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Antitumor Effects of TNF-β, 5-FU and Their Combinations on Cervical Carcinoma Cell Lines

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Abstract: Cervical carcinoma is one type of cancer with a high mortality rate in females. In this study, we investigated the cytotoxic effects of TNF-β, 5-FU and their combinations on human cervical carcinoma cell lines, ME180 and HeLa. The highest degree of cytotoxic effect was obtained with 5-FU on both cell lines. While high concentrations of TNF-β showed cytotoxic effects on both cell lines, the lowest dose induced growth. Combination treatment of 5-FU and TNF-β gave a slightly higher cytotoxic and antiproliferative response compared to individual use of 5-FU and TNF-β on both cell lines.

Key Words: TNF-β, 5-FU, ME180, HeLa, Cervical Carcinoma

Introduction

Tumor necrosis factor (TNF) is a product of activated macrophages that plays a central role in integrating and amplifying the host response to infection and malignancy (1,2). Through interactions with macrophages, fibroblasts and endothelial cells, TNF promotes immune response, local inflammatory processes and wound repair. TNF is also proposed to mediate the wasting (cachexia) that often accompanies disease states (3). TNF was first identified as an oncolytic agent that promotes the hemorrhanic necrosis and regression of some malignancies by inducing an inflammatory response in tumor capillary beds (2). TNF also acts directly on transformed cells, eliciting a cytotoxic response from some and inhibiting the proliferation of others; however, many transformed cells are resistant to the cytotoxic and cytostatic activities of TNF (4).

5-Fluorouracil (5-FU) is a fluoridized pyrimidine that belongs to the group of antimetabolites. It blocks the synthesis of DNA, thus arresting the cell cycle at S phase, by complexing to the enzyme thymidilate synthetase. 5-FU can also inhibit the synthesis of RNA by forming faultily structured ribonucleic acids incorporation into RNA (5).

TNF can induce the inhibition of cell proliferation and apoptosis in some cells (6). The present study shows the antiproliferative and cytotoxic effects of TNF-β and its combination with 5-FU on human cervical carcinoma cell lines, ME180 and HeLa.

Materials and Methods

Materials: Recombinant human TNF-β was a gift from Dr. Milton W. Taylor, Indiana University (USA). 5-FU was purchased from Sigma.

Cells and cells culture: ME180 and HeLa cervical carcinoma cell lines were obtained from Dr. Milton W. Taylor, Indiana University, Bloomington, Indiana, USA. These cell lines were maintained in DMEM supplemented with 10% FCS at 37°C in humidified 5% CO_2 atmosphere. Cells maintained at the exponential growth phase were harvested for the experiments.

Cytotoxic assay: ME180 and HeLa cells were grown to a monolayer in 96-well plates. Both types of cells were treated with various doses of TNF-β, 5-FU and variable concentrations of TNF-β (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml) with a fixed concentration of 5-FU (12.5 µg/ml). Cells were incubated for 72 hours at 37°C in humidified 5% CO_2 atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (8% in methanol), and dye absorbed by live cells was extracted with sodium citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm.

Antiproliferative assay: ME180 and HeLa cells growing at the exponential growth phase were treated with TNF-β (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml), 5-FU (50 µg/ml, 12.5 µg/ml, 3.125 µg/ml, 0.390 µg/ml) and with their combinations. Cells were incubated for 72 hours at 37°C, 5% CO_2 in DMEM medium. At the

end of the incubation period, the live cells were trypsinized and counted by trypan blue staining. The growth inhibition assay was expressed as the percentage increase in the number of treated cells relative to the increase in the number of untreated control cells.

Statistical analysis: Statistical significance analysis was carried out using the SPSS program (paired-samples t-test).

Results

TNF-β, 5-FU and their combinations show different cytotoxic effects

Although treatment of HeLa cells with a 100 ng/ml concentration of TNF-β killed 25% of cells, a 1.56 ng/ml concentration of TNF-β showed a growth stimulatory effect on HeLa cells (Figure 1). The other cell line, ME180 cells, gave an almost identical response to that of TNF-β. When these cells were treated with 100 ng/ml of TNF-β, the dead rate was 19%, whereas a 12.5 ng/ml concentration of TNF–β killed only 4.2% of the cells. Furthermore, as we observed in HeLa cells, the lowest dose of TNF-β (1.56 ng/ml) showed an even greater growth stimulatory effect on ME180 cells (Figure 1).

The cytotoxic effect of 5-FU appered to be higher than that of TNF-β on both cervical carcinoma cell lines ($p<0.05$). 5-FU showed a very strong cytotoxic effect on both cell lines in a dose-dependent manner. When HeLa cells were treated with a 50 µg/ml concentration of 5-FU, nearly 76% of the cells had died by the end of the 72-h incubation period. Even at the lowest dose of 5-FU (0.78 µg/ml), 44% of the cells were dead by the end of the 72-h incubation period indicating the fact that HeLa cells are very sensitive to the growth inhibitory effect of 5-FU (Figure 2). When ME180 cells were treated with 5-FU, we obtained even greater cytotoxic effects. With a 50 µg/ml concentration of 5-FU, nearly 90% of ME180 cells had died by the end of the 72-h incubation. Moreover, the lowest concentration of 5-FU (0.78 µg/ml) killed 50% of ME180 cells (Figure 2).

After determining the individual cytotoxic effect of TNF-β and 5-FU on both cell lines, we wanted to determine whether a combination of both would give greater cytotoxic effects on ME180 and HeLa cells. Therefore, we treated both cell lines with various doses of TNF with 12.5 mg/ml of 5-FU. However, no synergistic cytotoxic effects of TNF-β and 5-FU combinations were seen, but some additive effect was obtained with increasing concentrations of both chemicals (Figure 3).

Antiproliferative effects of TNF-β and 5-FU on ME180 and HeLa cells

After determining the cytotoxic effects, we wanted to determine the antiproliferative effects of TNF-β and 5-FU on HeLa and ME180 cells. When both cell lines were treated with TNF-β, HeLa cells showed 56% proliferation at a 100 ng/ml concentration, and the lowest dose of TNF-β gave a 96% proliferation rate compared to

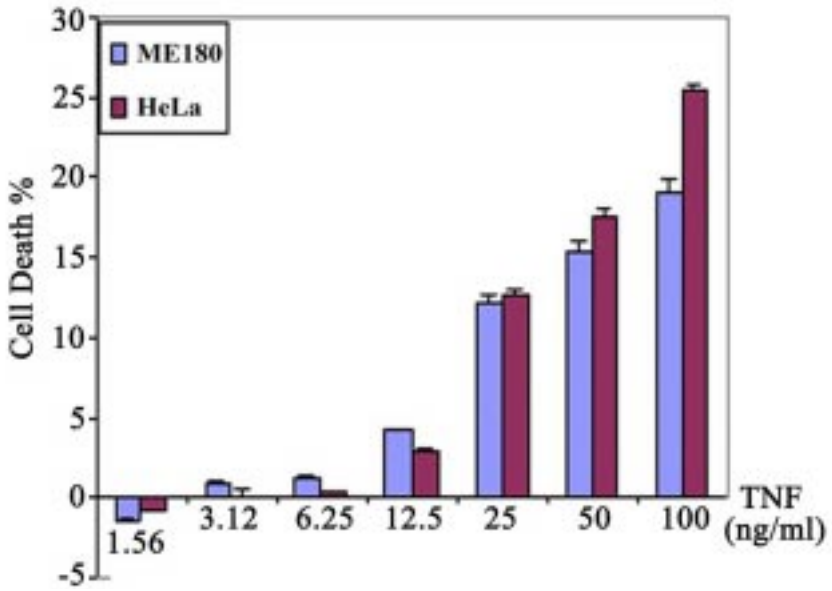

Figure 1. Confluent cultures of ME180 and HeLa were treated with various doses of TNF-β and incubated for 72 hours at 37°C in humidified 5% CO_2 atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (8% in methanol) and dye absorbed by live cells was extracted with sodium citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm. OD values of TNF-treated samples were divided to that of untreated control cells to determine the rate of cell dead. Results are average of three independent experiments.

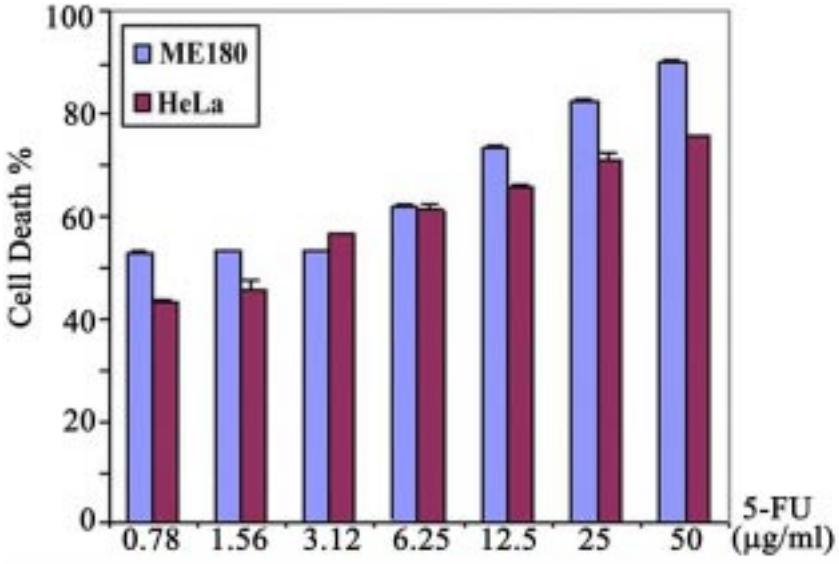

Figure 2. Confluent cultures of ME180 and HeLa were treated with various doses of 5-FU and incubated for 72 hours at 37°C in humidified 5% CO_2 atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (8% in methanol) and dye absorbed by live cells was extracted with Sodium Citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm. OD values of 5-FU-treated samples were divided to that of untreated control cells to determine the rate of cell dead. Results are average of three independent experiments.

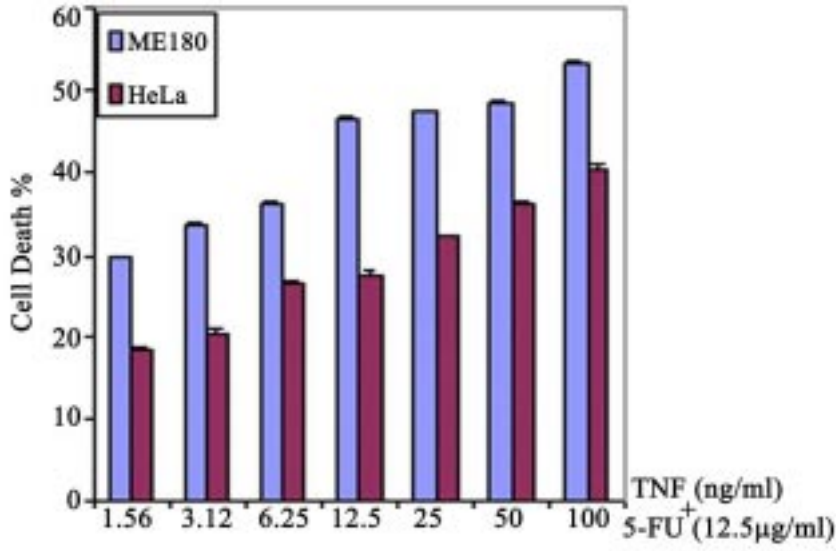

Figure 3. Confluent cultures of ME180 and HeLa were treated with various doses of TNF-β and fixed concentration (12.5 µg/ml) of 5-FU and incubated for 72 hours at 37°C in humidified 5% CO_2 atmosphere. Following incubation, floating cells were removed and attached cells were stained with crystal violet (8% in methanol) and dye absorbed by live cells was extracted with sodium citrate (0.1 M) in 50% ethanol. Absorbance was read at 600 nm. OD values of TNF+FU-treated samples were divided to that of untreated control cells to determine the rate of cell dead. Results are average of three independent experiments.

untreated cells. In ME180 cells, this effect was 78% at the 100 ng/ml TNF-β and 112.5% at a 1.56 ng/ml concentration of TNF-β (Figure 4).

The antiproliferative effect of 5-FU is much greater than that of TNF-β on both cell lines ($p<0.05$). When treated with 50 µg/ml of 5-FU, HeLa cells showed only a 7.4% proliferation rate compared to untreated culture; this rate was 58% at the lowest dose of 5-FU used. The other cell line ME180 gave the same proliferation rate, which was 7.5% at 50 µg/ml and 43% at the lowest concentration of 5-FU used (Figure 5). After determining the individual antiproliferative effects of TNF-β and 5-FU, we wanted to determine whether a combination treatment of both would yield greater effects. As shown in Figure 6, synergistic antiproliferative effects were detected when TNF-β and 5-FU ($p<0.05$) were combined.

Discussion

Chemotherapy is used primarily to treat advanced or recurrent cervical cancer. There are three major applications: primary therapy, as a radiation sensitizer, and neoadjuvant therapy. The four best single drugs with

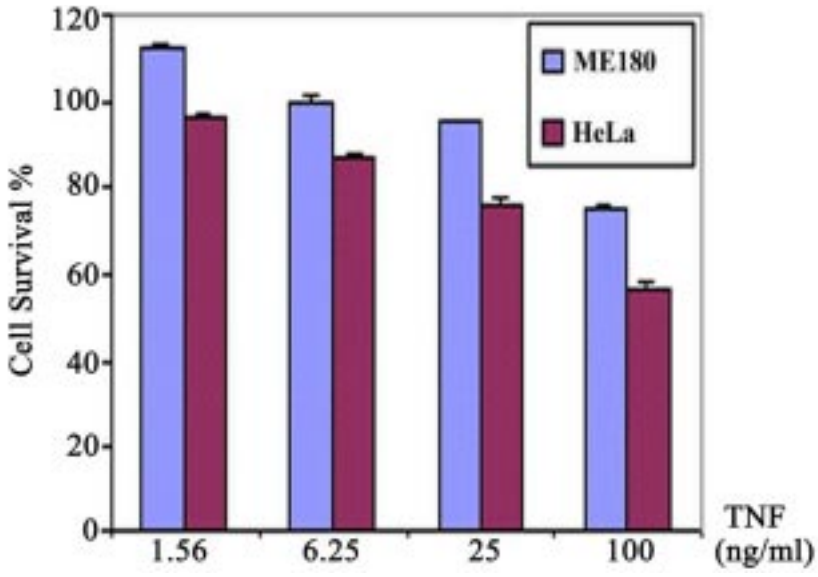

Figure 4. ME180 and HeLa cells growing at the exponential growth phase were harvested by trypsinization. Both cells were carried to 24-well plates ($1x10^5$ cells /well) then treated with TNF-β (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml) and incubated for 72 hours at 37ºC, 5% CO_2 in DMEM medium. At the end of the incubation period, the live cells were tripsinized and counted by trypan blue staining. The growth inhibition assays expressed as the percentage increase in the number of cells present in the TNF-treated cultures relative to increase in the number of untreated control cells. Results are average of three independent experiment.

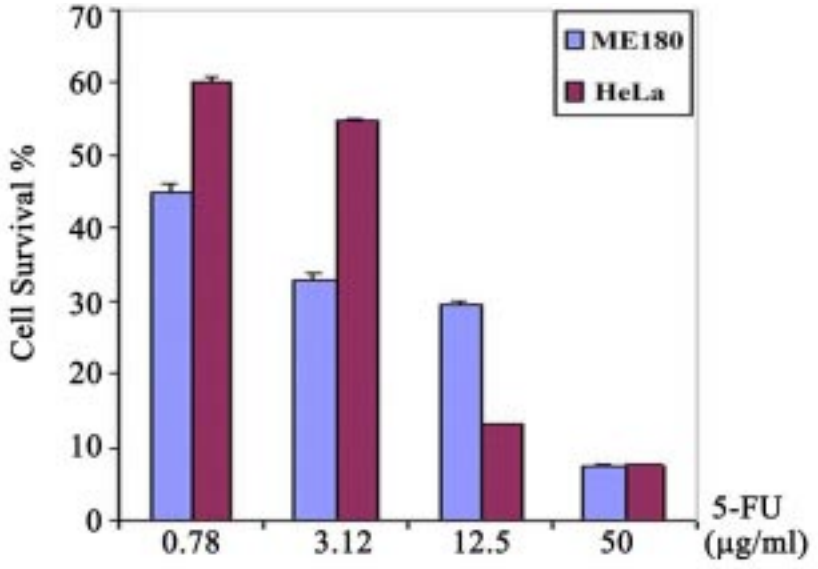

Figure 5. ME180 and HeLa cells growing at the exponential growth phase were harvested by trypsinization. Both cells were carried to 24-well plates ($1x10^5$ cells/well) then treated with 5-FU (50 µg/ml, 12.5 µg/ml, 3.125 µg/ml, 0.390 µg/ml) and incubated for 72 hours at 37ºC, 5% CO_2 in DMEM medium. At the end of the incubation period, the live cells were tripsinized and counted by trypan blue staining. The growth inhibition assays expressed as the percentage increase in the number of cells present in 5-FU-treated cultures relative to increase in the number of untreated control cells. Results are average of three independent experiment.

moderate activity against cervical cancer are cisplatin, ifosfamide, dibromodulcitol and adriamycin. Concomitant chemotherapy with hydroxyurea or a combination of cisplatin and 5-fluorouracil have been shown to enhance radiation response in several randomized trials. Hydroxyurea is the preferred radiation sensitizer because it offers less toxocity, ease of administration, and equivalent results. Various cisplatin combinations of mitomycin C, 5-FU, vincristine, and bleomycin have been used to shrink locally advanced cervical cancer (7).

What have not been described are the antiproliferative and cytotoxic effects of TNF-β, 5-FU and their combinations on cervical carcinoma cell lines, ME180 and HeLa. Therefore, we tested the antitumor and cytotoxic effects of TNF-β and its combinations with 5-FU on cervical carcinoma cell lines. Although high concentrations of TNF-β can induce cytotoxicity, the lowest dose of TNF-β used in this study induced cell proliferation (Figure 1). What was obtained here is in agreement with the previously published results of Manchester et al. They found that while high concentrations of TNF-α showed cytotoxic and antiproliferative effects on ME180 cell line, the lowest dose induced growth (4). Even though the single use of TNF-β did not show significant

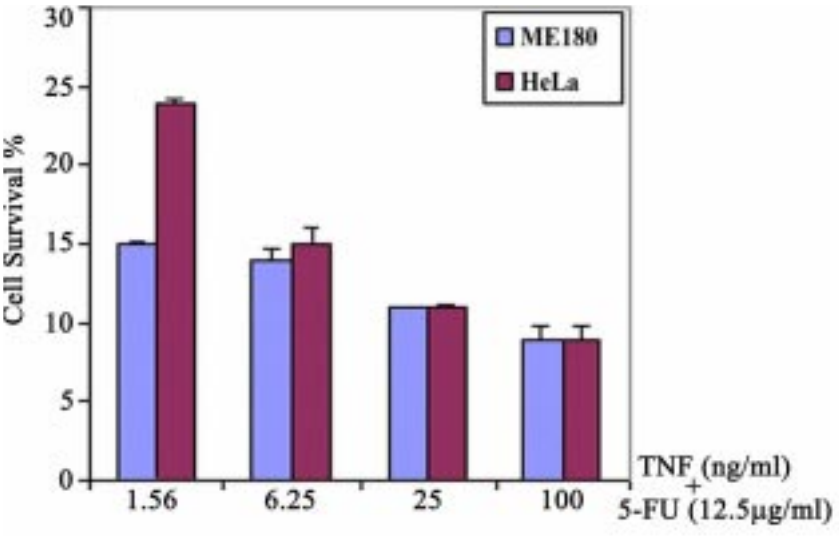

Figure 6. ME180 and HeLa cells growing at the exponential growth phase were harvested by trypsinization. Both cells were carried to 24-well plates ($1x10^5$ cells/well) then treated with TNF-β (100 ng/ml, 25 ng/ml, 6.25 ng/ml, 1.560 ng/ml) and with fixed dose of 5-FU (12.5 µg/ml) and incubated for 72 hours at 37°C, 5% CO_2 in DMEM medium. At the end of the incubation period, the live cells were tripsinized and counted by trypan blue staining. The growth inhibition assays expressed as the percentage increase in the number of cells present in TNF+5-FU-treated cultures relative to increase in the number of untreated control cells. Results are average of three independent experiment.

antiproliferative or cytotoxic effects, its combination with 5-FU gave a very high synergistic antiproliferative effect. Recently, similar observations were made by Ueda et al, (8). They showed that the 5-FU sensitivity of tumor cells can be increased in the presence of epidermal growth factor (EGF) or transforming growth factor alpha, TGF-α. These growth factors were shown to stimulate the activity of dihydropyrimidine dehydrogenase (DPD) and pyrimidine nucleoside phosphorylase (PyNPase). These are the enzymes that stimulate 5-FU anabolism and inhibit its catabolism, leading to the enhancement of the antiproliferative action of 5-FU. Even though we did not test the TNF-β activation of these enzymes, it is possible that potentiation of the antiproliferative effect of TNF-β could be due to activation of the above enzymes.

The antiproliferative and cytotoxic effects of 5-FU have been known for quite a long time. Different groups tested its cytotoxic and antiproliferative effects under *in vitro* and *in vivo* conditions and came up with results supporting what we are reporting here. Wadler et al. tested 5-FU along with IFN-a on an esophagus carcinoma patient (9). They found that 5-FU showed a dramatic effect and this was further increased by IFN-α combination. Sugimachi and Marsh et al. (10, 11) reported successful treatment rectal carcinoma patients by 5-FU. In addition, Gebbia et al reported that 5-FU and its combination with other chemotherapeutics gave successful treatment of pancreatic carcinoma patients (12). Reiter et al. tested cytotoxic and antiproliferative effects of 5-FU, IFN-α and their combinations on ME180, K562, Escol, and Kapopsi's sarcoma cell lines and found that 5-FU gave the highest cytotoxic effects on all cell lines (13). Furthermore, synergistic antiproliferative and cytotoxic effects of 5-FU and TNF-α combinations were shown on human pancreatic, colon,ovarian, and gastric cell lines (14-17). In addition to these, 5-FU is succesfully used to treat colon, rectal, and pancreatic carcinoma patients (18).

There are several hypothesis to explain cytotoxic and antiproliferative effects of 5-FU. It was recently shown by Aota et al that 5-FU can inhibit activation of Nf-kB pathway (19) thereby eliciting growth inhibitory effects. Tanaka et al showed that 5-FU can block the cell cycle and induce apoptosis in human xenograft (20), it was also reported that 5-FU can stop cell cycle at S phase and induce apoptosis in human colorectal carcinoma (21) and breast cancer cell lines (22).

The results we presented in this paper are in good agreement with the once published elsewhere previously and potentiation of antiproliferative effect of TNF-β, even at very low doses, with low dose of 5-FU can provide some advantages for developing new treatment strategies for cervical carcinoma patients.

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