^3$H-thymidine incorporation was used as angiogenic read-out.

FL-MSC-CM induced angiogenic tube formation of TNFα-stimulated MVEC. By ELISA it was observed that concentrations of CXCL1 (GROα), HGF, VEGF, IL-6, IL-8, MDK (midkine), SDF-1α and uPA are increased in MSC-CM compared to CM from MVEC or control medium. Proliferation experiments showed that MSC-CM can induce a 12-fold increase in proliferation of MVEC. By quantitative PCR it was shown that MSC-CM induced in MVEC downregulation of several inhibitors of angiogenesis (CXCL10 and IGFB1) and upregulation of VEGF. Comparable concentrations of VEGF or HGF in control medium resulted in a proliferation rate of only 3-4 fold. By blocking experiments with neutralizing antibodies against the measured proteins, only HGF and VEGF blocking reduced proliferation, although this was only 50%. The involvement of the other potentially mitogenic factors found in FL-MSC-CM (bFGF, CXCL1, IL-6, IL-8, MDK, SDF-1, and CXCL1) could not be demonstrated. A remaining candidate factor effect of MSC-CM is uPA, which is critical in the angiogenic tube formation in fibrin matrices. In summary, we have demonstrated that MSC of foetal origin produce angiogenic factors, of which VEGF and HGF play a significant role in the enhancement of MVEC proliferation by FL-MSC conditioned medium. Moreover, future experiments will be required to investigate other factor(s) secreted by FL-MSC that might be responsible for this angiogenic activation.
Introduction

Mesenchymal stromal cells (MSC) are multipotent cells, which are able to differentiate into bone, cartilage, adipose tissue, myocytes and tendon\(^1\). Furthermore, varying degrees of differentiation towards neuronal, hepatic and cardiac characteristics have been reported. They can be easily isolated and expanded, which make them good candidates for the repair and regeneration of many different tissues. In (pre)clinical studies, MSC were found to improve myocardial function and cerebral function after an infarction, and decrease liver damage, joint damage and bone diseases\(^2\). Due to their immunosuppressive effect, MSC have also been clinically applied to treat graft-versus-host disease in hematological stem cell transplantation, and in preclinical studies on solid organ transplantation and autoimmune diseases\(^3\). The therapeutic effect of MSC was originally thought to be attributed to their ability to physically participate in the formation of a supportive microenvironment of the bone marrow or by differentiation into different cell types that are needed to repair or replace damaged tissue. However, it is controversial whether MSC really engraft and undergo in situ differentiation. For example, bone marrow transplantation does not result in engraftment of donor stroma, although this has been subject of debate for many years\(^4\). Functional integration of MSC after injection into cardiac muscle has never been proven. Recent reports suggest that the observed and potential therapeutic effects of MSC might be (partly) mediated by paracrine factors secreted by these cells\(^5\)\(^-\)\(^9\). The factors released by MSC could well play a role in inhibiting fibrosis (scar formation), apoptosis, and in enhancing angiogenesis\(^10\)\(^,\)\(^11\). These, for tissue (re)generation very important roles, are summarized as "the trophic effect of MSC"\(^11\).

A positive role of paracrine factors from MSC on neovascularization has been described by a number of groups. It was shown that stromal cells from adipose tissue or bone marrow of mouse as well as human origin can produce factors, e.g. VEGF and HGF, that induce pro-angiogenic and anti-apoptotic effects of endothelial cells (EC) in vitro\(^5\)\(^,\)\(^12\). In an in vivo hind limb model in nude mice, transplantation of human MSC from adipose tissue resulted in improved neovascularization\(^13\)\(^,\)\(^14\). In a previous study, a significant improvement in hematopoietic engraftment was observed 6 weeks following transplantation of human MSC of foetal origin in combination with CD34\(^+\) cells into
NOD/SCID mice\textsuperscript{15}. Since MSC could not be traced in the BM of mice, the enhancing effect on hematopoietic engraftment was not due to engraftment of MSC, but might be due to the paracrine factors produced by MSC. We suggest that MSC facilitate HSC engraftment by stimulating vascular repair mechanisms of the (by irradiation) damaged bone marrow environment.

The aim of the present study was to investigate whether human MSC of foetal origin used in our previous \textit{in vivo} study; foetal lung-derived MSC (FL-MSC), indeed are able to activate endothelial cells towards a proangiogenic state, and to identify the factors secreted by the FL-MSC which mediate this angiogenic effect. Paracrine factors produced by MSC that might be involved in neovascularization are VEGF, HGF and bFGF\textsuperscript{5,9,16}. However, it is still unclear whether these are the only important factors and to what extent they contribute to the effect of MSC on endothelium. In the present study it was found that FL-MSC-CM induce angiogenic tube formation of micro vascular endothelial cells (MVEC) in a model for 3-dimensional tube formation. Gene expression and protein analysis showed that MSC from foetal origin produce key angiogenic factors. The use of neutralizing antibodies showed that VEGF and HGF produced by FL-MSC play a significant role in the enhancement of MVEC proliferation by FL-MSC conditioned medium, that their effect is additive and that other factors secreted by FL-MSC must be involved as well.

\textbf{Materials and methods}

\textit{Isolating and culturing FL-MSC}

FL-MSC were isolated and cultured as described before\textsuperscript{15}. Briefly, foetal lung tissue was obtained after informed consent from legal abortions according to a protocol that was approved by the institutional ethics committee (gestational age: 15-22 weeks). Single cell suspensions were made by using a cell strainer (100 \( \mu \)m, Falcon, Becton Dickinson). Cells were cultured in M199 (Gibco Laboratories, Grand Island, NY) supplemented with 10\% heat-inactivated FCS, 200\( \mu \)g/ml Penicillin/ Streptomycin (P/S), Endothelial Cell Growth Factor (ECGF) (20\( \mu \)g/ml, Roche Diagnostics GmbH, Mannheim, Germany), and
heparin (8 U/ml), which will be further indicated as M199 complete. Flasks were kept in
a humidified atmosphere at 37°C (5 % vol/vol CO₂). Medium was refreshed every week,
and cells were replated every two weeks.

**FL-MSC-conditioned medium (FL-MSC-CM)**
The medium was conditioned during a period of 5-7 days in the second week of culture
when cells reached confluency, and was collected just before detaching the cells for
replating. 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 previously¹⁷,¹⁸. Human dermis was digested in a 25%
dispase II/0.2% collagenase II solution for 2 hours at 37°C. The cell suspension was
subjected to 100 μm filtration to remove cell clumps and plated on fibronectin-coated
dishes. The heterogeneous cell suspension was cultured for 7 days in M199 medium
supplemented with 20% heat-inactivated human serum (HS) (PAA Laboratories), 10%
heat-inactivated new born calf serum (NBCS) (Life Technologies), 100 U/mL
penicillin/streptomycin, 2 mM L-glutamin, 5 U/mL heparin (MVEC medium) and 10
ng/mL bFGF (Peprotech). Endothelial cells were then isolated and purified by
fluorescence-activated cell sorting (FACS) using anti-CD31-PE (BD-Biosciences) (purity
97.8 ± 1.7%).

After purification, hMVEC were cultured in MVEC medium (M199, 10% human serum,
10% new born calf serum), supplemented with 150μg/ml crude endothelial growth
factor from bovine brain¹⁹ on 1% gelatin-coated dishes. MVEC were propagated in a
split-ratio of 1:3 and confluent cells were used from passage 8 to 10 for experiments. In
each experimental set-up, three different MVEC 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
previously²⁰. In short, highly confluent hMVECs (0.7x10⁵ 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, P/S, and TNFα. After 24 hours, the medium was replaced with FL-MSC-CM to which also TNFα was added. This was repeated every second day. As a positive control, VEGF (25 ng/ml) was used in combination with TNFα. Invading cells and tubular structures of hMVECs in the 3D fibrin matrix were analyzed by phase-contrast microscopy. The length and amount of the tube-like structures was determined using an Olympus-CK2 microscope equipped with a monochrome charge-coupled device camera (MX5) connected to a computer with Optimas image analysis software. Six fixed microscopic fields (7.3 mm² per field) per well were analyzed and used to calculate the total length of the tube-like structures, expressed as mm/cm².

Angiogenesis PCR array
A RT2 Profiler PCR Array from SuperArray (Bioscience Corporation, Frederick, MD, USA) was used to detect expression of genes related to angiogenesis. In this PCR Array, microarray profiling capabilities are combined with SYBR Green-Based Real-Time Quantitative PCR. The expression of 84 genes, five housekeeping genes (HKG: β-2-microglobulin, β-actin, hypoxanthine phosphoribosyltransferase 1, Ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase) and three RNA quality controls are profiled simultaneously in a 96 wells plate.

RNA was isolated from cultured FL-MSC as well as from MVEC cultured in control medium or MSC-CM, both supplemented with 10% human serum. TrizolTM B (Campro Scientific GMBH, Berlin, Germany) was used to isolate RNA according to the manufacturers’ instructions. Concentration and quality of RNA was determined spectrophotometrically and by using a Bioanalyzer (Agilent 2100, 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: Δ Ct sample = (Ct sample GENE) – (Ct of HKGs (average of 5 HKGs)).

Enzyme-Linked ImmunoSorbet Assay (ELISA)
Based on the results of the PCR array on FL-MSC, the concentration of several growth factors was determined by ELISA in different batches of FL-MSC-CM and culture media: bFGF, CXCL1 (GROα), HGF, PGE2, TGFβ1, VEGF (all Quantikine, from R&D Systems), IL-6,
IL-8, (Both from Pelikine, Sanquin Reagents), Midkine (MDK) (Peprotech Inc.), Urokinase was measured as described before\textsuperscript{20}.

**MVEC proliferation assay**

Proliferation of MVEC after stimulation with FL-MSC-CM as determined by \(^3\)H-thymidine incorporation: MVEC were seeded in 96 wells plates. Twenty-four hours after plating, cells were stimulated for 48hrs with FL-MSC-CM or with FL-MSC-CM supplemented with blocking antibodies against VEGF (100 \(\mu\)g/mL, Avastin (Hoffmann-La Roche Ltd), bFGF (10 \(\mu\)g/ml) HGF (1 \(\mu\)g/ml), CXCL1 (10 \(\mu\)g/ml) or Midkine (10 \(\mu\)g/ml) (all from R&D Systems) Anti-SDF1-\(\alpha\) (40ng/ml) anti-IL-6 (5\(\mu\)g/ml) and anti-IL-8 (5\(\mu\)g/ml) were a gift of L. Aarden (both 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 (bFGF (10 ng/ml) (Peprotech), HGF 30 ng/ml, VEGF 10 ng/ml) with or without the addition of its specific neutralizing antibody. Twenty-four hrs after stimulation, tracer amounts of \(^3\)H-thymidine were added (1 \(\mu\)Ci/well) and the cells were incubated for an additional 24hrs. Then the cells were washed with PBS, 100% methanol, and 5% trichloroacetic acid. Cells were lysed with 0.3 M NaOH, collected in a liquid scintillation tube and counted in a liquid scintillation counter.

**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 ± sd, unless mentioned otherwise.
Results

Enhanced tube formation in fibrin matrix after stimulation with MSC-CM and TNFα
MVEC growing on fibrin matrices are able to form tube-like structures under angiogenic conditions, as demonstrated before, this tube formation is TNF-α and VEGF concentration dependent. When MSC-CM was added to MVEC cultures, tube formation was observed (Fig1). This MSC-CM induced tube formation was also TNF-α dependent. Addition of MSC-CM in combination with 30ng/ml TNF-α induced a tube formation significantly higher than the medium control (153±24 vs. 27±8; p<0.05) or the medium with 25 ng/ml VEGF (153±24 vs. 133±24; p<0.05), both with the same TNF-α concentration.

MSC express angiogenesis related genes
MSC were analyzed by an angiogenesis specific RQ-PCR array, in table 1 the deltaCt from genes encoding soluble growth factors are shown. We selected factors of which it can be expected to have a positive effect on angiogenesis and that had a relevant transcript level as showed by a Δ Ct below 9. Subsequent studies were performed to elucidate the role of bFGF, CXCL1, HGF, IL-6, II-8, MDK, PGE2, TGFβ1, and VEGF.

Angiogenic factors/ cytokines as measured in MSC-CM by ELISA
To confirm the presence of the protein product of the genes mentioned above, the concentrations were determined by ELISA. Cytokines as measured in different batches of MSC-CM are shown in table 2 (mean ± sem pg/ml, n=3-8). bFGF and IL-8 were found in low levels, but high levels of CXCL1, HGF, IL-6, IL-8, PGE2, SDF-1α, TGFβ1, and VEGF were found. uPA levels in MSC-CM medium were remarkably high and undetectable (>100 ng/ml). Unfortunately we were nit able to measure MDK levels in MSC-CM. We only focussed on experiments neutralizing this protein (see below).
Figure 1: Angiogenic effect of MSC-CM on MVEC
Conditioned medium of FL MSC was added to MVEC cultures on a fibrin gel. A/B: After 4 days of culture in the presence of TNF, tube-like structures appear. C: Quantification of the tube length shows that this tube formation is influenced by TNF-α on a dose dependent manner. D: uPA concentrations were determined by ELISA and showed that an extremely high concentration of uPA is present in MSC-CM compared to M199 and MVEC-CM. *p<0.05; **p<0.01; #: tube formation by MSC-CM is lower than 25 ng/ml VEGF with the same TNF concentration, p<0.05; § P<0.05 MSC conditioned medium induces increased tube formation compared to 25 ng/ml VEGF in the presence of 30 ng/ml TNF.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Δ Ct</th>
<th>Gene</th>
<th>Δ Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANGPT1</td>
<td>11.59</td>
<td>IL8</td>
<td>6.81</td>
</tr>
<tr>
<td>ANGPT2</td>
<td>15.64</td>
<td>LEP</td>
<td>16.44</td>
</tr>
<tr>
<td>ANGPTL3</td>
<td>16.88</td>
<td>MDK</td>
<td>5.91</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>14.22</td>
<td>MMP2</td>
<td>3.06</td>
</tr>
<tr>
<td>CCL2</td>
<td>7.34</td>
<td>MMP9</td>
<td>-</td>
</tr>
<tr>
<td>CXCL1</td>
<td>5.79</td>
<td>PDGFA</td>
<td>13.78</td>
</tr>
<tr>
<td>CXCL10</td>
<td>14.01</td>
<td>PF4</td>
<td>22.70</td>
</tr>
<tr>
<td>EGF</td>
<td>16.45</td>
<td>PGF</td>
<td>13.37</td>
</tr>
<tr>
<td>FGF1</td>
<td>10.79</td>
<td>uPA</td>
<td>2.36</td>
</tr>
<tr>
<td>FGF2</td>
<td>6.93</td>
<td>PTGS1</td>
<td>7.06</td>
</tr>
<tr>
<td>HGF</td>
<td>8.32</td>
<td>SERPINF1</td>
<td>9.15</td>
</tr>
<tr>
<td>IFNA1</td>
<td>14.57</td>
<td>TGFA</td>
<td>14.24</td>
</tr>
<tr>
<td>IFNB1</td>
<td>17.59</td>
<td>TGFBI</td>
<td>5.18</td>
</tr>
<tr>
<td>IFNG</td>
<td>-</td>
<td>TGFBI2</td>
<td>10.19</td>
</tr>
<tr>
<td>IGF1</td>
<td>15.45</td>
<td>TNF</td>
<td>16.17</td>
</tr>
<tr>
<td>IL1B</td>
<td>9.98</td>
<td>VEGF</td>
<td>8.03</td>
</tr>
<tr>
<td>IL6</td>
<td>5.84</td>
<td>VEGFC</td>
<td>6.50</td>
</tr>
</tbody>
</table>

**Table I.** MSC express genes of soluble proteins that are involved in angiogenesis as determined by a PCR array

For almost all cytokines/angiogenic factors, the concentrations measured in unconditioned culture medium (M199 complete) are below the detection level, except for SDF-1α, PGE2 and TGFb1. It can therefore be excluded that the effect of MSC-CM is due to these factors. In addition, the levels of IL-8 in MSC-CM were modest and this cytokine is known to be produced by endothelial cells themselves\(^{21}\).

Although levels of bFGF were also low, its potency for endothelial activation is very high, thus in the following studies, the effect of bFGF in MSC-CM was tested, along with CXCL1, HGF, IL-6, MDK and VEGF.

**The proliferation inducing effect of MSC-CM in only partly mediated by VEGF and HGF**

Proliferation of MVEC was used as read-out for the effect of angiogenic factors\(^{22}\). Activation of MVEC with MSC-CM (figure 2A) resulted in a significant increase in \(^3\)H-TdR incorporation after 48hrs (12-fold increase as compared to control (M199 complete)). This proliferation was significantly higher than the proliferation induced by high concentrations of VEGF (10 ng/ml), bFGF (10 ng/ml) or HGF(30ng/ml) (resp 3.8-fold, 6.1-fold and 3.4-fold as compared to control) (figure 1B). MSC-CM contains this concentration of HGF, however, much lower concentrations of VEGF or bFGF were measured. Moreover, the MSC-CM induced proliferation was almost 4 times higher than
the maximal proliferation observed with the single cytokines. So, a combination of these cytokines and/or other factor(s) produced by FL-MSC are responsible for the enormous proliferation induction observed in the presence of MSC-CM.

We next determined which of the angiogenic factors found to be present in MSC-CM might contribute to proliferation. For this, neutralizing antibodies against bFGF, CXCL-1, HGF, IL-6, IL-8, MDK, SDF-1α, and VEGF, were tested for the ability to block the effect of MSC-CM. Neutralizing antibodies for bFGF, CXCL-1, IL-6, IL-8, MDK and SDF1α, did not result in an inhibition of proliferation by MSC-CM (fig 2B). As shown in figure 2B, only the antibodies against VEGF165 (relative proliferation 0.74±0.1, p<0.01) and HGF (relative proliferation 0.77±0.1, p<0.01) were able to block proliferation of MVEC. Combining these two antibodies resulted in an additive inhibiting effect on proliferation; 0.51±0.14 (p<0.01). Also, combining of antibodies against VEGF, HGF, CXCL1 and MDK did not enhance the inhibiting effect on proliferation. Since the antibody concentrations used in this assay were able to block much higher concentrations of the purified protein than present in the MSC-CM (fig 2A), other factor(s) with proliferation inducing potential for MVEC have to be present.
Figure 2: Effect of MSC-CM on proliferation of MVEC
A: MSC-CM induces a significant increase in proliferation of MVEC compared to control medium, but also compared to potent angiogenic growth factors, such as VEGF and bFGF. B: Blocking antibodies against VEGF, HGF and bFGF were tested for their ability to inhibit proliferation. Only antibodies against VEGF and HGF were able to block the proliferation induced by MSC-CM. However, although the blocking antibodies were able to block very high concentrations of the purified protein (A), no complete inhibition of the effect could be established (51%). This indicates that another, still unknown factor is likely responsible for the induction of proliferation of MVEC by MSC-CM.

*p<0.5 and **p<0.01 compared to M199 (A) or MSC-CM (B); $p<0.01$ MSC-CM vs M199+VEGF and vs M199 (A); $^\#p<0.01$ Inhibition of the blocking antibody of the effect of the targeted protein.
Regulation of genes involved in angiogenesis upon stimulation of MVEC

The effect of MSC-CM on the expression of angiogenesis related genes by MVEC (n=2) and HUVEC (n=3) was analysed by the same PCR array as used to study the expression of these genes in MSC. Endothelial cells were cultured for 48hrs in MVEC medium in the presence or absence of MSC-CM to which 10% normal human serum was added. MSC-CM induced consistent down regulation in MVEC as well as HUVEC only of CXCL10 and IGFB1 (both ~10 fold in MVEC), EFN2, ITGAV, MMP2 and TGFß2 (all three ~2 fold in MVEC).

Interestingly, VEGF was found to be slightly (~2 fold) upregulated in both MVEC and HUVEC. For all other genes no pronounced effects were observed in MVEC (data not shown).

Discussion

MSC conditioned medium of adult bone marrow or adipose tissue was reported to contain a number of potentially angiogenic growth factors\(^5,9,16\). In this study, we show that also MSC from human foetal lung secrete multiple growth factors with potent angiogenic inducing capacities. The angiogenic inducing potential of conditioned medium of FL-MSC was demonstrated \textit{in vitro} by its stimulation of tube formation in 3D fibrin matrices. In previous studies the angiogenic effect of MSC has been attributed to HGF or VEGF. Proliferation of endothelial cells needs to occur in angiogenesis, and here we show that the HGF and VEGF present in FL-MSC-CM are responsible for only half of the proliferation inducing effect. Although we demonstrated the presence of other angiogenic growth in the CM, the factor responsible for the additional effect could not be identified. The FL-MSC-CM affected the expression of genes in endothelial cells involved in angiogenesis. In particular, MSC-CM was shown to induce and increase in the expression level of the VEGF gene by MVEC.

Capillary-like structures can be formed in a 3D \textit{in vitro} angiogenesis assay\(^20\). FL-MSC-CM showed an angiogenic inducing potential on tube formation of MVEC, which was significantly higher as with VEGF (25ng/ml). For both stimuli, MVEC needed to be activated with TNF-α, however, a relatively high concentration of TNF-α was needed for
activation with FL-MSC-CM. This might indicate that an increase in receptors following activation improves susceptibility for cytokines in FL-MSC-CM.

To determine which angiogenic proteins are secreted by FL-MSC, we first analyzed the cells for expression of a panel of genes involved in angiogenesis. Additional to the angiogenic factors reported for adult MSC (VEGF, bFGF and HGF\textsuperscript{5,9,16}), we found relative high expressions for other genes; Angiopoietin-1, u-PA, CXCL1, aFGF, and Midkine (MDK). We subsequently determined the protein levels of most of the angiogenic factors in conditioned medium of FL-MSC. aFGF, which transcript was found to be relatively highly expressed, could not be measured in FL-MSC conditioned medium. In FL-MSC cell lysates, however, extremely high levels could be measured, indicating that aFGF is not released during culture by FL-MSC. The concentrations of VEGF, HGF, and bFGF measured in FL-MSC seem comparable with the levels as reported by Rehman and colleagues for adipose tissue derived-MSC\textsuperscript{16}.

Activation of MVEC with FL-MSC-CM, resulted in an impressive induction of endothelial cell proliferation (12-fold as compared to control). As angiogenesis occurs in the microvascular system, we used MVEC in these assays. Rehman et al also showed the proliferation inducing effect of adipose tissue derived MSC-CM on MVEC, however, they did not show the specific effect of HGF and VEGF by blocking experiments. Nakagami et al did show an effect of blocking HGF and VEGF, however, the used aortic EC, that are macro vascular and less likely to be involved in \textit{in vivo} angiogenesis. The potency of FL-MSC-CM to induce proliferation seemed to be much higher than that described for CM of BM-derived MSC of mice or of human\textsuperscript{9} or that of adipose tissue-derived MSC of human origin \textsuperscript{10,16}(4.5-fold, 5.5-fold and 3.5-fold as compared to control, respectively. By neutralizing HGF and VEGF in FL-MSC-CM with antibodies, the proliferation of MVEC was reduced, but only to a maximum of 50%. Since these antibodies were capable to completely inhibit higher concentrations of the recombinant factors then found in the CM, other factors should be involved. From our research it is also clear that the proliferation inducing effect of HGF and VEGF in the conditioned medium is rather an additive than a synergistic effect, although the later has been described by Van Belle et al\textsuperscript{23}.

Kinnaird et al demonstrated the involvement of VEGF as well as of bFGF in both human and mice BM derived-MSC conditioned medium-induced proliferation of endothelial
cells⁹. This is partly in contrast to our results, since we were not able to demonstrate any involvement of bFGF; the concentration measured in FL-MSC-CM was low and inhibition with anti-bFGF had no effect. Kinnaird and colleagues did not neutralize HGF; however, by inhibiting VEGF and bFGF they were also only partly able to attenuate the proliferative effect of MSC-CM. We found new candidate proteins in FL-MSC-CM, which might be involved in proliferation of micro vascular endothelial cells, namely CXCL1, MDK, and uPA. CXCL1 is known to positive correlate with angiogenesis²⁴, while, the biological significance of MDK in angiogenesis in general and its mechanism of action still remains to be clarified. MDK was found to modulate angiogenesis by abrogating VEGF-A-induced proliferation of MVEC in vitro through downregulation of proangiogenic cytokines and upregulation of tissue inhibitor of metalloproteinase ²⁵. The involvement of CXCL1 and MDK, in endothelial proliferation could not be shown by us. The significance of uPA in FL-MSC-CM still needs to be studied.

The results from this study strongly suggest that the mitogenic effects of MSC-CM are due or at least partly due to multiple cytokines. A recent study (Timmers et al. 2008, in press), in which cardioprotective properties of MSC-CM was demonstrated, suggested that the responsible paracrine factor of therapeutic option is likely a large complex of multiple components that may include proteins and lipids (>1000 kDa (100-220 nm)). In this complex, additional factors might be present that can explain the remaining 50% effect on proliferation in our study. Additional experiment need to be performed to confirm this. The study of Timmers, is the first in using stem cell secretions instead of stem cells for therapy. They showed that in a clinically relevant porcine model of cardiac ischemia and reperfusion injury CM-treatment was associated with a significant reduction (~60 %) in myocardial infarct size.

Although the experiments are based on the assumption that there is a direct relation between the induction with FL-MSC-CM and MVEC behavior, it might also be that activation of MVEC resulted in an autocrine production of cytokines by MVEC contributing to the process of angiogenesis. Then MSC-CM contributes indirectly to the pro-angiogenic activation of MVEC. By PCR array we found that MSC-CM induced consistent downregulation in endothelial cells of CXCL10 and IGFB1. CXCL10, also known as IP-10 (interferon-inducible protein 10), was found to be a potent inhibitor of angiogenesis in vivo²⁶. From IGFB1 (insulin-like growth factor B1), it is known that is a
regulator of vascular function, and may contribute to cardiovascular disease by causing endothelial cell dysfunction\textsuperscript{27}. Also members of the IGF family were reported to be frequently expressed in many types of tumors associated with malignant progression and poor prognosis. Our results, which show downregulation of these genes, might well fit in the conditions which create a balanced angiogenic “prone” environment for MVEC. The upregulated gene, VEGF, although to a moderate (2-fold) extent, further contribute to this since it is a factor known to stimulate angiogenesis.

Since we have demonstrated that FL-MSC-CM contains key angiogenic factors, our previous findings on an improved hematopoietic engraftment following cotransplantation of culture-expanded human MSC into NOD/SCID, might be explained by an improved neovascularization of the damaged bone marrow after irradiation. A additional study on the bone marrow microenvironment of NOD/SCID mice following transplantation will further confirm our hypothesis.
References


(9) Kinnaird T, Stabile E, Burnett MS et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004;94:678-685.


