

Wound healing properties of mesenchymal conditioned media: Analysis of PDGF, VEGF and IL-8 concentrations

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SUMMARY

Regenerative medicine has become a mainstay in recent times, and employing stem cells to treat several degenerative, inflammatory conditions has resulted in very promising outcomes. These forms of cell-based therapies are novel approaches to existing treatment modalities. Mesenchymal stem cells (MSC) are emerging as the most versatile amongst all the existing stem cell technologies as they are multipotent and have shown promise in treating several disease conditions. The mode of action of these cells can be based on cell-cell contact or by paracrine effects. In this study, we compared the concentrations of the cytokines PDGF, IL-8, and VEGF between conditioned and spent media of MSCs to evaluate their potential therapeutic properties for wound healing in inflammatory conditions. We hypothesized that conditioned media contains higher concentrations of wound healing cytokines compared to spent media. We found that while IL-8 and VEGF were present in highest concentrations in conditioned media, PDGF was present in maximal amounts in spent media.

INTRODUCTION

The wound healing cascade involves complex coordination of fibroblasts, keratinocytes, platelets, and mesenchymal stem cells that 'hone' in due to inflammation caused by a wound. This leads to tissue modelling, aided by the surrounding matrix to result in wound closure (1).

Mesenchymal stem cells (MSC) are multipotent stem cells, meaning they have the capacity for self-renewal and differentiation, which make them excellent candidates for regenerative medicine (2). These cells have a unique fibroblastic morphology and are plastic adherent. They have the ability to differentiate into osteoblasts, chondrocytes and adipocytes (3). They can also trans-differentiate into neurons and hepatocytes. MSCs can be found in many tissues such as bone marrow, the umbilical cord, and tooth pulp, and they are employed widely in the field of regenerative medicine to treat incurable diseases (4, 5).

The immunomodulatory mechanism of MSCs is mediated either by cell-cell contacts or by secretion of biologically active proteins. MSCs are known to secrete a large array of anti-inflammatory cytokines that suppress activated T cells, natural killer cells (NK cells) and B cells (6). These cytokines include macrophage colony stimulating factor (M-CSF), transforming growth factor (TGF) beta, vascular endothelial growth factor (VEGF), IL-1, IL-6, stem cell factor (SCF), stromal-cell derived factor (SDF-1), IL-8, and platelet-derived growth factor (PDGF) (7). These proteins are functionally diverse and have

a far-reaching impact in regenerative medicine. They can produce effects ranging from reduction of inflammation to wound healing and can be used as cell-free systems, thereby circumventing the need of cell-based systems (8). MSCs are therefore considered to be excellent candidates for treating inflammatory diseases and autoimmune disorders (9). PDGF, VEGF and IL-8 cytokines are well documented for their role in wound healing and can be harnessed for treating non-healing ulcers (10). Platelet-derived growth factors (PDGF) comprise a larger family of growth factors, sharing qualities similar to those observed in VEGF. They are primarily responsible for the regeneration of lost blood tissue during the wound healing cascade and have proven to be a potent mitogen, chemo-attractant and survival factor for cells of mesenchymal origin such as fibroblasts, smooth muscle cells or glial cells (11). Due to their regulation of actin microfilaments, these factors can also be responsible for changing the shape of cells in connective tissue (blood). Vascular endothelial growth factor (VEGF) is responsible for the regulation of vasculogenesis – the development of blood vessels from precursor cells during early embryogenesis – and angiogenesis – the formation of blood vessels from pre-existing vessels at a later stage. Its functions include pro-angiogenic activity, increasing vascular permeability and stimulation of cell migration in macrophage lineage and endothelial cells (12). Interleukin-8 (IL-8) or neutrophil activating peptide is a cytokine belonging to the CXC chemokine family. It is released by macrophages and is responsible for the recruitment of neutrophils. IL-8 is known as a significant chemo-attractant that aids the process of cell migration and chemotaxis and a major mediator of the inflammatory response *in vivo* (13). It is, in essence, a pro-inflammatory cytokine with pro-angiogenic, proliferative and pro-motility activities (14).

Media collected from growing MSCs is either known as conditioned media or spent media. While conditioned media is collected during the exponential growth phase of cells grown in serum-free media for less than 24 hours, spent media is derived from exponentially growing cells cultured in serum-containing media for at least three days. This investigation aimed to compare the cytokine concentrations of the two media as it is generally well-established that MSC-media is rich in growth factors which can be concentrated, characterized, and employed for treating several clinical conditions (15). In the present study, we hypothesized that bone marrow-derived MSCs (BMSC)-conditioned media contained higher levels of pro-angiogenic, wound healing cytokines – PDGF, VEGF, and IL-8 – than spent media.

RESULTS

Cytokine levels in BMSC-conditioned and spent media

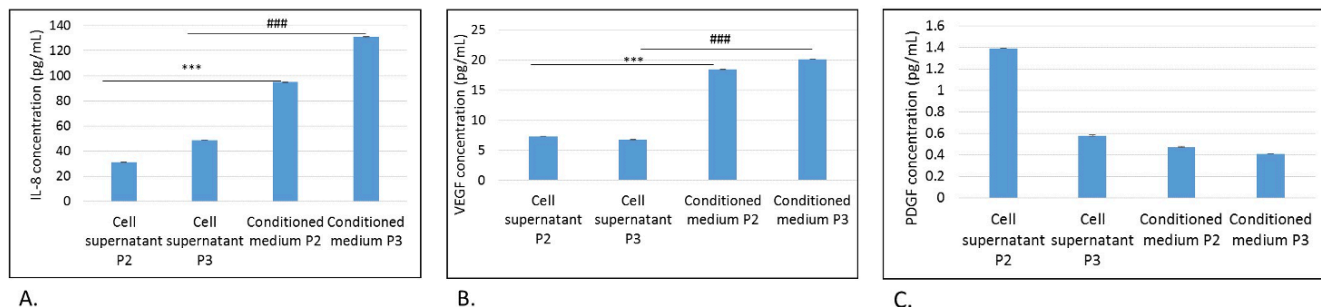


Figure 1: Bar graph showing the levels of IL-8, VEGF, and PDGF in BMSC-conditioned and spent media at P2 and P3 (N=3, mean \pm SD). Significance is shown as '***', and '###' for $p < 0.001$ when comparing the cytokine levels in conditioned media with the cell supernatant at P2 and P3, respectively.

This investigation aimed to compare the PDGF, VEGF, and IL-8 cytokine concentrations between conditioned and spent media via the use of ELISA. Conditioned media showed the highest IL-8 and VEGF levels, while PDGF levels were highest in spent media (**Figure 1**). The spent media contained 31.33 pg/mL of IL-8 at P2, which increased to 48.92 pg/mL at P3. However, the conditioned media showed significantly higher amounts of IL-8 with 94.9 pg/mL and 130.72 mg/mL at P2 and P3, respectively ($p < 0.001$) (**Figure 1A**). VEGF levels were 7.3 pg/mL and 6.8 pg/mL in cell supernatants as compared to 18.42 pg/mL and 20.16 pg/mL in the conditioned media at P2 and P3, respectively ($p < 0.001$). PDGF concentrations were 1.39 pg/mL and 0.57 pg/mL in cell supernatants at P2 and P3. However, PDGF levels were minimal in the conditioned media. Thus, while IL-8 and VEGF concentrations were the highest in conditioned media, PDGF was highest in spent media.

DISCUSSION

This investigation was based on the hypothesis that BMSCs-conditioned media contained higher concentrations of wound healing cytokines than BMSCs-spent media. In our study, we found that conditioned media derived from growing BMSCs had significantly higher levels of VEGF and IL-8. On the contrary, PDGF was present in higher concentrations in spent media compared to VEGF or/and IL-8. By extension, we determined conditioned media of BMSCs at P2 and P3 was enriched in IL-8 and VEGF cytokines, whereas PDGF levels were the lowest in conditioned media. These findings support our hypothesis to an extent in that conditioned media of BMSCs contains higher levels of certain cytokines than spent media. An investigation into the anomaly presented by the PDGF concentration between conditioned and spent media constitutes further study.

Our results suggest that BMSC-conditioned media can be further concentrated and used for clinical purposes, as the media is highly enriched in cytokines that are relevant to wound healing. Some of the key cytokines required for the wound healing process include VEGF, IL-8, and PDGF (16), which we found to be present in significant albeit varying degrees within BMSC-conditioned media.

Recent studies similarly showed significant increases in VEGF levels (12, 13) as well as an increase in IL-8 levels in BMSC-conditioned media (14, 15). Furthermore, literature suggests that a combination of two or more growth factors and cytokines is necessary to achieve optimal neo-angiogenesis

(17). Growth factors from conditioned media contain several cytokines and exosomes, which also are known to contain a variety of growth factors and cytokines necessary for a wide gamut of functions ranging from anti-inflammatory to neo-angiogenesis (18, 19). Exosomes are lipid vesicles secreted by a variety of cells, particularly MSCs, which are rich in proteins, miRNA and lipids (20). They are known for their tissue repair capabilities (21) and their therapeutic efficacy in treating Graft Versus Host Disease (22). The results have been extremely encouraging. As the results demonstrate the presence of wound healing cytokines, the present study serves as a preliminary attempt for their enrichment and further analysis.

MATERIALS AND METHODS

Maintenance of BMSCs

Bone marrow-derived mesenchymal stem cells (BMSCs) were obtained from Lonza. BMSCs were grown in serum-free and serum-containing media to collect conditioned media and cell-spent media, respectively, at passage 2 (P2) and passage 3 (P3). Passages are the number of times the cells are removed at confluency and re-seeded in a flask. BMSCs at passage 2 to passage 4 (P2-P4) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) (Invitrogen) in a six-well plate. The cultures were maintained in a humidified incubator with 5% CO₂ at 37°C.

Preparation of conditioned media

BMSC-conditioned media is obtained by culturing exponentially growing cells in serum-free media for a maximum of 24 hours. At 70-80% confluency of BMSC culture (P2 and P3), media was replaced with serum-free media and collected after 17 hours of incubation. The collected media was centrifuged at 1500 rpm for 10 minutes, and the supernatant was collected. Supernatants were aliquoted and stored at -80°C.

Collection of spent media

Spent media is obtained by culturing cells in serum-containing media for 3-4 days. Media was collected from 70-80% confluent BMSCs culture (P2 and P3) and centrifuged at 1500 rpm for 10 min. The supernatant was aliquoted and stored at -80°C.

Cytokine analysis

Enzyme-linked immunosorbent assays (ELISA) were used to

analyze the levels of interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) in the conditioned and spent media using kit-prescribed protocol (RayBio).

Statistical analysis

The data was analyzed using GraphPad Prism 8 software. Statistical differences were determined by using an ANOVA with Tukey's post-hoc test. Three independent experiments were performed. The mean values of the cytokines studied are represented in **Figure 1** as bar graphs with standard deviation (SD values) as error bars.

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