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Article

Modulation of VEGF and TGF beta in 5-FU induced inflammation in MCF-7 using an herbal formulation

Maanasi Vinay Nair[1], Ankita Umrao[2], Gururaj A. Rao[2], Jyothsna A. Rao[2]
[1] The International School Bangalore (TISB), Bangalore, India
[2] International Stemcell Services Limited, Bangalore, India

SUMMARY
Acquired drug resistance is an increasing challenge in treating cancer with chemotherapy. One mechanism behind this resistance is the increased inflammation that supports the progression and development of cancer that arises because of the drug's presence.
Integrative oncology is the field that focuses on including natural products alongside traditional therapy to create a treatment that focuses on holistic patient well-being. In this in vitro study, we hypothesized that the use of an herbal formulation, consisting of turmeric and green tea, alongside a traditional chemotherapeutic drug, 5-fluorouracil (5-FU), can reduce the expression of two pro-cancer cytokines in breast cancer cells.
Breast cancer cells were cultured and then treated with both 5-FU and the herbal formulation. We conducted enzyme-linked immunosorbent assays (ELISAs) to measure VEGF and TGFβ in the cell culture supernatant. The results clearly demonstrate that the use of the herbal formulation alongside the chemo drug significantly decreases the level of cytokines produced when compared to the levels produced when exposed solely to the chemo drug.
Hence, we conclude that this combination of treatment, based on the principle of integrative oncology, shows potential for reducing the resistance against treatment conferred through increased inflammation. Consequently, this suggests a prospective way forward in improving the efficacy of cancer treatment.

INTRODUCTION
At the end of 2020, 7.8 million women worldwide were diagnosed with breast cancer in the previous five years, making breast cancer the world's most prevalent cancer (1). Research into improving efficacy of treatment for breast cancer is essential to ensure that such women's lives are improved tremendously despite their diagnosis.

While chemotherapy is one of the most common first-line therapies for cancer, resistance and thus failure of treatment remains a large challenge. One way tumor cells confer resistance against chemotherapeutic drugs is through interactions with the micro-environment, mediated by cytokines, which are cell signaling proteins produced by the stroma, or the connective framework of the tumor. Contrary to its intended effect, the presence of a drug can actually induce tumor cells to produce signals that promote further inflammation, supporting tumor proliferation, angiogenesis, and even metastasis (2).

Chronic inflammation often creates an environment that supports tumorigenesis and tumor progression (3). This inflammation can be attributed to the excess reactive oxygen and nitrogen species, generated as a consequence of continuous inflammation producing mutagenic agents (4). Additionally, the inflammatory cytokines made by tumor-associated inflammatory cells can contribute to cancer progression (5).

5-Flurouracil (5-FU) is a popular chemotherapeutic drug used to treat a wide variety of cancers from colorectal to breast cancer. It works by inhibiting and impairing the DNA replication and repair process in cancer cells, leading to cytotoxicity (6). However, there have been many studies showing changes in the levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 and transcription factor NFκB, after 5-FU had been administered in both animal models and clinical samples (7, 8). Low-dose continuous 5-FU administration led to vascular endothelial growth factor (VEGF)-mediated angiogenesis and 5-FU treatment activated the transforming growth factor-beta (TGFβ) pathway both *in vivo* and *in vitro* (9, 10).

VEGF is an important growth factor for angiogenesis, which is the growth, and formation of new blood vessels. As a result, VEGF aids in maintaining chronic inflammation by bringing in inflammatory cells to the site of the tumor (11), promotes breast cancer cells by invasion and migration (12), and is also linked to the survival of breast cancer cells by inhibiting apoptosis (13). Its presence in breast cancers is well-known, produced by residing macrophages and the cancer cells themselves in a tumor (14).

TGFβ has a paradoxical role in cancer development and progression, having shown dual action as a tumor suppressor and promoter. In the early stage of pre-malignant cancer, this cytokine can induce cell cycle arrest and apoptosis (15). However, advanced tumors, such as breast cancer, can evade these tumor-suppressive effects through mutations in other pathways (16). During later stages, the cancer cells make use of TGFβ for further invasion and metastasis through epithelial to mesenchymal (EMT) promotion (17).

Hence, both VEGF and TGFβ are suitable for measuring how the addition of a chemotherapeutic drug can inadvertently progress the tumor further and for determining if the inclusion of an herbal formulation can reduce the subsequent inflammation to potentially improve the efficacy of the chemotherapy.

Directly targeting these cytokines and growth factors that contribute to the chronic inflammation supporting the cancer is an area of interest and has potential for successful cancer treatment. There have been developments of small molecules that can block the TGF pathway in cancer; however, these molecules show little efficacy as a monotherapy (18). The

same trend is seen in clinical trials of VEGF inhibitors used in cancer therapy due to the variable sensitivity of different types of tumors to such treatment (19, 20). In both cases, there is a need for better modulation of such cytokines with more current standard treatments like chemotherapy or radiation to improve efficacy.

With the rise in popularity of the active compounds from natural herbal sources as potent molecules to use alongside conventional treatments for cancer, Sri Raghavendra Biotechnologies Pvt Ltd in Bangalore, India developed an herbal formulation, HF1, consisting of green tea (*Camellia sinensis*) and turmeric (*Curcuma longa*) (21). Both green tea and turmeric are famed for their anti-inflammatory properties, and their active components, (-)-epigallocatechin-3-gallate (EGCG) and curcumin respectively also have been studied to have potential effects against cancer, ranging from lung to breast to stomach to hematopoietic cancers (20). Previous work also suggests that HF1 demonstrates potential in reducing inflammation-based resistance in breast cancer cell cultures (22).

In this study, Michigan Cancer Foundation-7 (MCF-7), a breast cancer cell line, was used as the *in vitro* model. 5-FU was the chosen chemotherapeutic drug to investigate the effect of treatment on the level of VEGF and TGFβ produced by the breast cancer cells. The study also investigated whether the addition of HF1, both together with 5-FU and after 5-FU, would be able to reduce levels of VEGF and TGFβ as hypothesized. We found the combination of HF1 and 5-FU significantly reduced the level of VEGF and TGFβ. This suggests that HF1 could be a potential complementary medicine to mitigate the inflammation caused by chemo-drugs like 5-FU.

RESULTS

We cultured MCF-7 cells under optimum conditions and then divided the culture into six groups for treatment as follows: 1) untreated MCF-7 culture for 48 hours, 2) MCF-7 cells treated with 5-FU for 24 hours, 3) MCF-7 cells treated with 5-FU for 48 hours, 4) MCF-7 cells treated with 5-FU and HF1 together for 24 hours, 5) MCF-7 cells treated with 5-FU and HF1 together for 48 hours, and 6) MCF-7 cells treated with 5-FU for 24 hours and HF1 for the next 24 hours. We then performed a sandwich ELISA, an assay that uses two antibodies for detection of a target antigen, for VEGF and TGFβ for each of these groups and expressed the quantity of measured cytokines in terms of fold expression compared to no treatment (Group 1).

There was a 2.5-fold increase in VEGF in 48 hours with 5-FU as compared to the untreated culture (Tukey p = 0.0.0002). Including HF1 in the treatment alongside 5-FU for both 24 and 48 hours showed a significant decrease in VEGF levels as compared to untreated culture (Tukey $p < 0.001$, $p < 0.001$, respectively). In 24 hours, the VEGF level was only about one-fourth of control levels. In 48 hours, the level of VEGF was greater than control but only increased by 1.8-fold, which is less than the VEGF level produced in 48 hours without HF1. However, using HF1 for 24 hours after 5-FU only for 24 hours brought down VEGF levels to 0.7-fold, even below control (overall ANOVA = 0.0003, Tukey $p < 0.001$) **(Figure 1)**.

In contrast to VEGF, the treatment of 5FU to MCF-7 cultures for both 24 hours and 48 hours did not significantly

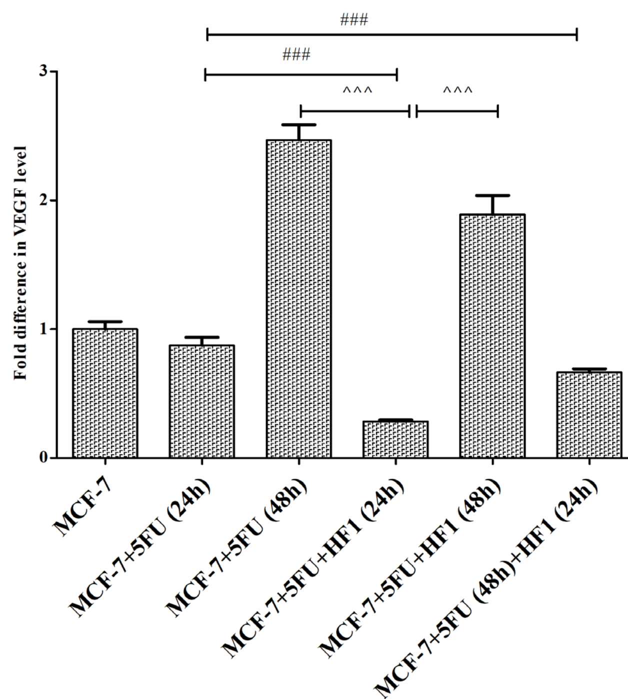

Figure 1: Fold expression of VEGF levels affected by 5-FU and HF1 in MCF-7 culture (n = 3 biological replicates; mean ± SEM). The concentration of 5-FU was 2 µg/ml each time it was used and the concentration of HF1 was 0.3 mg/ml each time it was used. '###' represents $p < 0.001$ for groups comparing 5-FU treated MCF-7 with and without HF1, and '^^^' represents $p < 0.001$ between timings used for HF1 treatment, with p = 0.0003 for overall ANOVA.

alter the level of TGFβ. Regardless, the inclusion of HF1 in the treatment did still decrease levels as compared to the untreated control in 24 hours. When used in conjunction with 5-FU for 24 hours, TGFβ dropped to 0.4-fold and when used in conjunction for 48 hours TGFβ remained at a level of 0.8-fold. Using HF1 for 24 hours after 5-FU for 24 hours resulted in a measurement of 0.6-fold TGFβ expression (Tukey $p < 0.001$, with the overall ANOVA value of 0.0007) **(Figure 2).**

DISCUSSION

The presence of 5-FU in the MCF-7 culture did result in an increase in VEGF levels, supporting the hypothesis that cancer cells can retaliate in response to treatment through inflammatory modulation. However, the same cannot be said for the TGFβ level, which may be due to the dual nature of this cytokine as mentioned earlier (15–17). Regardless, it is important to note that through this study, HF1 was successful in reducing the levels of cytokines VEGF and TGFβ in MCF-7 cells cultured with 5-FU after 48 hours. From these results, we can conclude that HF1 has the potential to improve the efficacy of chemotherapy on breast cancer, as an example of integrative oncology.

This study focused only on the development of resistance of MCF-7 against 5-FU by means of increased inflammation. However, to gain further insight and confidence about the success of HF1 in decreasing cytokine levels, further studies that broaden the data set would be required. This method can be repeated for different cancer cell types like HeLa cervical cancer or A549 lung cancer, as well as with

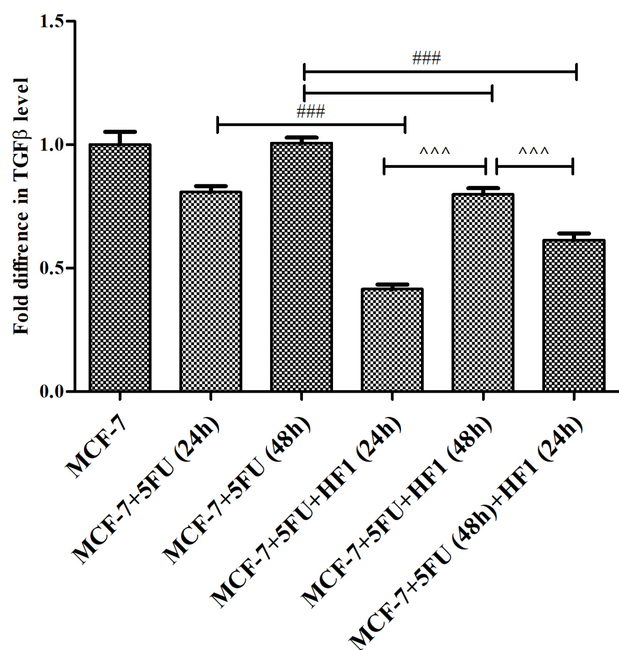

Figure 2: Fold expression of TGFβ levels affected by 5-FU and herbal formulation HF1 in MCF-7 culture (n = 3 biological replicates; mean ± SEM). The concentration of 5-FU was 2 μg/ml each time it was used and the concentration of HF1 was 0.3 mg/ml each time it was used. '###' represents $p < 0.001$ for groups comparing 5-FU treated MCF-7 with and without HF1, and '^^^' represents $p < 0.001$ between timings used for HF1 treatment, with $p = 0.0007$ for overall ANOVA.

different drugs such as paclitaxel or doxorubicin. Additionally, cytokine profiling before and after this integrative treatment could be improved by including the measurement of other cytokines, such as pro-inflammatory interleukins and tumor necrosis factor alpha (TNFα). Conversely, measuring anti-inflammatory cytokines could also shed light on whether HF1 is useful in combating resistance by improving immune response against cancer. If HF1 shows sustained efficacy in such research, then it can be tested using *in vivo* models as well.

MATERIALS AND METHODS

Culture conditions

MCF-7 cells, obtained from NCCS Pune, were cultured in DMEM-F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Thermo Fisher Scientific) in a humidified incubator at 37˚C with 5% CO_2. Cells were treated at 40% confluency and were harvested after the intended time frame. All treatments were included in the medium of cell culture from the very start of plating. Six well plates were seeded with 0.2 x 10^6 cells per well for all sets of treatments, and harvested numbers were counted to be around 1.0 x 10^6 cells per well.

Experimental groups

The first group was the MCF-7 control where the culture did not undergo any treatment for 48 hours. The second group, MCF-7 + 5-FU (24 hrs), was where the culture was treated with 2 μg/ml of 5FU for 24 hours. The third, MCF-7 + 5-FU (48 hrs), was similar to the second group except treated for 48 hours. The fourth group, labelled as MCF-7 + 5-FU + HF1 (24 hrs), consists of the culture that was co-treated with 2 μg/ml of 5-FU and 0.3 mg/ml of HF1 at the same time for 24 hours. Group 5, MCF-7 + 5-FU + HF1 (48 hrs), is the same as the fourth group but for 48 hours. Finally, the last group, MCF-7 + 5-FU (24 hrs) + HF1 (24 hrs), refers to the cultures that were treated with only 2 μg/ml of 5-FU for the first 24 hours and then treated with 0.3 mg/ml of HF1 for the next 24 hours, making the total treatment last for 48 hours.

Enzyme-linked immunosorbent assay (ELISA)

For each group, a 100 μl aliquot of the culture supernatant was used for sandwich ELISA (RayBio) to quantify the amount of VEGF and TGFβ present. Samples were loaded into the pre-coated wells, incubated for 2.5 hours at room temperature, washed with wash buffer, and then treated with 100 μl of the detection antibody. After incubating for an hour at room temperature and washing with buffer, the conjugate, substrate, and stop solution were added to complete the ELISA with a 30-minute incubation at room temperature between each. The resulting color produced in the wells was read at 450 nm with a Thermo Fisher Scientific spectrophotometer and then expressed as fold expression, taking the absorbance of untreated MCF-7 cells, as the baseline 1-fold. The same volume of aliquot was taken from the culture supernatant for every sample's ELISA.

Statistical analysis

Three independent sets of the experiments were conducted, and the experimental data are expressed as the mean and standard error of the mean (SEM). P-value < 0.05 was considered statistically significant. We analyzed the data by running ANOVA followed by Tukey's test using Graphpad Prism 6 software.

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