

Dual role of macrophages in the response of C26 colon carcinoma cells to 5-fluorouracil administration

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Abstract. Previous studies have demonstrated that tumor-associated macrophages (TAMs) are pivotal players in tumor progression via modulation of tumor angiogenesis, inflammation, metastasis and oxidative stress, as well as of the response of cancer cells to cytotoxic drugs. Nevertheless, the role of TAMs in the prognosis of colorectal cancer remains controversial. Therefore, the present study aimed to investigate how TAMs mediate the response of C26 colon carcinoma cells to the cytotoxic drug 5-fluorouracil (5-FU), upon TAM co-cultivation with these cancer cells *in vitro*. In this respect, 5-FU cytotoxicity was assessed in C26 cells in standard culture and in a co-culture with peritoneal macrophages, the production of NF- κ B was determined by western blot analysis, and the production of angiogenic/inflammatory proteins in each experimental model was evaluated by protein array analysis. To gain further evidence of the effect of TAMs on oxidative stress, malondialdehyde was measured through high-performance liquid chromatography, and the total nonenzymatic antioxidant levels and the production of nitrites were measured through colorimetric assays. The results demonstrated that TAMs exerted a dual role in the response of C26 cells to 5-FU administration in the co-culture model. Thus, on one side, TAMs sensitized C26 cells to 5-FU administration through inhibition of the production of inflammatory and angiogenic proteins in these cancer cells; however, they also protected cancer cells against 5-FU-induced oxidative stress. Collectively, the present findings suggest that the combined administration of 5-FU with pharmacological agents that

prevent TAMs to maintain the physiological range of tumor cell oxidative stress may highly improve the therapeutic potential of this drug.

Introduction

Administration of the cytotoxic agent 5-fluorouracil (5-FU) is the predominant therapeutic approach for the treatment of metastatic colorectal cancer (CRC) based on the capacity of its three active metabolites [fluorodeoxyuridine triphosphate (FdUTP), fluorouridine triphosphate (FUTP) and fluorodeoxyuridine monophosphate (FdUMP)] to induce cytotoxicity and cell death, by incorporation of FdUTP and FUTP into DNA and RNA, or by inhibition of the enzyme thymidylate synthase by FdUMP (1). Nevertheless, its clinical applicability is considerably limited by a number of disadvantages, particularly its low bioavailability, the high rate of 5-FU degradation (especially in the liver, at >80%) and the development of 5-FU resistance mechanisms in CRC cells (1,2). Furthermore, recent studies have demonstrated that the anticancer efficiency of various chemotherapeutic agents (including doxorubicin, docetaxel, cyclophosphamide, gemcitabine and 5-FU) can be modulated by cancer cells and also by tumor-associated macrophages (TAMs), via inducing chemoresistance or enhancing chemosensitivity to these cytotoxic agents in different tumor models, including leukemia, fibrosarcoma, pancreatic, breast and colon cancer models (3).

It is well known that, among the immune cell populations present in tumor microenvironment, TAMs are key protagonists in promoting and coordinating tumor growth (4) through their ability to modulate all processes involved in cancer progression, including the following: i) Tumor angiogenesis and inflammation [secretion of vascular endothelial growth factor (VEGF), platelet-derived growth factor, transforming growth factor- β (TGF- β), fibroblast growth factor and matrix metalloproteinases; and the cytokines and chemokines interleukin (IL)-1 β , IL-6, IL-8, IL-9, IL-10, chemokine (CC motif) ligand (CCL)17, CCL22, CCL18 and tumor necrosis factor α (TNF- α)] (5,6); ii) metastasis (production of IL-1 β and TNF- α) (7,8); iii) immunosuppression [secretion of immunosuppressive cytokines (IL-10, TGF- β) and

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prostaglandin E2] (9,10); and iv) oxidative stress [generation of reactive oxygen species (ROS) which are essential for the activation and expression of transcription factors responsible for the maintenance of a malignant phenotype] (11).

Therefore, the aim of the present study was to provide greater insight into the effect of the tumor microenvironment generated by the interaction between TAMs and C26 murine colon carcinoma cells on the response of these cancer cells to 5-FU treatment. In this respect, the cytotoxic effects of various concentrations of 5-FU were tested on C26 carcinoma cells cultivated alone as well as on those co-cultured with murine peritoneal macrophages. Furthermore, the role of TAMs in the antitumor effects of 5-FU on key molecules involved in tumor angiogenesis and inflammation, as well as in tumor oxidative stress, was addressed. The results demonstrated that TAMs orchestrate the response of C26 cells to 5-FU administration. Thus, on one side, TAMs render C26 colon carcinoma cells more susceptible to 5-FU treatment via inhibition of the production of inflammatory and angiogenic factors in these cancer cells; however, on the other side, they protect cancer cells from 5-FU-induced oxidative stress.

Materials and methods

Cell line and culture conditions. C26 murine colon carcinoma cells (Cell Line Services GmbH, Eppelheim, Germany) were cultured as a monolayer in complete RPMI-1640 medium (Lonza Group AG, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), at 37°C in a humidified atmosphere of 5% CO₂.

Co-culture of C26 tumor cells with macrophages. Peritoneal macrophages were isolated from 6 male BALB/c mice (Cantacuzino Institute, Bucharest, Romania). Experiments were performed according to the national regulations and were approved by the Babes-Bolyai University ethics committee (Cluj-Napoca, Romania; registration no. 32652/01.07.2014). Mice were previously injected intraperitoneally with 1 ml of 3% thioglycollate (Fluka) (12). After 3 days, chemically elicited macrophages (with inflammatory and antitumor action) were collected, and co-cultures were prepared by seeding C26 tumor cell suspensions on macrophage monolayers. The complex interaction of these macrophages with tumor cells in a co-culture system has been shown to enable tumor cell-macrophage crosstalk and to promote macrophage polarization into TAMs which favor tumor progression (12,13). In our experiments, co-cultures of macrophages and tumor cells were created at a cell density ratio of 1:4, as it has previously been demonstrated that this cell density ratio ensures the optimal cytokine interplay between tumor cells and macrophages, which provides an approximation of the physiological conditions of colon carcinoma development *in vivo* (13). In addition, the presence of the TAM-specific phenotype in this co-culture was verified by comparing angiogenic/inflammatory protein production in co-culture cell lysates with the production of the same proteins in cell lysates that resulted from the co-cultivation of IL-4-induced TAMs with C26 cells at the same cell density ratio as described above (data not shown). It was previously demonstrated that incubation of

peritoneal macrophages with 20 ng/ml of IL-4 (Sigma-Aldrich Chemie GmbH, Munich, Germany) for 24 h promotes the complete polarization of macrophages into TAMs (14).

Cell proliferation assay. The cytotoxicity of various concentrations of 5-FU (0.125–16 μ M) on C26 cells in standard cultures, as well as in co-culture with macrophages, was assessed. The antiproliferative effects of 5-FU at the aforementioned concentrations were determined using the Cell Proliferation ELISA, BrdU (colorimetric) immunoassay kit (#11647229001; Roche Applied Science, Mannheim, Germany), according to the manufacturer instructions and as described previously (15). This method is based on the incorporation of the pyridine analog bromodeoxyuridine (BrdU), instead of thymidine, into the DNA of proliferating cells. C26 cells (1x10⁴/well), cultured alone or together with peritoneal macrophages at a density ratio of 1:4, were seeded into 96-well plates and incubated with different concentrations of 5-FU for 72 h. Cells incubated only with medium were used as controls. Subsequently, cells were incubated with BrdU solution for 24 h and the culture medium was completely removed from each well. Following this step, the cells were fixed and the DNA was denatured. To detect the incorporated BrdU in the newly synthesized cellular DNA, a monoclonal antibody conjugated with peroxidase (anti-BrdU-POD, included in the kit) was added in each well. The antibody was removed after 1 h of incubation and the cells were washed three times with phosphate-buffered saline. A peroxidase substrate, tetramethyl-benzidine, was added to each well and the immune complexes were detected by measuring the absorbance of the reaction product at 450 nm with a reference wavelength of 655 nm. The effects of administration of 5-FU at various concentrations on C26 cells in the two culture conditions were determined in triplicate.

Preparation of cell culture lysates. To assess the effects of 5-FU on key molecules involved in tumor oxidative stress, inflammation and angiogenesis, as well as the role of TAMs in the antitumor effects of 5-FU, lysates from 4 μ M 5-FU-treated C26 colon carcinoma cells cultured alone or in combination with peritoneal macrophages were obtained. Cell cultures were lysed with lysis buffer containing 10 mM HEPES (pH 7), 200 mM NaCl, 1% Triton X, 10 mM MgCl₂, 1 mM dithiothreitol and protease inhibitor cocktail tablets (Complete, Roche Diagnostics GmbH, Mannheim, Germany). The homogenate was incubated for 30 min on ice and then centrifuged for 10 min at 12,000 x g, at 4°C and the supernatant was collected and stored at -80°C for further molecular measurements. The protein concentration was determined through a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA) (16).

Assessment of nuclear factor κ B (NF- κ B) production. To determine the effects of 4 μ M 5-FU on the levels of NF- κ B (a key transcription factor involved in tumor inflammation and angiogenesis) in the cell lysates obtained from standard C26 cell culture and from the mixed culture of C26 cells with peritoneal macrophages, western blot analysis was performed. Thus, 20 μ g of total protein from each lysate was loaded per lane onto a 10% polyacrylamide gel. Electrophoresis was performed at 95 mV and then the protein fractions were electrotransferred onto a nitrocellulose membrane at 100 mV

for 40 min. The membranes were blocked overnight at 4°C with 5% skimmed milk powder (Bio-Rad Laboratories, Inc.) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), under constant agitation. Subsequently, the membranes were incubated for 2 h at room temperature with a monoclonal mouse IgG anti-mouse NF- κ B p65 antibody (SC-56735; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:500 in TBS-T, with 5% skimmed milk powder (17). For the loading control, rabbit IgG anti-mouse β -actin antibody (SC-130656; Santa Cruz Biotechnology, Inc.) was used, diluted 1:500 with 5% skim milk powder in TBS-T. Membranes were washed with TBS-T and incubated at room temperature for 1 h with horseradish peroxidase (HRP)-conjugated goat IgG anti-mouse IgG antibody (SC-2005; Santa Cruz Biotechnology, Inc.) diluted 3,000-fold in TBS-T, with an additional washing step prior to detection. For β -actin determination, HRP-conjugated goat IgG anti-rabbit IgG antibody (SC-2004; Santa Cruz Biotechnology) diluted 4,000-fold in TBS-T was used. Proteins were detected by using Clarity™ Western ECL (Bio-Rad Laboratories, Inc.) and the membranes were exposed to a Kodak X-ray film for 2 min. The films were developed, photographed using a BioSpectrum Imaging System (Ultra-Violet Products, Ltd., Cambridge, UK) and analyzed using TotalLab Quant Software version 12 for Windows (TotalLab Limited, Newcastle, UK). NF- κ B expression levels in cell lysates following 5-FU treatment in the presence or absence of macrophages were compared with the levels of NF- κ B in untreated C26 cell lysates and untreated co-cultures, respectively. The final results are presented as the mean \pm standard deviation (SD) of two independent experiments.

Determination of inflammatory and angiogenic protein production. The effects of treatment with 4 μ M 5-FU on the expression levels of inflammatory/angiogenic factors in cell lysates obtained in standard or in co-culture conditions were investigated by performing a screening for 24 proteins involved in angiogenesis and inflammation using RayBio® Mouse Angiogenesis Antibody Array 1 kit (AAM-ANG-1-8; RayBiotech, Inc., Norcross, GA, USA) as described previously (15). One array membrane containing 24 types of primary antibodies against specific proteins was incubated with 200 μ g of proteins of cell lysates for 2 h at room temperature. Subsequently, a mixture of secondary biotin-conjugated antibodies against the same angiogenic factors as those for primary antibodies, was added on the membranes and incubated overnight at 4°C, followed by incubation with HRP-conjugated streptavidin for 2 h. Each incubation step was followed by five washing steps. Thereafter, the membranes were incubated with a mixture of two detection buffers for 1 min, exposed to an X-ray film (Kodak) for 2 min and then the films were developed. The protein expression level was quantified by measuring the intensity of the color of each spot on the membranes in comparison to the positive control spots already bound to the membranes, using TotalLab Quant Software version 12 for Windows. The expression of each angiogenic protein in cell lysates from each cell culture condition was determined in duplicate.

Quantification of malondialdehyde (MDA). MDA is the main product of ROS-mediated lipid peroxidation and is therefore a good indicator of overall oxidative stress (18). MDA levels were

determined as previously described through high-performance liquid chromatography (HPLC) (19). Following deproteinization with HClO₄, samples were centrifuged at 4,500 x g for 5 min and 100 μ l of each supernatant was used for HPLC analysis. The column type was RP18 (5 μ m) (Supelco, Inc., Bellefonte, PA, USA) and the mobile phase consisted of 30 mM KH₂PO₄/methanol in a volume ratio of 65:35. Flow rate was set at 0.5 ml/min and MDA was measured using a UV detector (UV-2070/2075; Jasco, Tokyo, Japan) set at 254 nm. The retention time of MDA was ~5.4 min. Data were expressed as μ M MDA and were normalized to the protein concentration from cell lysates. Each sample was determined in duplicate.

Determination of nitric oxide (NO) metabolites. The effect of 5-FU treatment on the production of NO in standard culture and in co-culture was assessed by measuring nitrites via colorimetric Griess assay, as previously described (19). NO is a key signaling molecule that becomes cytotoxic to cancer cells when produced in high levels, whereas low levels of NO exert tumor promoting properties (20). This assay relies on the diazotization reaction in which nitrite reacts under acidic conditions with sulfanilic acid (Sigma-Aldrich) to form a diazonium salt, which subsequently couples with *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich) to form a stable azo dye that can be measured spectrophotometrically at 548 nm. Sample deproteinization with ZnSO₄ was applied prior to the assay and each sample was determined in duplicate. Sodium nitrite (Sigma-Aldrich) was used as a standard. Data were expressed as nM nitrites following normalization to the protein concentration from cell lysates.

Statistical analysis. Data from the various experiments are expressed as the mean \pm SD. For statistical analysis of the effects of 5-FU on C26 cells or co-culture of C26 cells and macrophages, an unpaired *t*-test was used. The differences between the effects of 5-FU on the production of each inflammatory/angiogenic factor in cells from standard culture and co-culture were analyzed by two-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. All statistical analyses were performed by using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA). *P*<0.05 was considered to indicate a statistically significant difference.

Results

Effects of 5-FU on the C26 cell proliferation. The effects of different concentrations of 5-FU on the proliferation of C26 cells in standard culture and in the presence of peritoneal macrophages were expressed as the percentage of inhibition compared to the proliferation of the controls (untreated cells) (Fig. 1A and B). In the two culture conditions, C26 murine colon carcinoma cells were incubated with increasing 5-FU concentrations ranging from 0.125 to 16 μ M for 72 h. The results regarding the cytotoxic effects of 5-FU on C26 cell proliferation indicated that 5-FU at concentrations of \geq 4 μ M strongly inhibited the growth of C26 cells (by 75% compared to the proliferation of control cells) under standard culture conditions (Fig. 1A) and after co-cultivation with TAMs (Fig. 1B). As 4 μ M was the lowest concentration of 5-FU at

Table I. Effects of 5-FU treatment on the production of angiogenic/inflammatory proteins in standard culture of C26 cells as well as in co-culture of C26 cells and macrophages.

Angiogenic/inflammatory factors	Percentage of inhibition and stimulation of angiogenic/inflammatory protein production compared to controls	
	C26+5-FU	C26+ ϕ +5-FU
Granulocyte CSF	+7.07 \pm 0.37	-46.80 \pm 0.73 ^c
Granulocyte-macrophage CSF	+148.58 \pm 0.21 ^c	-48.13 \pm 2.69 ^c
Monocyte CSF	-2.30 \pm 10.67	-60.21 \pm 2.29 ^c
Insulin-like growth factor 2	+138.56 \pm 1.95 ^c	+11.09 \pm 5.37
IL-1 α	+117.25 \pm 1.33 ^c	-58.22 \pm 0.73 ^c
IL-1 β	+59.80 \pm 18.98 ^c	-70.84 \pm 0.44 ^c
IL-6	+1.96 \pm 5.15	-43.79 \pm 1.61 ^c
IL-9	+4.12 \pm 0.60	+15.27 \pm 1.33 ^c
IL-12p40	+53.29 \pm 11.95 ^c	-73.02 \pm 0.15 ^c
IL-13	+101.89 \pm 4.79 ^c	+10.82 \pm 1.80
Tumor necrosis factor α	+39.59 \pm 11.20 ^a	+19.53 \pm 14.65 ^c
Monocyte chemoattractant protein-1	+118.83 \pm 10.46 ^c	-54.80 \pm 1.68 ^c
Eotaxin	+10.11 \pm 9.18	-58.02 \pm 0.90 ^c
Fas ligand	+498.13 \pm 4.91 ^c	-44.23 \pm 1.18 ^c
Basic fibroblast growth factor	+315.23 \pm 12.42 ^c	-45.08 \pm 1.84 ^c
Vascular endothelial growth factor	-56.69 \pm 8.27 ^c	-75.84 \pm 6.96 ^c
Leptin	-45.53 \pm 3.11 ^b	-33.05 \pm 10.34
Thrombopoietin	-5.51 \pm 3.99	-52.67 \pm 1.77 ^c
TIMP-1	+75.99 \pm 0.03 ^c	-49.62 \pm 1.28 ^c
TIMP-2	+93.76 \pm 14.77 ^c	-48.97 \pm 0.11 ^c
Platelet factor 4	+85.30 \pm 35.92 ^c	-66.08 \pm 2.36 ^c
IL-12p70	+101.51 \pm 2.46 ^c	-56.46 \pm 0.45 ^c
Interferon γ	+216.83 \pm 4.55 ^c	-48.25 \pm 1.94 ^c
Monokine induced by interferon- γ	-59.64 \pm 10.89 ^c	-87.02 \pm 2.56 ^c

The protein levels after 5-FU treatment are compared to control levels of the same proteins. The results are expressed as % of the average inhibition (-) or stimulation (+) \pm standard deviation of two independent measurements. Statistical differences were evaluated by using two-way analysis of variance with Bonferroni correction for multiple comparisons, and significance is indicated as follows: ^aP<0.05; ^bP<0.01; ^cP<0.001. 5-FU, 5-fluorouracil; C26+5-FU, lysates from C26 cells incubated with 4 μ M 5-FU for 72 h; C26+ ϕ +5-FU, lysates from co-culture of C26 cells and macrophages incubated with 4 μ M 5-FU for 72 h; CSF, colony-stimulating factor; IL, interleukin; TIMP, tissue inhibitor of metalloproteinases.

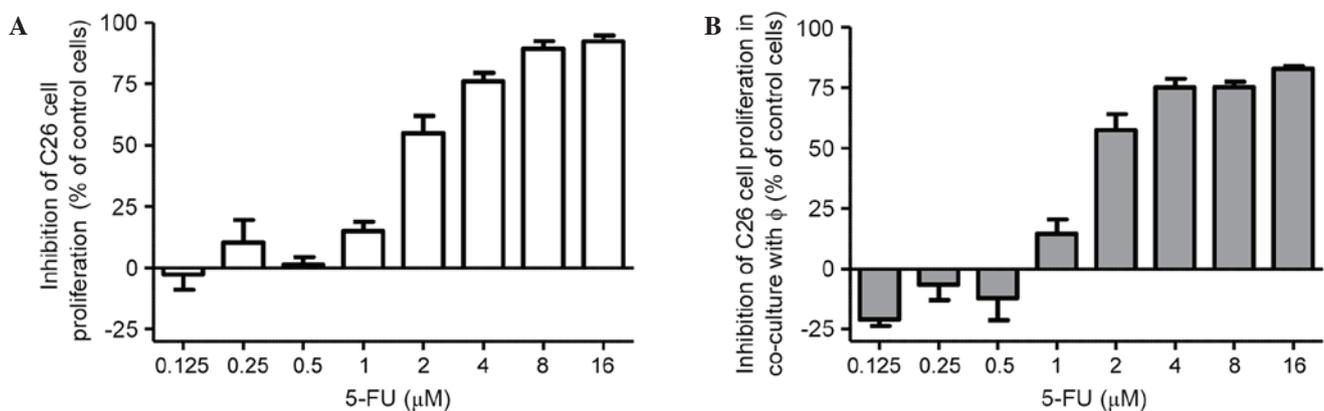


Figure 1. Effects of 5-FU on the proliferation of C26 murine colon carcinoma cells. The results show cell proliferation at (A) 72 h after incubation of C26 cells with various concentrations of 5-FU (0.125-16 μ M), and (B) 72 h after incubation of a co-culture of C26 cells with peritoneal macrophages (ϕ) with various concentrations of 5-FU (0.125-16 μ M). Data are presented as the mean \pm standard deviation of triplicate measurements. The results are expressed as a % of inhibition of C26 cell proliferation following 5-FU treatments compared to control cell proliferation (untreated cells). Control cells were (A) untreated C26 cells cultivated alone or (B) untreated C26 cells co-cultivated with peritoneal macrophages.

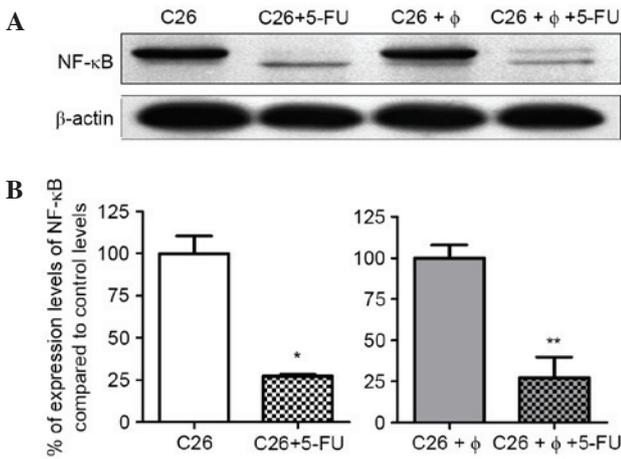


Figure 2. Effects of 5-FU administration on cell levels of NF-κB. (A) Western blot analyses of NF-κB levels in C26 cells in the following groups: C26, NF-κB levels in lysates from C26 cells without treatment for 72 h; C26+5-FU, NF-κB levels in lysates from C26 cells incubated with 4 μM 5-FU for 72 h; C26+φ, NF-κB levels in lysates from co-cultures of C26 cells and macrophages (φ) without treatment for 72 h; C26+φ+5-FU, NF-κB levels in lysates from co-cultures of C26 cells and macrophages incubated with 4 μM 5-FU for 72 h. β-actin was used as loading control. (B) Quantification of western blot data. The results are compared to the NF-κB levels in controls (represented by NF-κB levels in C26 cell lysates or NF-κB levels in lysates from co-cultures of C26 cells and macrophages). Data are expressed as the mean ± standard deviation of two independent measurements (*P<0.05 and **P<0.01 vs. controls). 5-FU, 5-fluorouracil; NF-κB, nuclear factor κB.

which strong cytotoxic effects were noted with regard to tumor cell proliferation (Fig. 1A and B), this concentration was used throughout the experiments performed for testing the modulatory actions of TAMs on the response of C26 colon carcinoma cells to 5-FU administration.

Influence of TAMs on the effects of 5-FU on NF-κB production. As several studies on colon carcinoma cell lines have demonstrated the role of constitutively activated NF-κB on the proliferative, antiapoptotic and angiogenic potential of these tumor cell lines (21,22), the present study was conducted to investigate whether cultivation of TAMs with C26 colon carcinoma cells could modulate the effects of 4 μM 5-FU on the production of this transcription factor. The results revealed that C26 cells constitutively expressed NF-κB (Fig. 2A and B). Notably, 5-FU exerted similar and strong inhibitory effects on the expression of NF-κB (70% inhibition compared to control levels) in lysates obtained under standard culture and under co-culture conditions (Fig. 2A and B).

Effects on the production of inflammatory/angiogenic proteins in response to the action of TAMs on 5-FU. To investigate whether TAMs modulate the action of 5-FU on the production of inflammatory and angiogenic proteins in C26 tumor cells co-cultivated with peritoneal macrophages, we performed a screening for 24 proteins involved in inflammation and angiogenesis by using RayBio® Mouse Angiogenic Cytokine Antibody Array kit. In addition, as a positive control for TAMs, we compared the inflammatory/angiogenic protein profile in co-culture lysates with the production of the same proteins in cell lysates where activated macrophages received 20 ng/ml IL-4 pretreatment for 24 h prior to incubation with

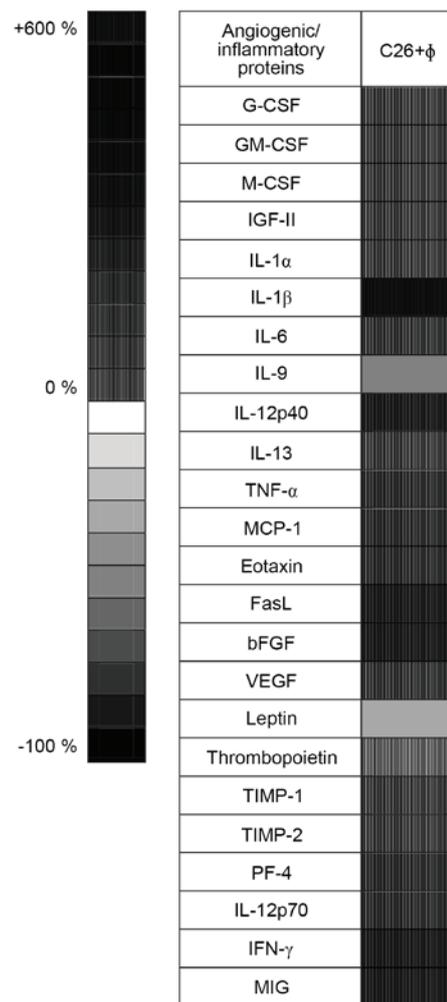


Figure 3. Effects of tumor-associated macrophages on the levels of angiogenic/inflammatory proteins in the cell co-culture lysates. Results are presented either as percentage (-) of protein levels [ranging from 0% (white) to 100% (black), shown as plain patterns] or as % of stimulation (+) of production of proteins [ranging from 0% (white) to 550% (black), shown as grid patterns] in cell lysates from untreated cell co-culture lysates (C26+φ) compared to levels of the same factors in lysates from untreated C26 cells. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, monocyte colony-stimulating factor; IGF-II, insulin-like growth factor 2; IL-, interleukin; TNF-α, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein-1; FasL, Fas ligand; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; TIMP, tissue inhibitor of metalloproteinases; PF-4, platelet factor 4; IFN-γ, interferon γ; MIG, monokine induced by IFN-γ.

C26 cells. Two-way ANOVA with Bonferroni correction for multiple comparisons was performed between the two inflammatory/angiogenic protein profiles and the statistical differences between each profile were not significant (data not shown). Thus, it is confirmed that upon co-culture of tumor cells with peritoneal macrophages, the latter were polarized into TAMs.

In accordance with previous studies (4-6,19), the overall production of the majority of the inflammatory and angiogenic factors in C26 cells co-cultivated with peritoneal macrophages was 2-fold higher than the production of the same factors in C26 cells cultivated alone (Fig. 3). More specifically, the co-cultivation of C26 cells with TAMs significantly stimulated the production of IL-6, TNF-α, monocyte chemoattractant

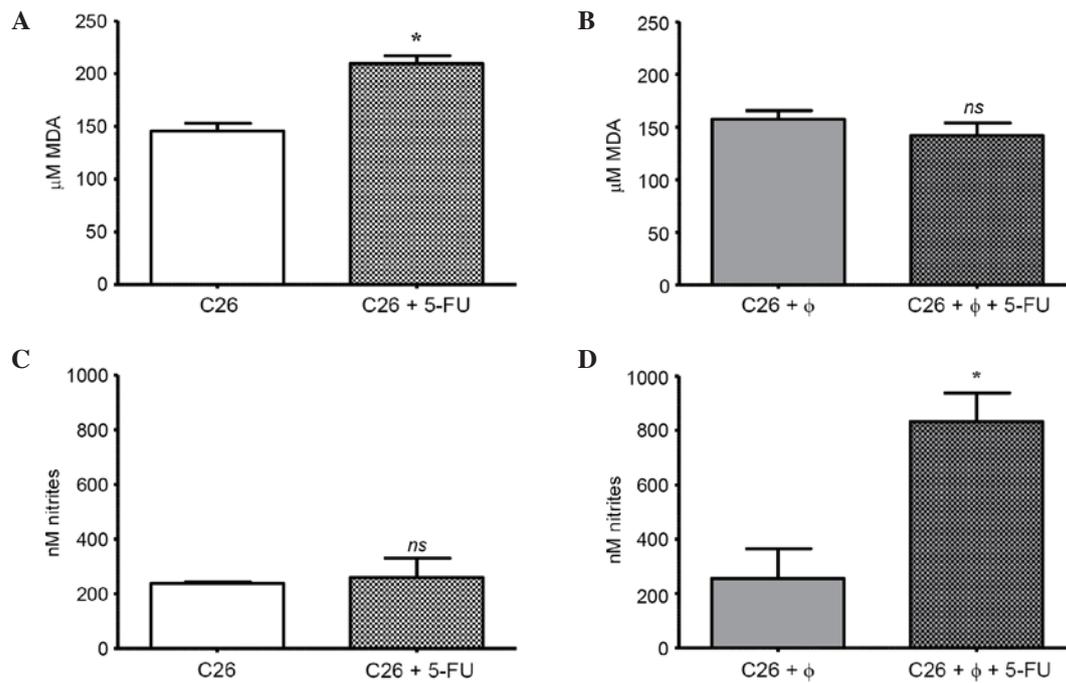


Figure 4. Effects of 5-FU treatment on the oxidative stress markers in lysates from standard cultures and co-cultures. (A) MDA levels in C26 cell lysates after 72 h of incubation with 4 μ M 5-FU; (B) MDA levels in cell lysates obtained from co-culture after 72 h of incubation with 4 μ M 5-FU; (C) nitrite levels in C26 cell lysates after 72 h of incubation with 4 μ M 5-FU; (D) nitrite levels in cell lysates obtained from co-culture after 72 h of incubation with 4 μ M 5-FU. Groups: C26, oxidative stress marker levels in lysates from C26 cells without treatment; C26+5-FU, oxidative stress marker levels in lysates from C26 cells incubated with 4 μ M 5-FU for 72 h; C26+ ϕ , oxidative stress marker levels in lysates from co-culture of C26 cells and macrophages (ϕ) without treatment; C26+ ϕ +5-FU, oxidative stress marker levels in lysates from co-culture of C26 cells and macrophages incubated with 4 μ M 5-FU for 72 h. Data are presented as the mean \pm standard deviation of two independent experiments. ns, not significant ($P>0.05$); * $P<0.05$ vs. control group. 5-FU, 5-fluorouracil; MDA, malondialdehyde.

protein-1 (MCP-1), eotaxin, VEGF, platelet factor 4 (PF-4) and IL-12p70 by 50-150%; the production of IL-12p40, Fas ligand (FasL), basic fibroblast growth factor (bFGF), interferon γ (IFN- γ), and monokine induced by IFN- γ (MIG) by 150-300%; and the production of IL-1 β by ~580%. In terms of inhibition, only the production of IL-9 was significantly inhibited, by 50% compared to its control level (Fig. 3).

Notably, when C26 colon carcinoma cells cultivated alone were incubated with 5-FU, the average production of the inflammatory and angiogenic proteins was increased by 84% compared to their levels in the untreated tumor cells ($P<0.0001$; Table I). Thus, the production of 19 out of 24 proteins studied was significantly stimulated. In particular, compared with control levels, the production levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor 2 (IGF-II), IL-13, MCP-1 and IL-12p70 were stimulated by 100-200% and the production of FasL, bFGF and IFN- γ was strongly stimulated by 200-500%. Only the levels of production of VEGF, leptin and MIG were significantly reduced, by 40-60% (Table I).

When macrophages were cultivated with C26 cells, 5-FU significantly reduced the production of inflammatory and angiogenic factors with an overall inhibitory effect of 44% ($P<0.0001$) compared to their control levels. Specifically, the levels of VEGF and MIG were strongly inhibited by 75-100%, the levels of monocyte colony-stimulating factor (M-CSF), IL-1 α , IL-1 β , IL-12p40, MCP-1, eotaxin, thrombopoietin, IL-12p70 and PF-4 were reduced by 50-75% and the levels of granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-6, FasL, bFGF, tissue inhibitor of metalloproteinases

(TIMP)-1, TIMP-2 and IFN- γ by 25-50% (Table I). The production levels of IL-9 and TNF- α were stimulated marginally, by 15-20% ($P<0.001$), following incubation of the cells in the co-culture with 5-FU.

Influence of TAMs on the effects of 5-FU on oxidative stress.

To determine whether TAMs are able to modulate the effects exerted by 4 μ M of 5-FU on oxidative stress in C26 cancer cells, important oxidative stress markers in cell lysates, such as MDA and nitrite levels (18,23), were quantified. The results are shown in Fig. 4A-D. Notably, the treatment with 5-FU significantly increased the level of MDA in C26 cells cultivated alone (by 45% compared to its control levels; $P<0.05$) (Fig. 4A). No significant differences between MDA levels in untreated cells under either culture condition were noted ($P=0.273$) (Fig. 4A and B). Furthermore, when C26 cells were exposed to 5-FU treatment in the presence of TAMs, there was no significant increase in the MDA level compared to the control level (Fig. 4B). This finding may suggest that TAMs counteracted the pro-oxidant action of 5-FU on C26 cells, as this effect could be noted only when C26 cells were cultivated alone (Fig. 4A and B).

To assess whether TAMs modulate the effects of 5-FU on NO production in C26 cells, the nitrite levels from cell lysates were determined, since nitrites are the major final metabolites of NO (23). The results are shown in Fig. 4C and D. The treatment with 5-FU did not affect the nitrite production in C26 cells under standard culture conditions (Fig. 4C). In the absence of 5-FU treatment, there was no difference between nitrite levels in cell lysates obtained under standard culture

and those obtained under co-culture conditions ($P=0.8499$; Fig. 4C and D). Notably, when 5-FU was administered in co-culture, there was a significant 4-fold increase in nitrite levels compared to their production in control lysates (lysates from co-culture without treatment; $P=0.0329$) (Fig. 4D).

Discussion

The controversial role of TAMs in colon carcinoma development directed our studies to further investigate whether this cell type influences the effects of 5-FU on the expression of the transcription factor NF- κ B, a constitutively activated protein in 67% of CRC cell lines (21). The results confirmed that C26 cells constitutively expressed NF- κ B (Fig. 2A) and this production was maintained in cells from the co-culture of C26 cells and TAMs (Fig. 2B). Since the intratumor constitutive expression and activation of NF- κ B are predominantly associated with tumor proliferation as well as with rescue of the cancer cells from cell death (21,24,25), inhibition of this transcription factor has been demonstrated to be a potent therapeutic strategy in CRC (26).

Notably, in the present study, 5-FU administration strongly reduced the levels of NF- κ B (by 70% compared to control levels) in cell lysates obtained under the two culture conditions (Fig. 2A and B). The suppression of NF- κ B production exerted by 5-FU may be linked to the high cytotoxicity of this drug on C26 cells (Figs. 1A and 2). This finding is also supported by previous reports of an association between the high cytotoxicity of several cytotoxic drugs (5-FU, doxorubicin, paclitaxel and bortezomib) on different cancer cell lines (human stomach cancer cells, human myeloid leukemia cells, human salivary gland cancer cells and C26 colon carcinoma cells) and the inhibition of NF- κ B activation and, finally, induction of apoptosis in these tumor cells (22,24,27).

Since NF- κ B also participates in the induction of several genes encoding for proteins that support tumor angiogenesis and inflammation (21,28), we assessed the expression levels of 24 proteins involved in these two pro-tumor processes via protein array analysis. In line with previous studies (4), our data confirmed that TAMs serve a crucial role in supporting tumor angiogenesis and inflammation, as co-cultivation of C26 cells with macrophages led to a doubling of the average production of the majority of the inflammatory and angiogenic factors compared to their production in C26 cells cultivated alone (Fig. 3). Notably, TNF- α and IL-1 β cytokines, which constitutively activate NF- κ B and finally emphasize the angiogenic and metastatic capacity of tumor cells (7,8,29-31), are overexpressed under co-culture conditions (Fig. 3). However, in the presence of macrophages, 5-FU treatment moderately to very strongly reduced the production of the majority of the pro-angiogenic (VEGF, eotaxin, thrombopoietin, bFGF) and pro-inflammatory (M-CSF, IL-1 α , IL-1 β , IL-12p40, MCP-1, G-CSF, GM-CSF, IL-6, FasL) proteins compared to their production in the untreated cell co-culture (Table I). The significant inhibition of these proteins could account for the high 5-FU cytotoxicity induced through the suppression of NF- κ B since the aforementioned proteins have also been associated with the proliferation, survival and metastasis of cancer cells (7,8,10,31,32). Furthermore, NF- κ B signaling has been described as the main mechanism for maintaining the pro-tumor phenotype of

TAMs (33,34). Interestingly, in the absence of macrophages, 5-FU considerably stimulated the C26 cell production of 19 out of 24 angiogenic and inflammatory proteins (Table I). Although 5-FU treatment strongly reduced VEGF expression (>50%), the levels of bFGF and FasL were significantly increased (by 315% for bFGF and ~500% for FasL) compared to their production in the untreated C26 cells cultivated alone (Table I). These data are supported by previous findings related to the upregulation of the angiogenic factor bFGF as a result of suppression of VEGF-regulated signaling pathways (35). Furthermore, the overexpression of bFGF and FasL was previously associated with the increase of the aggressiveness and metastatic potential of cancer cells (35,36). In conclusion, these data may suggest the limitation of the 5-FU cytotoxicity on C26 cells, probably due to the presence of efficient scavenger mechanisms in these cancer cells via enhancing their angiogenic and inflammatory capacity (Table I). It is noteworthy that the cultivation of macrophages with C26 cells counteracted the escape mechanisms of the carcinoma cells from cytotoxic drug effects, since the suppression of most of the angiogenic and inflammatory protein levels was noted after 5-FU administration.

Additionally, as many studies have demonstrated the role of TAMs in maintaining the physiological range of oxidative stress necessary for tumor cell proliferation and metastasis (37-39), we assessed the levels of two important oxidative stress markers in cell lysates (MDA and nitrites, as stable final products of NO metabolism) (18,23) after incubation of C26 cells in standard culture and co-culture with 5-FU. As previously shown in other colon carcinoma cell lines (40-42), 5-FU exerted pro-oxidant effects on C26 cells cultivated alone. In the present study, the significantly increased MDA levels following 5-FU treatment (+45%; Fig. 4A) compared to the levels in untreated C26 cells, could account for 5-FU cytotoxicity on C26 cells, as several studies already demonstrated that an increased oxidative stress over the physiological range induced inhibition of cell proliferation and finally cell death via ROS-induced apoptosis (40,42,43).

Nevertheless, this pro-oxidant effect of 5-FU may be responsible for stimulating the production of the angiogenic and inflammatory proteins in the remaining C26 cancer cells mentioned earlier (Table I). This finding is also supported by several studies that suggest that an essential feature of the aggressive phenotype, acquired by the undestroyed cancer cells exposed to high ROS levels, is the ability to enhance the production of angiogenic and inflammatory proteins (44). However, when 5-FU was administered in the co-culture, there was no effect on MDA levels in cell lysates (Fig. 4B). This finding suggested that TAMs may protect cancer cells against 5-FU-induced oxidative stress and, subsequently, from ROS-induced cytotoxicity. Furthermore, our data regarding the NO amount in cell lysates revealed significant increases in nitrite levels (4 times higher than in controls or 5-FU-treated C26 cells alone) only after 5-FU administration in C26 cells cultivated with TAMs (Fig. 4C and D). Nevertheless, the increase of NO production noted following co-cultivation of C26 cells with TAMs did not exceed the physiological range of NO production in tumors (nM range) described previously (20). This result is likely related to the cytoprotective effects of TAMs against pro-oxidant effects of 5-FU on C26 cells (Fig. 4A and D).

As other studies previously suggested, the increased production of NO could be a phenomenon strictly related to the tumor cell-macrophage interaction (12). Previous studies demonstrated that nanomolar concentrations of NO produced by inducible/endothelial nitric oxide synthases in human colon and ovarian carcinoma cells as a result of administration of cytotoxic drugs other than 5-FU, such as doxorubicin or cisplatin, ensure protection against ROS-induced apoptosis in these cancer cells (39,45,46). Although TAMs protect C26 cells against the pro-oxidant effect of 5-FU, this action may avoid the acquisition of the aggressive phenotype of the remaining C26 cells, since the production of the majority of the angiogenic and inflammatory proteins was reduced notably in cells under co-culture conditions (Table I).

Taken together, the results suggest that TAMs have a dual role in the modulation of the response of C26 cells to 5-FU treatment. On one side, TAMs increase chemosensitivity of these cancer cells to 5-FU treatment via mediating an overall strong reduction of inflammatory and angiogenic factors; however, on the other side, TAMs protect cancer cells against the pro-oxidant effect of 5-FU by maintaining ROS levels in the physiological range of C26 cell oxidative stress.

Finally, the current results suggest that therapeutic strategies of CRC should further exploit the intrinsic oxidative stress of cancer cells by combining the administration of 5-FU with pharmacological agents that prevent TAMs to maintain the physiological range of tumor oxidative stress.

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