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RESEARCH ARTICLE

Modulation of Cytokines and Apoptotic Genes in HeLa Cells and CAFs by Herbal Formulation HF20

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ABSTRACT: Cancer, a condition managed through surgeries, chemotherapies, and radiation, often encounters challenges such as drug resistance and relapse during treatment. The need for higher drug dosages to prevent relapse contributes to drug resistance and increased toxicity. Combining herbal formulations with standard treatments, complementary and alternative medicine (CAM) offers an approach to mitigate resistance and toxicity associated with standard treatments, ultimately improving outcomes. The body's balance, which has been disturbed by chemotherapy and radiation, is restored by these natural/herbal compositions. In this study, we hypothesized that the herbal formulation (HF20), derived from *Amaranthus spinosus* parts, can reduce the levels of Interleukin-6 (IL-6), a pro-inflammatory cytokine. Additionally, the research also examined the role of Transforming Growth Factor-beta (TGF-β) and Vascular Endothelial Growth Factor (VEGF) in inducing the transformation of bone marrow mesenchymal stem cells (BM-MSCs) into cancer-associated fibroblasts (CAFs). A comprehensive understanding of HF20's anticancer and anti-inflammatory properties is essential. Also, HF20 brought down IL-6 levels in HeLa Cells. Investigating cytokine expression using different conditioned media (CM) with HeLa cells and BM-MSCs, the study found that BM-MSCs secreted higher concentrations of cytokines when co-cultured with HeLa cells. Additionally, HF20 reduced the levels of VEGF and TGF-β, indicating its potential in countering tumorigenesis and cancer metastasis.

KEYWORDS: Biomedical and Health Sciences; Nutrition and Natural Products; HF20; Complementary and alternative medicine; TME; CAFs; IL-6; TGF-β; VEGF.

Introduction

Cervical cancer poses a significant risk to women's health and is one of the leading causes of mortality worldwide.[1] Conducting comprehensive research on the origins of cancer is imperative, given predictions that by 2050, deaths from cancer could surpass those resulting from heart disease.[2] India's primary traditional medical system, Ayurveda, synthesizes insights from various historical healing traditions, presenting a unique opportunity for the investigation of herbal therapies.[3]

As contemporary medicine advances, it is becoming increasingly important for researchers to close the gap between traditional and modern medical perspectives.[4] Utilizing herbal remedies is a noteworthy aspect of complementary and alternative medicine (CAM), which is gaining popularity in managing various health conditions, including cancer.[5] Integrative oncology is a framework that integrates traditional medical treatments with herbal remedies to maximize their therapeutic effects. This strategy can potentially lessen the adverse effects of conventional cancer treatments while offering additional physiological benefits.[6] Ayurveda stands out as an influential traditional medical system in India, offering a rich knowledge base for investigating herbal medicines. Integrating conventional and modern medical procedures becomes increasingly important as patients use complementary and alternative medicine (CAM), such as herbal remedies, to supplement conventional medicines.[7] Using a unique approach, herbal medications may deliver benefits, including reduced toxicity and reduced drug resistance, addressing chemotherapy's limits.

Amaranthus spinosus, a plant originally from South America but now cultivated in Asian countries, is the source of the extracts used to make HF20, an herbal medicine.[8-10] South Asian nations frequently eat this plant as a vegetable, which has medicinal value in Ayurveda and traditional Asian medicine.[10] Scientific research has focused on HF20 because of its conceivable antioxidant and anticancer properties.[8,9]

Vascular endothelial growth factor (VEGF) is a potent signaling molecule that plays a crucial role in the formation of new blood vessels. Angiogenesis is a fundamental process in tumor growth and progression, as tumors need a sufficient blood supply to receive nutrients and oxygen.[11]

The "seed and soil" hypothesis highlights the crucial role that the tumor microenvironment (TME) plays in promoting the growth of tumor cells and the development of cancer.[12] This environment comprises a wide variety of cellular and non-cellular elements. It consists of blood vessels, the extracellular matrix (ECM), stromal cells, immune cells, adipocytes, macrophages, fibroblasts, bone marrow mesenchymal stem cells (BM-MSCs), mesenchymal stromal cells (MSCs), and signaling chemicals such as cytokines and growth hormones.[13] Cancer-associated fibroblasts (CAFs), a significant subset of the TME, are crucial in promoting metastasis and tumor growth. CAFs accomplish this by altering the ECM and releasing signaling chemicals.[14] The healthy cells in the TME

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and the nearby organs are the source of these aggressive cells.[15] The healthy cells that comprise the tumor microenvironment (TME) and surrounding organs are the source of these aggressive cells.[16] In the TME, the transforming growth factor-beta (TGF-β) is essential for mediating cell connections. It starts the process that turns fibroblasts and MSCs into cancer-associated fibroblasts (CAFs) necessary for developing tumors.[17] In later stages of cancer, TGF-β also promotes tumor invasion and the epithelial-mesenchymal transition (EMT), which both help tumors grow.[18] Additionally, CAFs secrete higher amounts of cytokines like VEGF, which promote angiogenesis and promote the growth of tumor cells.[19] Notably, TGF-β significantly affects cell dynamics within the TME and is a crucial initiator of converting fibroblasts and MSCs into CAFs.[20] TGF-β significantly encourages EMT and tumor invasion, two processes that fuel the development of tumors in an advanced stage of cancer.[21] CAFs contribute significantly to the development of tumors by producing high levels of cytokines like VEGF, which promote angiogenesis and the growth of new blood vessels and aid tumor cell growth.[22]

BM-MSCs are multifunctional stromal cells that have lately gained recognition for their functions in the tumor microenvironment (TME), immunological modulation, and tissue repair.[23] BM-MSCs migrate towards tumor cells in response to stromal cells' production of cytokines, particularly CAFs, creating an environment favorable for metastasis.[24] The expression of VEGF by CAFs, which promotes the growth of new blood and lymphatic vessels, is controlled by TGF-β activity, essential for BM-MSC differentiation into CAFs.[25] BM-MSCs are drawn into the TME by inflammatory reactions in cancer-related situations, where they alter tissues and promote tumor growth.[26] Conditioned media (CM), which contains an abundance of cytokines and growth factors released by cells, is useful for analyzing the pathways involved in wound healing and regeneration.[27]

In one study, CM was used to examine how it affected angiogenesis and the TME. Researchers were able to observe the activation of pathways involved in re-establishing blood vessels. They noted increased secretion of VEGF and TGF-β by co-cultivating the HeLa cancer cell line with BM-MSCs.[28] This study made important discoveries about the complex dynamics of the tumor microenvironment.[28]

Briefly stated, interactions that take place within the tumor microenvironment have a significant impact on how cancer progresses. Cancer-associated fibroblasts (CAFs), fueled by substances including TGF-β and VEGF, are essential to tumor growth and metastasis.[29] By using conditioned media such as HeLa-CM, it is possible to stimulate other cultured cells, such as normal cells, to examine how they respond to cancer-related substances.[30] It is conceivable to mimic the TGF-β and VEGF-rich environment that characterizes the tumor microenvironment and draws BM-MSCs using conditioned media derived from tumor cells.[30] As a result, BM-MSCs exhibit noticeable modifications in their morphology and start to resemble CAFs. This method sheds light on the complex dynamics of tumor development and the part the microenvironment plays in it.[31] This study examines the cytokine modulation of IL-6 in HeLa cells when treated with HF20. Also, we hypothesized that HF20 can modulate VEGF and TGF-β.

Our studies reflected a decrease in both cytokine expressions due to HF20 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.

Methods

Preparation of the combination HF20:

The formulation of HF20 was prepared using leaves and inflorescence extracts of *A. spinosus* based on phytochemical analysis. The formulation of these two extracts showed a higher cytotoxic effect than the individual ones. So, for further studies, we have used this formulation.

Cell culture:

The HeLa cervical cancer cell lines (passage 1) used were obtained from the National Centre of Cell Science (NCCS) in Pune, Maharashtra. They were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% Fetal Bovine Serum (FBS) (Gibco). We maintained cells in an incubator at 37°C and 5% CO_2 saturation. The cells used were used with passage 8 for conducting the experiments. The BM-MSCs were grown in-house and cultured in the same conditions as HeLa.

Preparation of BM-MSC Conditioned Media (BM-MSC-CM) and HeLa Conditioned Media (HeLa-CM):

For the preparation of BM-MSC-CM and HeLa-CM, BM-MSC and HeLa cells were grown in 10% FBS (FBS; Gibco) in DMEM (DMEM; Gibco) until they achieved 80% confluency. The media was changed with plain DMEM after 20-hour incubation to induce cytokine expressions. The supernatants were collected, centrifuged for 10 minutes at 1500 rpm, and stored at -80°C.

To prepare HeLa-HF20-CM, 80% confluent HeLa cells were cultured with HF20 (18.18μg/mL; half of IC50 value on HeLa) for 48h in 10% FBS containing DMEM. The cells were washed, and fresh plain DMEM was added for 20 hours. The supernatant was collected and centrifuged at 1500 rpm for 10 min. The supernatant was stored at -80°C until use.

Groups used for cytokine analysis study:

Previously cultured passage 7 HeLa cells and passage 4 BM-MSCs were trypsinized, and cells were counted. One million cells were cultured and incubated for 24 hours. After incubation, half of the IC50 (18.18μg/mL) of HF20 conditioned media (CM) was added to the respective groups with the medium. Groups were incubated for 48 hours to observe the anti-inflammatory (IL-6) cytokine analysis. Supernatants (sup) were collected for IL-6 cytokine studies.

Seven groups of cells were cultured for VEGF and TGF-β such as HeLa, BM-MSCs, HeLa + BM-MSCs CM, HeLa + BM-MSCs (coculture), coculture + HF20, BM-MSCs + HeLa CM, BM-MSCs + (HeLa+HF20) CM, HeLa +HF20

respectively. Supernatants were collected after 48 hours of incubation.

Cytokine studies:

Interluekin-6 (IL-6) concentrations were calculated using the BD Bioscience kit (cat. No.555220). The concentrations of Vascular Endothelial Growth Factor (VEGF) (cat. No. E-EL-H0111) and Transforming growth factor beta (TGF-β) (cat. No. E-EL-0162) in different groups were assessed using commercially available ELISA kits (Elabsciences) according to the manufacturer's recommendations. The samples' Optical Density (OD) values were then calculated using the Lisa Quant ELISA Plate.

The equation y = mx + c, derived from plotting the standard and blank values in MS Excel, was used to determine the concentration of each growth factor in picograms per milliliter (pg/mL). The standards were part of the positive control, whereas the sample diluent was the negative control. By deducting the corresponding blank values, the OD values of the samples were corrected. Notably, in this process, VEGF and TGF-β had their folds diluted.

Statistical analysis:

The data was analyzed using Microsoft Excel software. The statistical difference between groups was examined using a paired t-test. n=3, p - value<0.05 considered significant.

Result and Discussion

IL-6 (Pro-inflammatory cytokine) levels:

HF20 brought down IL-6 in HeLa cells, demonstrating that HF20 has an anti-inflammatory effect on HeLa (**Figure 1**).

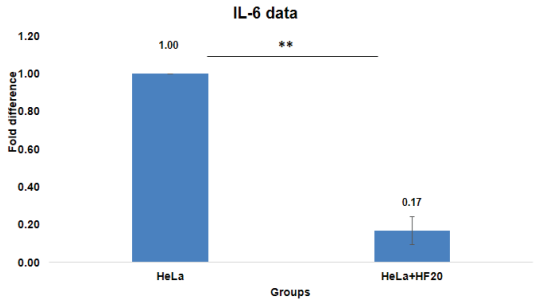

Figure 1: IL-6 cytokine levels. Downregulation in IL-6 cytokine levels subsequent to HF20 treatment in HeLa cells indicates the potential of HF20 as an effective anti-inflammatory agent. These findings highlight HF20's capacity to modulate inflammatory responses, presenting a promising formulation for therapeutic intervention in inflammatory conditions.

HF20 treatment decreased interleukin-6 (IL-6) cytokine levels on HeLa (n=3, **p<0.01), showing that HF20 has an anti-inflammatory effect on HeLa. The fold difference was calculated by dividing the treated group (HeLa +HF20) by the control group (HeLa).

As IL-6 is a pro-inflammatory cytokine, the reduction in IL-6 levels indicates a modulation of the immune response, which may contribute to the HF20's anti-inflammatory properties.

VEGF cytokine levels:

This experiment investigated whether HeLa and BM-MSC interactions corresponded to high VEGF expression and whether HF20 lowered it.

There was no significant increase in VEGF in the group of BM-MSCs-CM compared with BM-MSCs alone. VEGF was upregulated in the group where BM-MSCs were co-cultured with HeLa compared to BM-MSCs and HeLa alone group and brought down by HF20 (n=3, p<0.05) (**Figure 2**). VEGF was higher in BM-MSCs treated with HeLa-CM and brought down by HeLa HF20-CM with HeLa (n=3, p<0.05) (**Figure 2**).

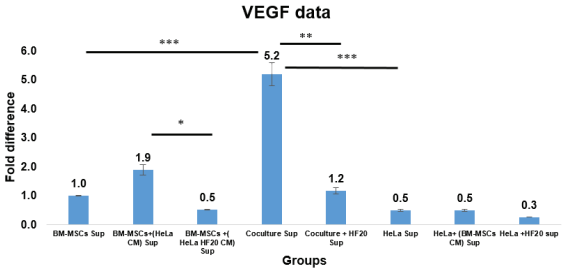

Figure 2: VEGF cytokine levels. In coculture and BM-MSC+ (HeLa CM), an increase in VEGF levels was observed, potentially attributed to CAF formation. However, coculture with HF20 resulted in a decrease in VEGF levels, indicating that HF20 might inhibit angiogenesis by reducing VEGF expression.

VEGF concentration across groups shows the fold difference in VEGF expression between the groups. Fold difference was calculated by dividing the treated groups into the control group. (n=3, *p< 0.05, **p<0.01, ***<0.001).

Our results demonstrated an increased production of VEGF in BM-MSC grown with HeLa-CM, thereby supporting the hypothesis of CAF formation. HeLa-HF20-CM was able to bring down the levels of VEGF significantly in BM-MSC. In a coculture, there was a notable elevation in VEGF. However, this heightened VEGF level was substantially reduced in the presence of HF20.

TGF-β cytokine levels:

This experiment investigated whether HeLa and BM-MSC interactions corresponded to high TGF- β expression and if HF20 lowered the TGF- β expression.

However, there was no marked difference in the overall expression of TGF-β concentrations. TGF-β was less in BM-MSCs but increased in the BM-MSCs cultured in HeLa CM (**Figure 3**). TGF-β was upregulated slightly in the group where BM-MSCs were co-cultured with HeLa compared to the BM-MSCs and HeLa alone group and brought down were HF20. However, there was no significant change in overall cytokine expression (n = 3, p>0.05=NS).

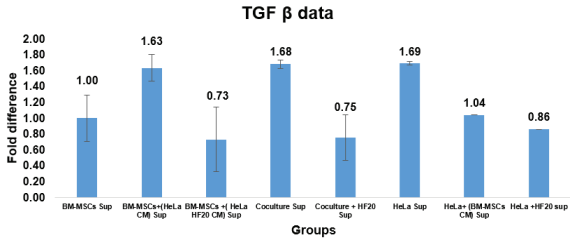

Figure 3: TGF-β cytokine levels. Despite slight alterations in TGF-β expression, there was no significant overall change observed in cytokine expression levels, suggesting a selective effect of HF20 on specific signaling pathways.

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TGF concentration across groups shows the fold difference in TGF expression between the groups. Fold difference was calculated by dividing the treated groups into the control group. Fold difference was calculated by dividing the treated groups into the control group (n=3, p>0.05=NS).

Our results demonstrated that HF20 could downregulate TGF-β levels in BM-MSC grown with HeLa but did not show significant change that HF20 could downregulate TGF-β levels.

Conclusion

Our results conclusively support our hypotheses by showing that HF20 was able to bring down IL-6 cytokine levels. HF20 inhibits VEGF and TGF-β concentrations across all applied groups. HF20 predominantly comprises potent phytochemicals such as saponins, alkaloids, polyphenols, and flavonoids, which are under study for their future employment in integrative oncology studies. However, further research needs to be conducted on the same.

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