JOURNAL OF
EMERGING INVESTIGATORS

Article

Herbal formulation, HF1 diminishes tumorigenesis: a cytokine study between MCF-7 and BM-MSCs.

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SUMMARY

Tumorigenesis is a result of favorable interactions between the tumor microenvironment (TME) and tumor cells. The microenvironment hosts several cytokines, soluble factors, and signaling factors secreted by stromal cells which accelerate the progression of cancer. Bone marrow mesenchymal stem cells (BM-MSCs) invade the TME and are hypothesized to differentiate into cancer associated fibroblasts (CAFs) by the action of Transforming growth factor beta (TGF-β) and Vascular Endothelial growth factor VEGF, some of the key cytokines involved in this process.

This study aimed to investigate expression of cytokines with different conditioned media using the MCF-7 breast cancer cell line and BM-MSCs. Furthermore, we also studied the effectiveness of a herbal formulation (HF-1) in countering the expression of these cytokines. BM-MSCs secreted significantly higher concentrations of the cytokines when in co-culture with MCF-7 cells. Furthermore, the diminished concentrations of VEGF and TGF-β observed post-addition of HF-1 demonstrates its potential applications in preventing tumorigenesis and cancer metastasis.

INTRODUCTION

Cancer is one of the leading causes of death, particularly in the current global context with breast cancer being the leading cause of death in women (1). The rising danger cancer poses to the human population, threatening to upstage heart disease as the primary cause of death by 2050, mandates extensive study into its pathophysiology and development. The “seed and soil” hypothesis states that tumor cell proliferation and cancer progression occur due to the complex interactions between the tumor cells and the tumor microenvironment (TME). Tumor cells can only metastasize under favorable conditions, underscoring the role TME plays in cancer progression. Consequently, cancer therapy targeting the TME is an area of extensive study (2). The TME is heterogeneous in nature, comprising both cellular and non-cellular components. Stromal cells, such as immune cells, adipocytes, pericytes, macrophages, fibroblasts/bone marrow mesenchymal stem cells (BM-MSCs), mesenchymal stromal cells (MSCs), the extracellular matrix (ECM), the surrounding blood vessels, and the soluble factors, like cytokines and growth factors, combined make up the TME (3, 4).

Cancer-associated fibroblasts (CAFs) are the most potent and aggressive cells present in the TME and lead to metastasis and tumor proliferation through the secretion of soluble factors and remodeling of the ECM. They are formed from previously healthy cells present in the TME and surrounding organs, such as epithelial cells, endothelial cells, MSCs, BM-MSCs, resident fibroblasts, adipocytes, pericytes, and fibrocytes.

The significance of CAF and Transforming growth factor beta (TGF-β) crosstalk underscores the importance of further study into elucidating TME mechanisms. TGF-β acts as a tumor promoter in late-stage cancer cells by inducing invasion of cancer cells through the basement membrane, thus allowing them to invade the circulatory system, and promoting epithelial to mesenchymal transition (EMT). TGF-β also plays a significant role in the interaction of cells in the TME, promoting the conversion of fibroblasts and MSCs into CAFs (5). TGF-β has historically proven to contribute hugely to the development of CAFs through the MiR-21/Smad 7 pathway (6,7,8).

CAFs, in turn, secrete an increased number of cytokines, such as Vascular Endothelial Growth Factor (VEGF) (9). VEGF is a critical mediator in angiogenesis, resulting in an increased supply of nutrients and oxygen to the tumor, stimulating tumor cell proliferation (10).

BM-MSCs are multipotent stromal cells. They have been studied in the context of immunoregulation, tissue regeneration and more recently, specifically in the TME. BM-MSCs move towards tumor cells in the TME in response to the expression of cytokines secreted by stromal cells, particularly CAFs. They then help to create a favorable microenvironment for metastasis through differentiation. It has been established that these BM-MSCs support the growth of cancer cells, and thus promote tumorigenesis (11).

Tumors are made up of both tumor and stromal cells, which both, along with extracellular factors, make up the TME. CAFs are fundamental part of tumor progression and invasion, and promote, either directly or indirectly, oncogenic processes such as angiogenesis and immunosuppression. They also have an altered gene expression as compared to normal fibroblasts (12, 13). While there have been numerous studies attempting to differentiate normal fibroblasts from CAFs, the distinction remains largely unclear due to the lack of exact means to distinguish between the two cell types (14). While it can be argued that CAFs are theoretically fibroblasts

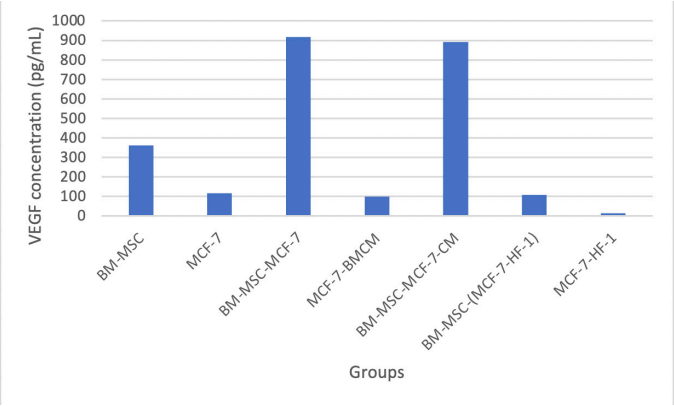

Figure 1. Bar graph showing VEGF concentration in different groups and the modulatory effect of HF-1 (n = 3, mean ± SEM). BM-MSC + MCF-7-CM and BM-MSC + MCF-7 groups showed significant increase ($p < 0.001$), shown as '***' with respect to BMMSC control and was significantly lowered ($p < 0.001$) by HF-1, shown as '###'. (n = 3)

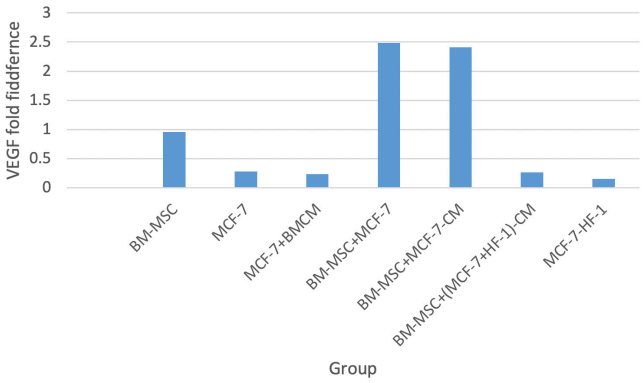

Figure 2: Bar graph showing the fold difference in VEGF expression across different groups and the modulatory effect of HF1 (N=3, mean±SEM). BMSC+MCF-CM and BMSC+MCF-7 groups showed significant increase ($p<0.001$), shown as '***' with respect to BMSC control and was significantly lowered ($p<0.001$) by HF-1, shown as '###'.

interacting with a TME to form CAFs by Notch signaling, this mechanism is yet to be confirmed by consistent testing (15). This said, it is possible that CAFs and fibroblasts possess similar transcription regulatory mechanisms by virtue of the former stemming from the latter. CAF formation could also be triggered by high Interleukin-1 (IL-1) signaling, via the STAT pathway (16). Nonetheless, CAFs have consistently proven to possess a spindle shaped morphology (17).

In the TME, there exists a pool of growth and soluble factors, secreted by the tumor, which aid in cancer progression and tumor invasion. One of these factors, TGF-β, has been shown to promote the differentiation of progenitor cells such as BM-MSCs into CAFs, and directly regulate the expression of VEGF by CAFs in the ECM, promoting angiogenesis and lymph-angiogenesis (17). TGF-β also plays an integral role in EMT and thus, aids in the creation of more CAFs (18). Moreover, CAFs have been found to secrete such cytokines and growth factors, creating a positive feedback loop of cytokine expression (19). Thus, increased expression of cytokines stimulates and is a characteristic of CAF formation. Research has shown that metastatic tumors manipulate the regenerative function of progenitor cells such as BM-MSCs and recruit them into the TME, where a significant portion proliferate and differentiate stromal cells. Inflammatory reactions in cancer cause the recruitment of BM-MSCs, as they are often associated with tissue remodeling (16, 20). The response of the BM-MSCs to the TME is guided by inflammatory cytokines. CAFs can be targets of these cytokines, as well as induce tumor promoting activation signals and secrete soluble factors which in turn aid in remodeling of the TME (21). CM from malignant tumor cells can be used to mimic the cytokine saturated environment of the TME, which attracts BM-MSCs. The CM also induces a change in morphology of the BM-MSCs. The BM-MSCs also acquire a similar morphology to that of a CAF (22).

Conditioned media (CM) is broadly defined as the cell secretome, rich in intracellular proteins and those stemming from the cell surface. The significant concentrations of growth factors and cytokines present in CM deem it an increasingly viable resource for wound-healing studies (23). CM can be applied in countering degeneration and promoting regenerative pathways (24). Considering this, CM was used in this study to determine if it, in any way or form, contributed to angiogenesis and abetted the amelioration of the TME.

MCF-7 is the breast cancer cell line, originating from a 69-year-old with the disease in 1970 and is the first cancer cell line to respond to hormones (12). BM-MSCs and MCF-7 were co-cultured in this study due to the propensity of the latter cell line to undergo revascularization pathways, thereby contributing to cancer metastasis (25). Additionally, given that MCF-7 is a highly regulated by growth factors present in plasma, the co-culture of MCF-7 and BM-MSCs would serve as a secondary metric for the increased secretion of VEGF and TGF-β (26). This provided us with the rationale to utilize MCF-7 in this study.

We hypothesized that MCF-7 interactions which BM-MSC have contributed to a potential enhancement of tumorigenic properties in vitro e.g., increasing vascular permeability via VEGF and contributing to chemotaxis via TGF- β. This was performed to imitate – in part – the true complexity of oncogenesis and TME in vivo. Further studies in consideration of more parameters are surely mandated, this investigation aimed to observe cell-cytokine interactions. In testing herbal formulation (HF-1) with relation to cancer cell proliferation and the widely hypothesized BM-MSC differentiation into CAFs in the TME, we were interested in considering if cell-MSC interactions could be curbed by HF-1 alongside studying the extent to which the aforementioned hypothesis could be validated in parallel (27).

Given that the formulation comprises Epigallocatechin-3-gallate (EGCG) and curcumin, substances which are currently being studied extensively for their potential potent

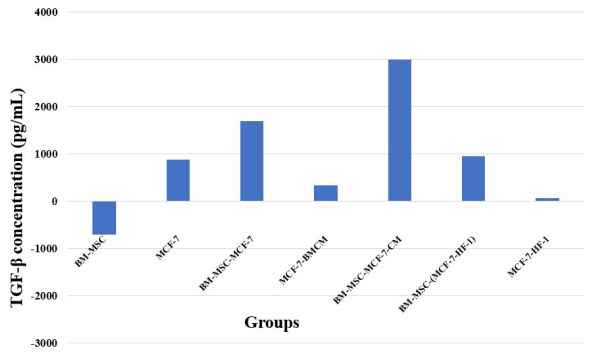

Figure 3: Bar graph showing TGF-β concentration in different groups and the modulatory effect of HF1 (N=3, mean±SEM). BMSC+MCF-CM and BMSC+MCF-7 groups showed significant increase (p<0.001), shown as '***' with respect to BMSC control and

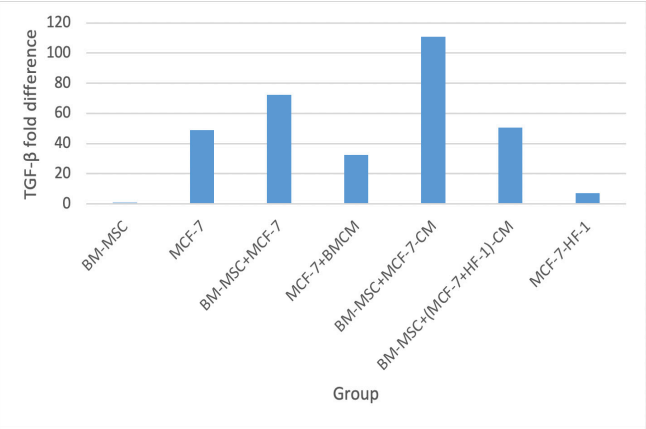

Figure 4: Bar graph showing the fold difference in TGF-β expression across different groups and the modulatory effect of HF-1 (n = 3, mean ± SEM). BM-MSC + MCF-7-CM and BM-MSC + MCF-7 groups showed significant increase (p < 0.001), shown as '***' with respect to BM-MSC control and was significantly lowered (p < 0.001) by HF-1, shown as '###'. (n = 3)

anti-cancer properties, we were interested in determining the extent to which it inhibited the TME (28). EGCG and curcumin have proven potent antioxidant, immunomodulatory, and anticancer properties which makes them particularly relevant in the field of integrative oncology (29, 30). Curcumin has previously inhibited tumor progression and angiogenesis in ovarian cancer via the nuclear facto- κB pathway, while EGCG has been indicated to be the most potent anticancer compound present in green tea (31, 32). While the full molecular characterization of these compounds with regards to cancer remains to be explored, our results are affirmed considerably by the existing literature on the subject.

Our studies reflected a decrease in both cytokine expressions due to HF-1 addition. It is to be noted that this finding can be extended to further studies conducted in a similar vein. These can include the consideration of varied experimental coordinates: gene expression studies, other cytokine studies involved in cancer processes, and delving into cancer's immunosuppressive properties.

RESULTS

This experiment aimed to investigate whether MCF-7 and BM-MSC interactions corresponded to high TGF-β and VEGF expression and if HF-1 lowered the TGF-β and VEGF expression

VEGF concentration across groups while **Figure 2** shows the fold difference in VEGF expression between the groups. VEGF was doubled in the groups where BM-MSCs were co-cultured with MCF-7 and were treated with MCF-7-CM (**Figure 1**). VEGF was 95.6 times higher in BM-MSCs co-cultured with MCF-7 (**Figure 2**).

However, there was a marked difference in the overall expression in the two cytokines, reflected by **Figure 3**, exhibiting TGF-β concentrations. TGF-β was significantly less (p value < 0.001) in BM-MSCs but was in abundance in the BM-MSCs cultured in MCF-7 CM (**Figure 3**). From **Figure 4,** displaying the fold differences between the groups in TGF-β expression, the value of BM-MSCs treated with MCF-7 was 146 times higher than in the control group (**Figure 4**).

For both cytokines, expression in MCF-7 and BM-MSC control groups were minimal. Moreover, TGF-β and VEGF production was much higher in the groups where MCF-7 cells were co-cultured with BM-MSCs (p value < 0.001), indicating that the soluble factors secreted by MCF-7 cells stimulated differentiation of BM-MSCs into CAFs.

Our results demonstrated an increased production of VEGF and TGF-β from BM-MSC grown in MCF-7 CM, thereby supporting the hypothesis of CAF formation. The use of HF-1 diminished concentrations of both TGF- β and VEGF, as seen by significantly lower concentrations of both cytokines post-treatment. The p value < 0.001 mirrors extreme statistical significance of the obtained findings. This substantiated our second hypothesis significantly.

DISCUSSION

In this study, the Marquis reagent tested with the poppy seThis study was conducted to determine the effects of co-culture between MCF-7 and BM-MSCs by quantifying TGF-β and VEGF concentrations. Similar results were found in the group treated with HF1.

Our results demonstrated substantial backing for both hypotheses. For the purposes of clarity, we will be discussing the implications of the obtained results for each individual cytokine.

We demonstrated that the highest concentration of TGF-β was obtained in BM-MSCs grown with the MCF-7, thereby promoting tumorigenesis (33-35). Our research shows that

it is potentially possible that the cytokines expressed by malignant MCF-7 cells can be linked to the differentiation over time of BM-MSCs into CAFs, as marked by increased expression of TGF-β and VEGF cytokines. This strongly supports the hypothesis that soluble factors and signaling molecules facilitate BM-MSC differentiation into tumor progressor phenotypes.

The low amounts of TGF-β found in BM-MSCs can be attributed to the amelioration of their regenerative properties including their characteristic maintenance of the ECM alongside their increased self-proliferation, evident in a rat model of lipopolysaccharide-induced acute lung injury (36). In this manner, the diminished concentrations of TGF-β allows for the rejuvenation of the defining BM-MSC regenerative capacity (37). The difference between expression of TGF-β in MSC and MCF-7 can potentially be attributed to the difference between the rates of division of both cells, apart from the general higher TGF-β by growing cancer cells (38).

These results were echoed in the case of our tests for VEGF expression in the cells, with the CM group demonstrating the highest concentrations of VEGF. According to literature, this finding is justified on the basis that in the presence of migrating BM-MSCs in the TME, the MCF-7 cells morph into a significantly more aggressive phenotype (39). Cancer has proven to recruit MSCs into the TME to facilitate vasculogenesis, thereby accelerating the spread of cancer and the further amelioration of the TME (40, 41). Given the considerable regenerative properties of BM-MSCs, the VEGF concentration observed in this case is justified. However, the VEGF concentration in MCF-7 alone is lower than the co-culture group, albeit significant, which can be attributed to the intrinsic migratory properties of cancer.

Our results conclusively support our hypotheses via showing that HF-1 inhibits VEGF and TGF-β concentrations across all applied groups. HF-1 predominantly is comprised of EGCG and turmeric, potent phytochemicals which are under study for their future employment in integrative oncology studies (42). A study by Wang et al. demonstrated that EGCG and turmeric co-interactions ameliorated the other's intrinsic anticancer properties, as observed (43). Both compounds possess benzyl rings and hydrophobic groups (-CH3) which are key facets of their anti-proliferative capacities (44, 45). The high electrophilicity thus conferred to each molecule independently has been speculated to aid in prompting the apoptotic cascade in tumor cells by chelating with DNA via ions such as Cu2+, Ni2+, and Fe2+ (46, 47). While the efficacy of HF-1 can be attributed to these properties to a large extent, further investigations are necessary to truly map out the interactions between EGCG, turmeric, and the TME (48).

Insofar as our study has demonstrated that addition of HF-1 has a positive counter effect against MCF-7 cell and mesenchymal interactions based on two cytokines, multiple other elements have yet to be included to truly emulate the complexity of TME interactions. Further studies can include exploring not only cytokine but also gene expressions to arrive at a more realistic portrayal of the true complexity of cancer interactions in vivo. Furthermore, experimentation with different cancer cell types would allow for an enhanced scope of the current study.

MATERIALS AND METHODS

Cell lines

The MCF-7 breast cancer cell lines used were obtained from the National Centre of Cell Science (NCCS) in Pune, Maharashtra, and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% Fetal Bovine Serum (FBS) (Invitrogen). We maintained cells in an incubator at 37°C and 5% CO2 saturation. Cells used were in the range of passage 5-9 for conducting the experiments.

The BM-MSCs were grown in house on 3D collagen scaffolds (in-house), which mimics a 3D environment. The environment that the scaffold provided was similar to the TME.

IC_{50} Calculation

The IC_{50} value for HF-1 was arrived at using IC_{50}.tk software (49).

IC_{50} value for HF-1 was 1.2mg/mL. Half the IC_{50} concentration was utilized for the experiment to observe cytokine expressions as higher concentrations would have led to cell cytotoxicity.

Preparation of MCF-7 Conditioned Media (MCF-7-CM) and BM-MSC Conditioned Media

(BM-MSC-CM) and MCF-7 with herbal formulation conditioned medium (MCF-7-HF-1-CM) MCF-7 cells were grown in 10% FBS (FBS; Invitrogen) in DMEM (DMEM; Invitrogen) until they achieved a high degree of confluency (~75%). The media was not changed for a period of 18h, to induce enhanced cytokine expressions, prior to the formation and collection of the MCF-7-CM. The supernatant was collected, centrifuged for 10 minutes at 1500 rpm, and stored at -80°C. BM-MSC-CM was prepared in the same manner.

For the preparation of MCF-7-HF-1-CM, 80% confluent MCF-7 cells were cultured with HF-1 (0.6 mg/mL; half of IC50 value on MCF-7) for 48h in 10% FBS containing DMEM. The cells were washed and fresh DMEM without FBS was added for 18h. The medium was collected, centrifuged at 1500 rpm for 10 min. The supernatant was stored at -80°C until use.

Herbal Compound (HF-1) Preparation

We placed 0.2 g of HF-1 powder mixed with 1 mL of water at 50°C for 30 minutes, vortexed, and centrifuged at 3000 rpm for 20 min. After syringe filtration, the extract was used for experimentation.

Cell Treatment

The following groups were formed and cultured for 48h.

The MCF-7 control group had MCF-7 cells cultured in 10% FBS containing DMEM while the BM-MSC control group had

BM-MSCs cultured in 10% FBS containing DMEM.
MCF-7 cells were co-cultured with BM-MSCs in 10% FBS containing DMEM. The condition media group comprised MCF-7 cells cultured in 50% BM-MSC-CM and 50% of 10% FBS containing DMEM. Furthermore, BM-MSCs were cultured in 50% MCF-7-CM and 50% of 10% FBS containing DMEM

Lastly, the HF-1 group consisted of MCF-7 cells co-cultured with BM-MSCs in 10% FBS containing DMEM with HF-1: BM-MSCs cultured with 50% MCF-7-HF-1-CM and 50% of 10% FBS containing DMEM. MCF-7 cells were also grown in HF-1 media.

Cytokine determination

The supernatants were thawed and used for determining cytokine levels. Not more than two freeze thaw cycles were performed for each sample. Levels of VEGF and TGF-β was analyzed using Enzyme Linked Immunosorbant assay (ELISA) (RayBio).

To the precoated wells, 100 µL of standards, samples and blank were added and kept for incubation at 37°C for 90 minutes. The liquid was removed and 100 µL of Biotinylated Detection Antibody was added to all the wells and kept for incubation at 37°C for 1 hour.

Post washing thrice, 100 µL of HRP Conjugate Solution was added to the wells and kept for incubation at 37°C for 30 minutes. The plate was washed five times, post which 90 µL of substrate was added and kept at 37°C for 15-30 minutes. Finally, 50 µL of stop solution was added to all the wells and determined the Optical Density values of the samples at 450 nm using Lisa Quant ELISA Plate Reader.

The concentration of each growth factor in pg/mL was estimated using the equation y = mx + c obtained after plotting the standard and blank values in MS Excel. We used sample diluent as negative control, standards as positive control, and the OD values of the samples were subtracted with blank values.

Statistical analysis

GraphPad Prism software was used to analyze the data. A one-way ANOVA followed by Tukey's test was performed to find the statistical difference between the groups. The experiment was performed at three independent times. The data were represented as mean ± SEM.

Received: October 15, 2020
Accepted: November 15, 2020
Published: April 11 2022

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