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A Contact-Based Method for Differentiation of Human Mesenchymal Stem Cells into an Endothelial Cell-Phenotype

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Abstract

Adult stem cells such as mesenchymal stem cells (MSC) are known to possess the ability to augment neovascularization processes and are thus widely popular as an autologous source of progenitor cells. However there is a huge gap in our current knowledge of mechanisms involved in differentiating MSC into endothelial cells (EC), essential for lining engineered blood vessels. To fill up this gap, we attempted to differentiate human MSC into EC, by culturing the former onto chemically fixed layers of EC or its ECM, respectively. We expected direct contact of MSC when cultured atop fixed EC or its ECM, would coax the former to differentiate into EC. Results showed that human MSC cultured atop chemically fixed EC or its ECM using EC medium showed enhanced expression of CD31, a marker for EC, compared to other cases. Further in all human MSC cultured using EC-medium, typically characteristic cobble stone shaped morphologies were noted in comparison to cells cultured using MSC medium, implying that the differentiated cells were sensitive to soluble VEGF supplementation present in the EC-medium. Results will enhance and affect therapies utilizing autologous MSC as a cell source for generating vascular cells to be used in a variety of tissue engineering applications.

Keywords

Direct cell–cell contact; Stem cell; Differentiated cell; Chemically fixed substrate; Decellularization

Introduction

Stem cell-based tissue engineering therapies are a promising alternative for treating ischemic disease and chronic wounds. Adult mesenchymal stem cells (MSC) are capable of modulating the neovascularization processes necessary for wound healing and are thus widely popular as an autologous source of progenitor cells. Recently, efforts have been made

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in differentiating human MSC into endothelial cells (EC) for testing their utility in wound healing [1, 2]. Results from these studies [1, 2] have shown that MSC cultured within polyethylene glycolylated fibrin gels exhibited an endothelial phenotype as evidenced by higher levels of endothelial markers including von Willebrand factor, vascular endothelial cadherin, and vascular endothelial growth factor [1, 2]. However, these differentiated EC networks formed from MSC lacked platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) expression, and were not sensitive to soluble vascular endothelial growth factor (VEGF) supplementation which indicated incomplete differentiation towards an EC type [1, 2]. Others have shown that MSC differentiation is influenced by direct contact with differentiated cells [3]. When MSC were cocultured with vascular EC, direct cell–cell contact induced changes in the MSC suggesting induction of their differentiation into EC-type cells [3]. The changes observed in the cultured MSC were effects induced by direct contact with the extracellular matrix (ECM) rather than soluble ECsecreted factors [4]. So, direct contact with distinct differentiated cells may be a critical determinant of MSC cell fate in blood vessel and other tissue engineering applications [3]. Collectively, from findings of all these studies, it can be implied that MSC can be harnessed as a potential source for differentiation into EC-like cells. However the mechanisms involved in modulating the differentiation of MSC into EClike cells is not clear, and a complete differentiation of MSC into EC is yet to be achieved. This motivated us to explore further by culturing MSC onto chemically fixed layers of EC. In recently published studies [5, 6], the effectiveness of chemically fixed differentiated cells used in culturing and maintaining viability of stem cells was shown. Resulting from these studies it was concluded that stem cells thrive on superficial protein–protein contacts and do not necessarily require contact with viable cell-feeders to retain their pluripotency and viability [5]. Based on this outcome, we hypothesized that culture of MSC atop fixed EC would coax the differentiation of the former into EC-type. Furthermore, we speculated that decellularized and chemically-fixed ECM derived from EC would also induce MSC differentiation as suggested by others [7, 8]. Contrary to this idea of using chemically-fixed cell-derived ECM, there have been many reports on the use of decellularized ECM as a tissue engineering scaffold in vivo, without chemical fixation [9]. The small intestinal submucosa derived ECM, a concept pioneered by [10] has applications, mostly for invivo tissue engineering [11-15]. For all such in-vivo tissue engineering applications, the use of decellularized ECM has already proven to be an effective means for tissue regeneration. However, we have pioneered the concept of chemically fixed ECM in-vitro, as demonstrated in our published works [5, 6, 16-18]. There are several advantages in using chemically fixed ECM as implied for this in-vitro study, as well. First, no specialized culture medium is required to maintain the viability or intactness of the chemically fixed cell-substrate used to culture and differentiate cells atop. Moreover, we have observed in previous studies that the decellularized ECM cannot be retained without fixation atop the tissue culture dishes, in-vitro, which might not be the case in-vivo. Additionally, the cells and the ECM when chemically fixed as a substrate, are treated using a very mild concentration of 2.5% formaldehyde or glutaraldehyde which retains the fluidity of the cell membranes [6]. So based on these rationale, human MSC were cultured atop chemically fixed layers of EC and, atop decellularized and chemically fixed ECM derived from EC respectively. Results will help develop a simple and extremely feasible technique for the differentiation of MSC into EC, based upon a direct contact-based culture method.

Materials and Methods

Culture and Passaging of Human MSC

Human adipose derived mesenchymal stem cells (MSC) were obtained from Lonza at passage = 3 (Lonza, Allendale, NJ, USA) and cultured according to manufacturer's recommendations. Certification was obtained and filed to ensure that the purchased human MSC (Lonza) were verified to be of the correct lineage and uncontaminated by other cell types or organisms. For the human MSC culture, a complete growth medium (Lonza), specifically MSCGM human mesenchymal stem cell (HMSC) growth BulletKit™ Medium (Lonza) was used for maintaining the mesenchymal stem cells in an undifferentiated state. This medium will be referred to as complete growth medium for HMSC henceforth. Prior to cell seeding, T-75 culture flasks were coated with 0.1% gelatin (Sigma Aldrich, St. Louis, MO, USA) and incubated (37 °C, 1 h). After this, the cell suspension in complete culture medium was transferred to a gelatin coated T-75 flask and incubated for 1 h (37 °C, 5% CO₂ and 95% RH). Prior to cell culture, the gelatin solution used for coating of the flasks was aspirated. After 70% confluency in culture was attained, cells were trypsinized and passaged for further experiments. Normal morphology and phenotype of the cultured cells were compared with other's published images of human MSC [3].

Culture and Passaging of Human EC

Human dermal microvascular endothelial cells (EC) obtained from Lonza at passage = 8 were a generous gift from Dr. Laura Suggs at UT Austin. Verification of the identity of these EC was done by short tandem repeat profiling, and documentation maintained in Dr. Suggs's Laboratory at UT Austin. Procured EC were cultured as described elsewhere [19] for at least three passages in endothelial basal medium (EBM-2) (Lonza) supplemented with EGM-2 BulletKit (Lonza) prior to being used in this study (37 °C, 5% CO₂ and 95% RH). For preparing a confluent monolayer of EC, cells were seeded at a concentration of approximately 50,000 cells per unit well of a 24-well plate. After the cells appeared to be 70% confluent, they were trypsinized and passaged for further experiments. Normal morphology and phenotype of the cultured cells were compared with others' published images of human EC [19].

Preparation of Chemically Fixed EC and Decellularized, Chemically Fixed ECM Derived from EC

For preparation of chemically fixed EC, visually confluent EC (in passages 8 and 9 only) cultured in 24-well plates were fixed with 2.5% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 12 h at 4 °C [5, 6]. Fixed cells were washed thrice using 1× PBS and used for further experiments or stored at 4 °C until further use. For preparation of chemically fixed ECM, a confluent layer of EC was permeabilized and removed by treatment with a 10% (w/v) commercially available NP-40 detergent solution (RT, 10 s) (Thermo-Fisher Scientific, Waltham, MA, USA). To confirm complete removal of cells after detergent treatment, matrices were stained with DAPI and imaged using a fluorescence microscope (Zeiss Axiovision, Zeiss, NY). EZBlue™ (a Coomassie Brilliant Blue G-250 colloidal protein pre-mixed stain, Sigma Aldrich) was used to stain and visually confirm the presence of decellularized ECM. Briefly, the chemically fixed ECM was washed thrice with 1×PBS

and then incubated with EZBlue™ Staining Reagent for 10 min at RT on a rotary shaker. Following this, the staining solution was removed and the stained ECM washed three times in double distilled water. The stained ECM matrices were imaged using bright field imaging (EVOS). After cellular removal from the wells was confirmed, the remnant ECM (decellularized ECM) was fixed using 2.5% formaldehyde for 12 h at 4 °C [5, 6]. Fixed ECM matrices were washed thrice using 1× PBS and used for further experiments or stored at 4 °C until further use. In addition, control substrates were prepared by coating wells of 24-well plates using complete growth serum (Lonza) for culturing human MSC.

Culture of Human MSC to Promote Differentiation into EC-Type

In order to promote their differentiation towards an EC-type by direct contact, human MSC (in passage 6–8) were seeded onto 3 types of substrates including chemically fixed EC (Plate-1), chemically fixed decellularized ECM derived from EC (Plate-2) and controls, respectively, at a concentration of approximately 50,000 cells per unit well of 24- well plates (including Plate-1, Plate-2, and controls). For the first 48 h of culture (37 °C, 5% CO₂ and 95% RH), all cells were cultured using complete growth medium for human MSC and their changes in morphology tracked using phasecontrast microscopy (EVOS Imaging System, Thermo- Fisher). After 48 h of culture, medium was changed to EBM-2 (EC media) in one set of experimental substrates including Plate-1, Plate-2, and controls. Controls for this experiment (Plate-1, Plate-2, and controls) continued to receive complete growth medium for human MSC, instead of EBM-2. Phase-contrast imaging was performed again after 72 h and at the end of 7 days of culture (37 °C, 5% CO₂ and 95% RH).

CD31 Staining to Detect Differentiation of Human MSC into EC-Type

To detect differentiation of human MSC into an EC-type, immunofluorescence detection for CD31 (or PECAM-1), an integral membrane glycoprotein that is expressed at high levels on endothelial cells, was performed [20]. For the immunostaining, a primary anti-CD31 monoclonal antibody and a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Abcam, Cambridge, UK) was used in immunofluorescence detection of the CD31 marker expression in human MSC cultured atop various substrates (including Plate-1, Plate-2, and controls). Stained cells were imaged using a Zeiss Axiovision confocal fluorescent microscope (ZEISS LSM 700 confocal, Germany).

Flow Cytometric Analysis to Quantify the Expression of CD31 Positive Cells

To determine the percentages of CD31 positive cells in the human MSC cultured atop various substrates, cells cultured for 7 days were washed and dissociated with TrypLE Express (12604039; Invitrogen, Carlsbad, CA, USA) to obtain single cell suspensions [5]. Isolated single cells were fixed with BD Cytofixation buffer (4% paraformaldehyde, Sigma), followed by permeabilization with 1 × BD Perm/Wash buffer. The cells were then stained with a primary anti-CD31 monoclonal antibody and a secondary FITC-conjugated goat anti-mouse IgG (both from Abcam, Cambridge, UK) following manufacturer's protocols. A FACSCanto™ instrument (Becton Dickinson, Franklin Lakes, NJ) was used to quantify the CD31 positive cells. The instrument was calibrated before each analysis with Rainbow Calibration Particles (BD). Before analysis of the stained cells, BD CompBead Plus positive and negative beads were analyzed to facilitate application setup. Nonviable cells were

excluded by staining with 0.1% (v/v) propidium iodide (Sigma). Average percentage of CD31 expression was reported for each case analyzed.

Experimental Sample Sizes and Statistical Analysis

All samples were present in triplicate for all experiments and all experiments were repeated once, unless otherwise mentioned. Numerical data is expressed as mean $\pm$ standard deviation. Student's *t*-test was performed to determine if the averages of any two sample datasets compared were significantly different. *p*-values less than 0.05 were considered significant.

Results

Both human EC and MSC were cultured, passaged and stabilized in-vitro prior to exposing them to experimental conditions after which confluent EC layers were fixed (chemically fixed EC (Plate-1), Fig. 1a) or decellularized, ECM extracted and fixed (chemically fixed decellularized ECM derived from EC (Plate-2), Fig. 1b). Chemical fixation did not alter the CD31 expression in the basal matrices formed by the EC (Supplementary Fig. 1) and retained the fibril-like structure of ECM elements after decellularization (Supplementary Fig. 2). Mild fixation using 2.5% formaldehyde is known to retain the membrane fluidity and biological activity of treated cells thereby enabling them to interact directly with other cells cultured via direct contact [6]. Others have demonstrated the role and importance of decellularized, extracted ECM in the regulation of cell functions as it possesses all the intrinsic cues necessary to communicate with and influence cellular differentiation [7, 8]. Likewise, we expected the chemically fixed EC and its ECM to influence and modulate the differentiation of human MSC towards an EC-type. Human MSC cultured atop chemically fixed EC (Fig. 1c), ECM (Fig. 1d), or controls (Fig. 1e) adhered and exhibited normal MSC morphology with typical spindle-shaped morphology and abundant ECM. Interestingly, for the human MSC cultured atop the chemically fixed EC (Fig. 1c), cell clustering and network formation was noted as early as 48 h of culture probably suggesting their differentiation initiation. Beyond 48 h, human MSC that were cultured with EBM-2 medium (Fig. 2a, b, c) or with HMSC medium (Fig. 2d, e, f) atop chemically fixed EC (Fig. 2a, d) or its ECM (Fig. 2b, e) continued to depict distinctly unique morphologies including cluster formation, and cells appearing cobble stone shaped and reduction in the amounts of secreted ECM. For the controls (Fig. 2c, f) that were cultured using EBM-2 (Fig. 2c) appeared to exhibit cobblestone morphologies (EC-type) compared to cells that were cultured using HMSC medium (Fig. 2f). This highlighted the combined effect of underlying substrate and soluble growth factor present in culture medium in inducing differentiation in cultured human MSC.

After 7 days of culture, cultured human MSC visually seemed to have proliferated when assessed atop all substrates confirming that culturing MSC with EBM-2 did not adversely affect them. CD31 expression was highest in human MSC cultured atop fixed EC (Figs. 3, 4a, Supplementary Fig. 3) and its ECM (Figs. 3, 4b, Supplementary Fig. 3) using EBM-2, and in cells cultured atop fixed EC using HMSC medium (Figs. 3, 4d, Supplementary Fig. 3) compared to other wells (Figs. 3, 4, Supplementary Fig. 3). This was a unique and interesting finding as both soluble and immobilized growth factors are required for cell

differentiation and in the absence of the former (soluble), immobilized growth factors can still have substantial effects on cell morphology and differentiation in culture [17, 21]. Besides in all MSC cultured using EBM-2 distinct cobble stone shaped morphologies was noted (Fig. 3a, b, c) in comparison to cells cultured using HMSC medium (Fig. 3d, e, f). Further, the observation that human MSC cultured with EBM-2 (Fig. 3a, b, c) appeared to mimic more closely the cobblestone morphology of EC-type cells implies that the differentiated EC-type cells were sensitive to VEGF supplementation (Fig. 3a, b, c). Quantitative assessment of percentage of CD31 positive cells further confirmed this observation (Fig. 5; Table 1). However the percentage of CD31 expression was apparently higher (Table 1), compared to immunofluorescence results in MSC cultured atop chemically fixed EC, or its ECM (using EBM-2 or HMSC medium) since there might have been cross mixing of fixed EC, or its ECM with the cultured MSC in the mixture used for analyzing via flow cytometry. So better isolation techniques that would refine and lead to selective removal of only the cultured MSC from the bottom fixed EC layer will need to be implemented.

Discussion

Autologous cell-based therapies are a developing strategy to engineer or rebuild defunct, diseased tissues to promote perfusion of oxygen and nutrients to otherwise deprived tissues. For all these applications such as in wound healing, the end-goal is to achieve revascularization, which is often the rate-limiting step in engineering new tissues. Establishing a robust blood vessel network is essential to sustain the immense metabolic demands of inflammation and neotissue engineering and promotes more rapid resolution of the damaged tissue [1]. While autologous EC can be derived from biopsies and cultured for this purpose, there are two major limitations that using stem cells such as human MSC, can overcome. The first is that adult cells do not have the same proliferative capacity as stem cells. The second is that these cells are not easily accessible in large enough quantities via biopsy. So our proposed work will directly affect and enhance therapies utilizing autologous MSC as a cell source for generating vascular cells to be used for wound healing or engineering healthy or replace diseased tissues.

As ECM is known to impact differentiation, the most direct method to differentiate MSC would be to contact them with EC or its derived ECM [3, 4, 7]. We proposed to do exactly that via a chemically fixed bed of EC and their decellularized ECM. This kind of direct contact with distinct differentiated cells may be a critical determinant of MSC fate as suggested by others attempting to co-culture MSC with EC [3, 4, 22].

Interestingly many studies have explored the effects of co-culture of human EC and MSC in 3D culture systems [23]. In one such study, human umbilical vein endothelial cells co-cultured with human bone marrow MSC in a 3D spheroid co-culture system, inhibited adipogenic differentiation and proliferation of MSC and selectively promoted their osteogenic differentiation [23] on the other hand. In another recent study, human MSC inhibited EC proliferation and angiogenesis via direct cell-cell contact in a 3D culture system [24] further demonstrating the possibility that human MSC can also lead to stabilizing an EC layer and promote a stable lining formation leading to a neoblood vessel

formation in vitro [25]. So, it is clear from these recent reports [23, 24] that both cell types, MSC and EC interact with each other, the results of which can be beneficial for tissue engineering applications. In this study, we showed that non-viable chemically fixed EC or its ECM can also modulate the differentiation of human MSC towards an EC-type by a simple direct culture method. This observation is in line with our earlier published work [5, 6], where we highlighted the role of residual cell-membrane mobility in feeder cell layers post chemical-fixation for culturing pluripotent stem cells. For the first time, our pioneering work revealed the role of biological activity of cell-derived substrates for maintaining an undifferentiated state in induced pluripotent stem (iPS) cells [6]. Membrane fluidity was shown to be an important parameter for cell culture substrates necessary for modulating mechanisms responsible for maintaining pluripotency or inducing differentiation in cells cultured atop such substrates [6]. Likewise, from the results of our current study, it can be inferred that mild chemical fixation did not possibly alter the biological activity of the substrate EC or EC-derived ECM, which allowed them to interact and induce differentiation in human MSC cultured atop them.

Although our results are promising, a complete comprehensive protocol for differentiation of MSC into EC is yet to be elucidated via this route. Others have highlighted the role of collagen IV in the ECM [26] essential for differentiation towards an EC-type. Further we did not have a scope in this study to sort cells using the marker Flk-1 for characterization of EC-type cells [26]. Also the role of specific growth factors such as VEGF in soluble vs. immobilized mode for further expansion and maturation of differentiated MSC into a mature EC phenotype, need to be explored [27, 28].

Conclusion

In this study it was shown that a simple direct contact culture of human MSC atop non-viable chemically fixed EC or its ECM can modulate the differentiation of the former into the latter cell type. This finding is novel, the method is scalable and can be easily adopted or modified to test paracrine interactions among other cell types as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Upper panel: **a** Confluent EC were either chemically fixed (chemically fixed EC (Plate-1)), or **b** first decellularized and then the remnant ECM was histologically fixed (chemically fixed decellularized ECM derived from EC (Plate-2)) after 7 days of culture. Controls included wells coated with growth serum for HMSC culture. Shown above are phase-contrast images in which scale bar in both images depicts 100 μ m. Lower panel: Human MSC seeded in Plate-1 (**c**), Plate-2 (**d**) and control wells (**e**) respectively (from left to right) imaged after 48 h of culture. Upto 48 h all wells were cultured using complete growth medium for human MSC. Human MSC grown atop fixed EC cells in Plate-1 adopted slightly different morphologies (circled) compared to cells grown in Plate-2 and control wells. Not much difference was noted in the morphologies of HMSC grown in Plate-2 and controls. Scale bar in all images depicts 100 μ m

Fig. 2.

Human MSC grown in Plate-1 (a), Plate-2 (b) and control (c) respectively (from left to right) imaged after 72 h of culture. For the first 48 h HMSC were cultured using growth medium for HMSC, after which the medium was replenished with EC culture medium (top row). As a control for this experiment, HMSC were cultured atop substrates identical to Plate-1 (d), Plate-2 (e) and control (f) respectively for which cells were cultured using HMSC medium for the first 48 h after which the medium was replenished with fresh HMSC medium (bottom row). Scale bar in all images depicts 100 μm

Fig. 3. Human MSC grown in Plate-1 (**a, d**), Plate-2 (**b, e**), and controls (**b, f**) respectively (from left to right) imaged after 7 days of culture. In the top row shown are cells cultured using EC medium and in the bottom row are cells cultured using HMSC medium. Cells were stained using antibody for CD31 (green) and imaged using both phasecontrast and fluorescence. Scale bar in all images depicts 100 μ m (color figure online)

Fig. 4. Human MSC grown in Plate-1 (**a, d**), Plate-2 (**b, e**) and controls (**c, f**) respectively (from left to right) imaged after 7 days of culture. In the *top row* shown are cells cultured using EC medium and in the *bottom row* are cells cultured using HMSC medium. Cells were stained using antibody for CD31 (white) and imaged using both fluorescence. Scale bar in all images depicts 150 μm (color figure online)

Fig. 5.

Human MSC grown in Plate-1, Plate-2, and controls, cultured using EC- or MSC-medium were analyzed for % CD31 expression using FACS analysis. One characteristic plot is depicted for each sample per case

Table 1

Human MSC grown atop various substrates were analyzed for their expression of CD31 using flow cytometry after 7 days of culture, results of which are summarized

Cultured using	Substrate	Percent (%) of cells out of total cells expressing CD31
EBM-2 EC-medium	Chemically fixed EC (Plate-1)	75.8 ± 1.2^a
EBM-2 EC-medium	Chemically fixed ECM derived from EC (Plate-2)	70.2 ± 3.8^a
EBM-2 EC-medium	Control (coated with growth serum)	14.1 ± 1.8
HMSC media	Chemically fixed EC (Plate-1)	85.4 ± 14.2^a
HMSC media	Chemically fixed ECM derived from EC (Plate-2)	26.2 ± 2.2
HMSC media	Control (coated with growth serum)	1.18 ± 0.15

^a Indicates values significantly different compared to other non-denoted values. However all significant data points did not have any significant difference among themselves