MCF7-MSC co-culture assay: approach to assess the co-operation between MCF-7s and MSCs in tumor-induced angiogenesis

Ş. Comşa¹, Felicia Ciuculescu², R. Henschler³, M. Raica¹

¹Department of Histology
²Medical student
³Johann Wolfgang Goethe-University Frankfurt, Institute of Transfusion Medicine and Immune Hematology, German Red Cross Blood Donor Center, Frankfurt, Germany

Abstract
The multipotent stromal cells (MSCs) exhibit a broad differentiation potential. MSCs might participate in neovascularization through their ability to migrate and generate capillary-like structures. These processes were shown to be modulated by tumor angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF). The aim of our study was to define the way the MCF-7 cell line (MCF-7s) influenced the MSCs’ recruitment for the tumor-induced angiogenesis, and to assess the role of VEGF in this process. We tested the chemotactic potential of plasma or VEGF, but also of MCF-7s or their conditioned medium (CM) in the MSCs’ transmigration. We compared the migratory potential of MSCs, MSCEs (MSCs cultured in endothelial cell growth medium) and HUVECs. Recombinant VEGF has been shown to chemoattract MSCs, although to a lesser degree than plasma or serum containing medium alone. Moreover, it changed the MSCs’ morphology, stimulating the appearance of longer and thinner prolongations as compared to plasma. MCF-7s or their CM both directly induced migration of MSCs. Surprisingly, CM augmented with MCF-7s attracted less cells than the control medium itself, but CM augmented or not with MCF-7s changed the morphology of MSCs in a manner similar to VEGF. The migratory behavior of the MSCEs was comparable to that of HUVECs, while their morphology could be considered intermediate between MSCs and HUVECs, as they developed shorter prolongations than MSCs, but much longer than HUVECs in the corresponding wells. In conclusion, both tumor cells and VEGF alter the migration behavior of MSCs in a transmigration model, indicating a role of tumor cell-derived VEGF to modulate the recruitment of MSCs into sites of angiogenesis.

Keywords: angiogenesis, breast cancer, MSC, MCF-7, transmigration.

Introduction
According to the International Society for Cellular Therapy, a multipotent stromal cell (MSC) is defined by the following criteria: (a) its property of adherence to plastic; (b) its phenotype: CD14- or CD11b-, CD19- or CD79α-, CD34-, CD45-, HLA-DR-, CD73+, CD90+, CD105+; and (c) its capacity to be differentiated into three lineages, namely chondrocyte, osteoblast, and adipocyte [1]. Although the MSCs are defined by their capacity to be differentiated toward these three cell lineages, they display a broader differentiation potential [2].

MSCs express several proangiogenic factors, including vascular endothelial growth factor (VEGF) [3, 4] and induce the expression of their specific receptors on the target cells [5]. In addition, it is suggested that MSCs might participate in the formation of new vessels through their ability to migrate and generate capillary-like structures and these angiogenic properties seem to be modulated through growth factor-mediated paracrine regulation [6]. MSCs have the capability to acquire endothelial-like characteristics in pro-angiogenic environments [7]. MSCs are actively recruited during tumor neovascularization and engraft into established tumor lesions [8]. MSC-derived fibroblasts are thought to have the capacity to (trans) differentiate into endothelial-like cells and pericyte-like cells, which would stimulate tumor growth by the formation and stabilization of tumor blood vessels [9]. The inhibition of tumor growth by MSCs has been observed in different types of animal models, while others reported no effect on tumor growth [2]. It was shown that murine MSC could reduce metastasis in a model of Lewis lung carcinoma and B16 melanoma [10], while a more recent study with human MSCs showed that MSCs could increase the metastasis rate of breast cancer cells [11]. It is also interesting to note that cancer cells can modify the growth or migration of MSCs, making the picture even more complex [2].

The potent angiogenic stimulant VEGF is known to regulate MSC mobilization and recruitment to sites of neovascularization, as well as directing the differentiation of MSCs to a vascular cell fate [12]. Recent studies show that MSCs do not express VEGF receptors, but VEGF can promote MSC proliferation and migration by activating platelet-derived growth factor (PDGF) receptors in MSCs [8].

MCF-7, an established human metastatic breast...
cancer cell line, was designed in the Michigan Cancer Foundation in 1973, from a pleural effusion and is the most commonly used breast cancer cell line worldwide [13].

Fierro FA et al [14] showed that changes were detected in the morphology, proliferative capacity and aggregation pattern of MCF-7 cells after co-culture with MSC. They also suggested that VEGF and IL-6 mimic the effects produced by MSC or its products on the proliferation and aggregation properties of MCF-7.

In this study, we aim to define the way the MCF-7 cell line influences the MSCs’ recruitment for the tumor-induced angiogenesis and to assess the role of VEGF in this process.

Materials and Methods

Chemicals and reagents

The following materials were purchased from the indicated sources. Insulin, cell culture tested bovine serum albumin (BSA) and gelatin (from porcine skin) were purchased from Sigma (Steinheim, DE). The basic fibroblast growth factor (bFGF) and human recombinant VEGF were from R&D Systems Inc. (Minneapolis). Human plasma was produced from venous blood of voluntary persons and was stored at -20\degree C before use. Penicillin-Streptomycin solution, trypsin EDTA (0.25% solution) were from Gibco (Karlozhuhe, DE). May-Grünwald solution, Giemsa solution and methanol were purchased from Merck (Darmstadt, DE).

Cell sources

Human mesenchymal stromal cells (MSC) were isolated from bone marrow of patients undergoing hip replacement surgery, after their informed consent. The cells stained negatively for the hematopoietic marker CD45 and positively for the mesenchymal marker CD73, CD90, and CD105, as determined by flow cytometric analysis. Their pluripotency was confirmed by successful induction of osteoblastic, chondrogenic, and adipogenic differentiation upon addition of the respective media under standardized conditions.

MSC were culture expanded and trypsinized when they reached 70–80% confluence. Cells were seeded at 2–3×10^3/cm² in T75 flasks (Becton Dickinson, Walkersville, MD). After three weeks of culture, both MSCs and MSCEs were tested for the endothelial cell growth medium (EGM-2) from Lonza (Walkersville, MD). After three weeks of culture, both MSCs and MSCEs were tested for the endothelial marker CD31, using flowcytometric analysis, and then, the cells were used for the experiments.

HUVECs were obtained from Cambrex Bio Science (Walkersville, CC) and were cultured in EGM-2, into T75 flasks, which had their bottom coated with gelatin 0.1%. The medium removal and the passage protocols for HUVECs were similar to those for the MSCs.

MCF-7 cells (HTB 22) were purchased from ATCC (Wesel, Germany). Cells were seeded in T-75 flasks at 1×10^6 cells/flask in low glucose DMEM containing 10% FBS, 2 mM glutamine, 0.01 mg/mL insulin and 1% Penicillin/Streptomycin mix (MCF-7 medium) and incubated at 37°C in an atmosphere of 5% CO\_2. The medium renewal was performed twice per week, while cells were weekly passaged at a sub-cultivation ratio of 1:3.

Collection of conditioned medium

To detect the presence of chemokines potentially mediating any paracrine effects of MCF-7s on MSCs/ MSCEs/HUVECs, conditioned medium (CM) from MCF-7s was collected and analyzed in the transmigration assay. CM refers to medium that was incubated in the presence of cells for 24 to 48 hours.

The transmigration assay

For chemotaxis assays, MSCs were trypsinized and, then, kept in migration medium – low glucose DMEM supplemented with 0.5% Bovine Serum Albumine (BSA) (Sigma, Munich, Germany) at 37°C, before using them in the experiment. The same treatment was performed for the MSCEs and for the HUVECs.

Ninety-six well chambers 8-µm PVP-free membranes from NeuroProbe (Gaithersburg, MD) were used. The lower wells were filled with different potential migration-inducing medium: migration medium containing plasma or VEGF, MCF-7 medium, CM supplemented or not with MCF-7 cells, at a total volume of 430 µL. After filling the lower wells, filters were placed avoiding air bubble formation. The chambers were closed by careful tightening of the screws. The upper wells were then filled with 300 µL of cell suspensions (5×10^4 MSCs/ MSCEs/HUVECs per well) in the migration medium (Figure 1). After mounting them, the chambers were placed into a humidified incubator at 5% CO\_2, 37°C.

Figure 1 – Schematic presentation of the trans-migration experiment. The lower wells were filled either with plasma/VEGF diluted in DMEM+0.5% BSA or MCF-7 medium/CM±MCF-7 cells. Then the 8-µm pore-membrane was placed on the top of these wells. The chambers were closed by careful tightening of the screws and the upper wells were then filled with 300 µL of cell suspensions (MSCs/ MSCEs/HUVECs) in migration medium (5×10^4 cells/well).
After a period of 16–20 hours, medium was carefully removed from the upper wells. Subsequently, membranes were removed and were immediately rinsed with PBS on both sides. Cells on the membrane were fixed by immersion in 100% methanol (Merck, Darmstadt, Germany). The cells from the upper side were wiped off with a wiper apparatus (NeuroProbe) and rinsed with PBS. Cells from the lower side were stained with May–Grünwald/Giemsa (Merck, Darmstadt, Germany) and then washed in PBS. The membranes were well dried before examination.

Evaluation of completed transmigration was performed on the fixed filters under an inverted light microscope (Olympus CK-2), at 40/100-fold magnification. MCF-7s from the bottom of the corresponding wells were also evaluated (magnification, 40-fold). Photos of representative fields were taken to document the findings using a digital camera.

**Statistics**

Statistical evaluation was performed using Student’s *t*-test and Pearson’s correlation coefficient in Microsoft Excel software, and *p*<0.05 was considered as significant.

**Results**

In order to evaluate the potential changes in the phenotype of MSCs after having been culture-expanded in EGM-2, we compared them with the MSCs that were culture-expanded in their regular medium. We noticed no difference between them after 24 hours of culture, but after three weeks, the two cell cultures presented a different morphology, suggesting a change in the phenotype of MSCs after being culture-expanded in EGM-2. Interestingly, after the 3-week culture in the specified media, both the control MSCs and MSCEs were negative for the endothelial marker CD31, as determined by flow cytometric analysis.

We tested the ability of different media to induce migration of MSCs using a Boyden chamber (transwell) assay. The cells were seeded into the upper wells of the transmigration chambers and incubated with plasma, VEGF and CM in the presence or absence of MCF-7 cells, in the lower wells. MSCs migrating to the bottom surface of the membrane were fixed and counted. To ensure that only cells from the lower side of the filter were counted, the correct focal plane was determined as it follows: the shape and appearance of the pore holes were round and clearly outlined openings on the upper filter sides, whereas pores on the lower face of the membrane appeared to be more oblique and with frail margins. The cells from the lower side of the membrane were counted in three different fields for each chamber and an average was calculated.

As a positive control, MSCs responded to the plasma induced stimuli and migrated towards the plasma source as detected by their migration to the lower side of the membrane (Figure 2).

VEGF in different concentrations (20 ng/mL; 50 ng/mL) attracted less cells than the control solution (DMEM+0.5% BSA) or plasma. The transmigration of the MSCs was not significantly influenced by the concentration of VEGF in the lower well (Figure 2).

Chemoattraction by VEGF also changed the morphology of the MSCs as longer prolongations appeared, comparing to the cells incubated with the control solution or plasma (Figure 3).

In order to evaluate the angiogenic profile of the chemokines released by MCF-7s, we tested the chemotraction of MCF-7 medium alone, conditioned medium of MCF-7 tumor cells (CM) in the presence of MCF-7 cells and CM alone (obtained by selecting the supernatant after a double centrifugation of the MCF-7 cell suspension). CM in the presence of MCF-7s, attracted significantly more cells than the MCF-7 medium alone (*p*<0.05). No difference in attracting MSCs either between CM alone and MCF-7 medium or CM alone and CM in the additional presence of MCF-7 cells was noted (Figure 4). Interestingly, CM supplemented or not with MCF-7s determined a change in the MSCs’ morphology, which resembles with the morphology of the cells in contact with VEGF (Figure 3).

The MSCEs showed a very similar behavior with HUVECs when they transmigrated through the membrane. There was a clear correlation between the migration rates of MSCEs and HUVECs (*p*<0.05). Interestingly, MSCs migrated better towards DMEM+0.5% BSA served as control. Plasma proved to be a potent chemoattractant, while addition of VEGF resulted in a reduced transmigration rate, compared to both, control and plasma. All values were determined as averages of three independent experiments. Error is represented by SD. (*Student *t*-test *p*-value is significant: *p*<0.05).

Figure 2 – Transmigration of MSCs induced by plasma and VEGF. MSCs were added to the top well and were allowed to migrate overnight. DMEM+0.5% BSA served as control. Plasma proved to be a potent chemoattractant, while addition of VEGF resulted in a reduced transmigration rate, compared to both, control and plasma. All values were determined as averages of three independent experiments. Error is represented by SD. (*Student *t*-test *p*-value is significant: *p*<0.05).
Figure 3 – The morphology of the MSCs after transmigration towards the lower side of the membrane. The cells in contact with VEGF developed longer and thinner prolongations comparing to the cells in contact with plasma. The changes performed by CM/MCF-7 cells on the transmigrated MSCs were similar to those performed by VEGF. The text boxes refer to the content of the lower wells. Magnification, 100×.

Figure 4 – Transmigration of MSCs induced by CM±MCF-7 cells. MSCs were added to the top well and were allowed to migrate overnight. MCF-7 medium served as control. CM supplemented with MCF-7 cells attracted less cells than the control. No significant differences could be noticed neither in the case of the control and CM, nor in that of CM and CM supplemented with MCF-7 cells. All values were determined as averages of the counts in three independent experiments. Error is represented by SD. (*Student t-test p-value is significant; p<0.05).

Figure 5 – Comparison among the morphology of the MSCs, MSCEs and HUVECs after transmigration induced by CM. The images represent photos of the cells from the lower side of the membrane. The MSCEs presented an intermediate morphology between MSCs and HUVECs, as they developed shorter prolongations than MSCs but much longer prolongations than HUVECs. Their morphology seems to be closer to MSCs, than to HUVECs. The text boxes refer to the content of the upper wells. Magnification, 100×.

Discussion

We have described the conditions that allow the assessment of chemotactic migration of adherent human MSCs, MSCEs and HUVECs. We noticed that plasma was a very good chemotactic agent, while Annabi B et al. [6] proved that MSCs’ migration was strongly induced by serum. Rüster B et al. [15] found a dose and time-dependent induction of MSC migration when using human plasma as a stimulus. And Jaganathan BG et al. [16] showed that MSCs had an optimal migration percentage between 0.5 and 1% human plasma. Based on these results, we allowed MSCs to transmigrate towards the lower side of the membrane overnight and we established the 1% plasma as the positive control of our assay.

We have evaluated the chemotactic potential of VEGF at concentrations of 20 and 50 ng/mL, and we noticed no significant differences between the transmigration rates obtained for the two concentrations. Ritter E et al. [17] observed that the degree of VEGF-
induced migration of MSCs was similar at a concentration of 25 or 50 ng/mL. In agreement with the results of Annabi B et al. [6], we found that only a small amount of the MSCs transmigrated through the membrane, comparing to the number of plasma-attached cells, independent of the VEGF concentration. Beside VEGF, serum contains several other polypeptide growth factors that regulate cell motility [18] and this could be an explanation for the migratory behavior of MSCs. The fact that VEGF attracted fewer cells than the control solution made us think of the hypothesis of an inhibitory action of VEGF in the MSCs’ transmigration. Opposite to these results, Ritter E et al. [17] noticed that VEGF had a significant migration-inducing effect: 15-fold over serum-free or serum-supplemented medium.

When MCF-7s were cultivated in DMEM with 10% FBS, media conditioned by MCF-7 cells enhanced migration by 15- to 25-fold over that of serum-free or serum-supplemented medium [17]. Surprisingly, our results show that CM augmented with MCF-7s attracted significantly less cells than the MCF-7 medium alone (DMEM containing 10% FBS, 2 mM glucose, 0.01 mg/mL insulin and 1% Penicillin/Streptomycin mix). It seems that the addition of MCF-7 cells either consumes a MSC chemoattractant from the medium or it determines the appearance of an element which inhibits the transmigration of MSCs. As MCF-7s secrete VEGF and as our results show that VEGF inhibits the MSC transmigration, we could think that this element could be VEGF.

The phenotypic plasticity of MSCs into endothelial-like cells provides a rationale for their potential role in neovascularization and microvascular network remodeling [19, 20]. Interestingly, these processes were shown to be modulated in response to tumor angiogenic factors [21], while several studies have shown that MSCs can be induced to differentiate towards endothelial cells, following exposure to VEGF [22, 23]. Our results show that, although VEGF inhibits the transmigration of MSCs, it changes their morphology, determining the appearance of longer and thinner prolongations comparing to those of the MSCs in contact with plasma. Surprisingly, MCF-7s or CM have a similar effect on the MSCs’ morphology and so, the tumor-secreted VEGF seems to play a role in modulating the recruitment of MSCs for angiogenesis. Besides changing the morphology of the MSCs, VEGF could also perform some other phenotypic changes in MSCs, which could lower their migratory potential and activate them for the new vessels’ formation.

Similar to the results obtained by Hombauer H and Miguell JJ [24], we demonstrated in our study that most of the MCF-7s co-cultured with MSCs/MSCs/HUVECs adhered to the bottom of the wells and were to be seen as single cells, without organizing in clusters.

Our study suggests that the migratory behavior of MSCEs is similar to that of HUVECs. Taking into account the fact that MSCs started to express endothelial markers when they were cultured in the presence of endothelial growth supplements [22] we might say that this migratory behavior could be the result of the differentiation of MSCEs into endothelial-like cells. A direct comparison of MSCs’ and MSCEs’ behavior on Matrigel, in the absence of VEGF, sustains this supposition, as, after three hours, the MSCEs partially formed cord- or tube-like structures, whereas the MSCs had not invaded the Matrigel [7]. In addition, the MSCEs present a higher affinity to tumor chemotraction comparing to MSCs, so that cultivating MSCs in endothelial growth medium seems to be a “preconditioning” process for the interaction of the MSCs with the tumoral environment.

We noticed that MSCEs had an intermediate phenotype between MSCs and HUVECs, but closer to MSCs. Liu JW et al. [7] also concluded that the morphology of confluent MSCEs was different from that of MSCs. It remains to be shown whether this morphological difference is related or not to the cells’ angiogenic profile.

To our knowledge, this is the first article, which reports the appearance of specific morphological changes in the MSCs, after in vitro transmigration towards different chemoattractant media, through an 8-µm-pore polycarbonate PVP-free membrane.

Conclusions

Both tumor cells and VEGF alter the migration behavior of MSCs in a transmigration model, indicating a role of tumor cell-derived VEGF to modulate the recruitment of MSCs into sites of angiogenesis. The MSCEs represent a hybrid cell population, with a transmigration behavior similar to HUVECs, but with a morphology that situates them much closer to MSCs than to HUVECs. Future research is needed to investigate the correspondent changes in the morphology, the migratory potential, the markers’ profile and the gene expression acquired by MSCs in pro-angiogenic tumor/non-tumoral environments, in order to define the “angiogenic switch” in the phenotype of these cells.

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References


Corresponding author
Marius Raica, Professor, MD, PhD, Department of Histology, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40256–204 476, e-mail: raica@umft.ro

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