Cell Wall Membrane Fraction of *Chlorella sorokiniana* Enhances Host Antitumor Immunity and Inhibits Colon Carcinoma Growth in Mice

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Abstract

A colon cancer growth inhibitor partially purified from the isolated cell wall membrane fraction of *Chlorella sorokiniana*, here referred to as *Chlorella* membrane factor (CMF), was evaluated for its antitumor and immunomodulatory effects in cell culture and in a colon carcinoma mouse model. The CMF treatment dose- and time-dependently inhibited colon carcinoma cell growth in 2-dimensional cultures. Treatment with CMF also significantly inhibited the growth of colon carcinoma spheroids in 3-dimensional cell culture in coculture with T lymphocytes. In a mouse CT26 colon carcinoma peritoneal dissemination model, intraperitoneal injection of CMF (10 or 30 mg dry weight/kg body weight, every other day) dose-dependently and significantly attenuated the growth of tumor nodules via induction of tumor cell apoptosis. Evaluation of immune cell populations in ascites showed that CMF treatment tended to increase T lymphocytes but lower granulocyte populations. The present study suggests that the cell wall membrane fraction of *Chlorella sorokiniana* contains a bioactive material that inhibits colon carcinoma growth via direct cell growth inhibition and stimulation of host antitumor immunity. Hence, it is suggested that the *Chlorella* cell wall membrane extract or a bioactive substance in the extract is an attractive complementary medicine for cancer therapy.

Introduction

In the United States, colon cancer is the second leading cause of cancer death in both sexes combined and there were an estimated 101 420 new cases and 51 020 deaths in 2019.[1] Because of improvements in early detection and treatment, the current 5-year survival rate is 90% in patients diagnosed with early-stage colon cancer. However, survival rates of patients diagnosed with regional and distant metastases are 71% and 14%, respectively.[2] Therefore, colon cancer still comprises a significant portion of cancer-dependent mortality and morbidity. Accordingly, finding a better therapy is an urgent necessity.

Chlorella is a unicellular green algae detected in fresh water throughout the world. *Chlorella* whole cell powder or crushed cell body powder is taken as a nutritional and functional dietary supplement due to its high nutritional value.[3,4] In addition, water or alcohol extracts of *Chlorella vulgaris* and *C pyrenoidosa* have been shown to have therapeutic value against multiple cancers.[5-12] Although these studies suggest that an antitumor effect associated with *Chlorella* extract is related to the stimulation of host antitumor immune responses,[6,9,11] its molecular mechanism is yet

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to be fully understood. Furthermore, the origin of the bioactive component/components is unclarified.

The *Chlorella* cell wall is a thick membrane composed of a large amount of insoluble polysaccharide, a relatively small amount of protein/glycoprotein, and unidentified materials.[13,14] Polysaccharides consist primarily of mannose and glucose.[13] Since the *Chlorella* cell wall is unique in structure and composition and makes up a relatively large portion of the *Chlorella* body, it is of interest to study the biological activities of the water extract from the *Chlorella* cell wall in the field of cancer prevention and therapy. In this article, we report for the first time that the colon cancer growth inhibitor in the cell wall membrane fraction of *C sorokiniana* inhibits the growth of human and murine colon carcinoma cells in vitro in cell culture and in vivo in a mouse colon cancer allograft model via direct growth inhibition and stimulation of host antitumor activity through T lymphocyte activation.

Materials and Methods

Animals

Female Balb/c mice were obtained from Charles River Laboratories International, Inc. All mice were housed in a clean facility and acclimatized for 10 days. All animal experiments adhered strictly to protocols approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 3857) and Institutional Biosafety Committee (Protocol # 1050).

Materials

The mouse colon carcinoma cell line CT26.CL25 (CRL-2639); human colon carcinoma cell lines SW620 (CCL-227), HT29 (HTB-38), COLO 205 (CCL-222), and Caco-2 (HTB-37); and human lymphoblast cell line Jurkat (TIB-152) were purchased from American Type Culture Collection (ATCC; Manassas, VA). RPMI (Roswell Park Memorial Institute) 1640 and Eagle's minimal essential medium (MEM) was purchased from Mediatech, Inc (Manassas, VA). Macoy's 5A modified medium was from Sigma (St Louis, MO). Fetal bovine serum was from EQUITECH-BIO Inc (Kerrville, TX). Penicillin-streptomycin stock was obtained from Lonza Rockland, Inc (Allendale, NJ). Lipopolysaccharides (LPS) from *Escherichia coli* O111:B6 were purchased from Sigma. Fluorescent conjugated antibodies targeting CD4 (H129.19), CD8b (YTS156.7.7), CD19 (6D5), dendritic cells (DCs) marker (33D1), LY6G (1A8), CD68 (FA-11), and mouse IgG (immunoglobulin G) isotype were obtained from BioLegend (San Diego, CA).

Chlorella *Membrane Factor Preparation*

The *C sorokiniana* cell wall membrane fraction was isolated from a culture of whole *Chlorella* by the proprietary method developed by the euglena Co Ltd (Tokyo, Japan). Briefly, the whole *Chlorella* was cleanly cultured in a commercial plant and the cell wall membranes spontaneously suspended in *Chlorella* culture media were separated from intact cell bodies of *C sorokiniana* by 2 centrifugations at 8700g for 10 minutes. The resultant *Chlorella* cell wall membrane fraction was washed with deionized water 3 times and freeze dried.

To extract the colon cancer growth inhibitor from the *Chlorella* cell wall membrane fraction, the lyophilized membranes were rehydrated with phosphate-buffered saline (PBS) at a concentration of 40 mg/mL and incubated at 4°C for 12 hours and later at 37°C for 30 minutes with a 30-second sonication and vortex mixing at every 10 minutes. Insoluble materials composed of *Chlorella* cell wall membranes were removed by centrifugations at 2300g first and the resultant supernatant was further centrifuged at 11 800g, at room temperature for 10 minutes each. Insoluble materials larger than 200 to 300 nm diameter in the supernatant fraction was removed by a 0.22-µm disk filter (Midwest Scientific, Valley Park, MO), and the filtrate was stored at −20°C until use. This fraction was designated the *Chlorella* membrane factor (CMF) and subjected to the experiments described below.

Electron Microscopy

The transmission electron microscopy (TEM) of the freeze-dried *Chlorella* cell wall membrane fraction was carried out after rehydration of the membranes with a small amount of PBS. These rehydrated membranes were fixed with Trump's fixative (pH 7.4) overnight at 4°C, post fixed with 1% osmium tetroxide in a 0.2 M phosphate buffer for 1 hour, and dehydrated with a graded ethanol solutions several times. The *Chlorella* cell wall membranes were washed with acetone and embedded in Spurr resin, followed by polymerization of sample block in flat embedding molds. The sample block was thin-sectioned at a thickness of 700 to 900 Å using a Leica UC7 ultramicrotome and placed on a 200 mesh copper TEM grid. Ultrathin sections were analyzed using a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope at an accelerating voltage of 80 kV. Electron micrographs were taken with a Tecnai 12 (FEI) microscope, equipped with a Gatan CCD camera. The electron micrograph of rehydrated *Chlorella* cell wall membranes indicated that the experimental material used for the present study was composed of exclusively various size membranes but not any intact *Chlorella* bodies (Figure 1).
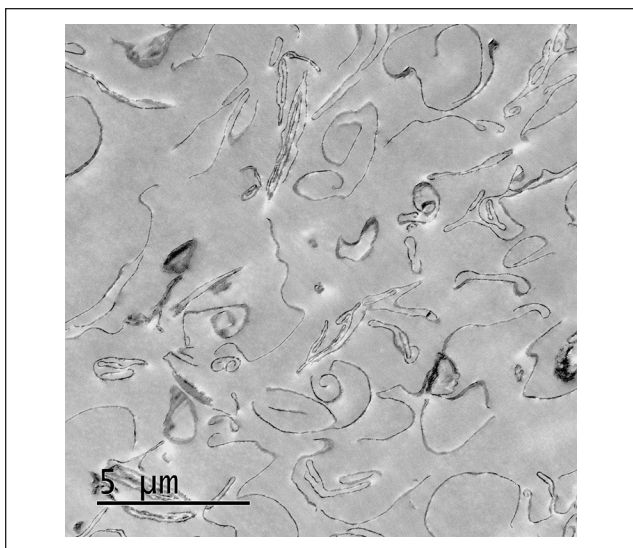
Figure 1. Transmission electron micrographs of freeze-dried *Chlorella* cell wall membrane fraction.

Cell Culture

The CT26 murine colon carcinoma cells, SW620 and COLO 205 human colon carcinoma cells, and Jurkat cells were cultured in RPMI 1640. The HT29 human colon carcinoma cells were cultured in Macoy's 5A modified medium. Caco-2 human colon carcinoma cells were cultured in MEM. Each medium was supplemented with 10% v/v fetal bovine serum and 1% v/v penicillin-streptomycin. These cells were cultured at 37°C in a humidified air atmosphere containing 5% CO_2.

Effect of CMF on the Growth of Human and Murine Colon Carcinoma Cells in 2-Dimensional Cell Culture

The murine (CT26; 1000 cells/well) and human (SW620, HT29, COLO 205, and Caco-2) colon carcinoma cells (3000 cells/well) were seeded into a 96-well plate with 100 µL growth medium, followed by treatment with CWME after 24 hours. The time- and dose-dependent effects of CMF were evaluated by measuring cell growth for cells treated with 1, 10, and 100 µg/mL CMF at 48 and 72 hours after treatment. Cell proliferation was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[15] PBS served as a negative control.

Effect of Combination Treatment by CMF and Jurkat Cells on the Growth of CT26 Cell Spheroid in In Vitro 3-Dimensional Spheroid Culture

To evaluate the combined effect of CMF and immune cells, a 3-dimensional (3D) spheroid assay was performed as described previously with slight modifications.[16,17] The cells were treated with CMF (25 µg/mL) on Day 1 and Day 4. The image of spheroids was taken at Day 7 by an inverted microscope IX51 (Olympus America Inc, Center Valley, PA) equipped with cellSens Dimension software (Olympus). Growth of the tumor spheroids was evaluated by measuring the spheroid volume.

The activation status of Jurkat cells was evaluated by morphological change and IL-2 (interleukin-2) expression. IL-2 expression in Jurkat cells treated with 25 µg/mL CMF was measured by 1-step reverse transcriptase quantitative polymerase chain reaction as described as previously.[17] The sequences of primers used are described in Table 1.

Effect of CMF Treatment on Colon Carcinoma Tumor Growth in the Peritoneal Cavity

The antitumor effect of CMF was evaluated in Balb/c mice using a CT26 murine colon carcinoma allograft. Mice were anesthetized with isoflurane and injected intraperitoneally with a suspension of 2.5×10^5 CT26 cells in 200 µL PBS. The intraperitoneal administration of CMF (10 or 30 mg/kg) was carried out at 3 days after CT26 inoculation for 8 days (every other day, totaling 5 injections). The PBS control was injected intraperitoneally with the same schedule. The mouse body weights were monitored at 2-day intervals. All mice were sacrificed by exposure to saturated CO_2 followed by cervical dislocation 2 weeks after CT26 inoculation. Tumor nodules contained in the omenta and pancreases were collected to examine tumor growth. The

Table 1. Primers Used for RT-qPCR.

Primer		Sequence	Size
Human IL-2	Forward (5′-3′)	ATGAGACAGCAACCATTGTAGAATTT	87 bp
	Reverse (5′-3′)	CACTTAATTATCAAGTCAGTGTTGAGATGA	
18S	Forward (5′-3′)	GAGGTTCGAAGACGATCAGA	315 bp
	Reverse (5′-3′)	TCGCTCCACCAACTAAGAAC	

Abbreviation: RT-qPCR, reverse transcriptase quantitative polymerase chain reaction.

tumor nodules contained within the 2 organs were weighed and fixed in 10% formalin for histological analysis. Because the weights of PBS- and CMF-treated mouse omenta and pancreases were similar among the mice, tumor nodule weights were normalized by subtracting the average weights of the omentum (425.2 ± 37.3 mg) and the pancreas (161.1 ± 27.2 mg).

Analysis of CMF Treatment-Associated Apoptosis of CT26 Cell Tumor Cells by Immunohistochemistry

A TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay and immunohistochemistry of cleaved caspase-3 was carried out to evaluate apoptosis in tumors from either PBS- or CMF-treated mice. The TUNEL assay was conducted using the APO-BRDU-IHC (TUNEL) Apoptosis Kit (Novus Biologicals, Centennial, CO). Immunohistochemistry of cleaved caspase-3 was carried out as described previously.[17] The average number of TUNEL or cleaved caspase-3 positive cells in 10 random fields (n = 6) was calculated.

Flow Cytometry Analysis of Leukocytes in the Ascites of CMF-Treated Tumor-Bearing Mice

Leukocytes in ascites of CMF-treated tumor-bearing mice were collected at the end of the mouse study (2 weeks after CT26 inoculation). Five milliliters of saline was injected into the abdominal cavity using a 22G 1¼″ Surflash I.V. Catheter (Terumo Medical Corporation, Somerset, NJ) and ascites fluids were collected via catheter. Ascites collection was repeated one additional time. Following the removal of red blood cells using an ACK lysing buffer (Lonza Walkersville, Inc, Walkersville, MD), the leukocytes were immunostained using anti-CD4 (helper T cells), anti-CD8b (cytotoxic T cells), anti-CD19 (B cells), anti-DC marker (33D1, dendritic cells), anti-LY6G (neutrophil), and anti-CD68 (macrophage) antibodies, and their population distributions were evaluated by flow cytometry. Mouse IgG was used for the isotype control. PBS served as a negative treatment control. The changes of cell populations were analyzed by flow cytometry (BD LSRFortessa X-20; BD Biosciences, San Jose, CA) and analyzed by BD FACSDiva software (BD Bioscience).

Statistical Analysis

All values are expressed as the mean ± standard deviation of mean. For all in vitro and in vivo experiments, statistical significance was assessed by an unpaired *t* test or ANOVA (analysis of variance) followed by Tukey's test. All experiments were conducted with multiple sample determinations with several samples (n = 3-6). Statistical significance was set at *, $P < .05$.

Results

CMF Treatment Attenuated the Growth of Both Murine and Human Colon Carcinoma Cells in Cell Culture

An MTT assay was carried out to evaluate the effect of the *Chlorella* cell wall membrane water extract (CMF) on the growth of murine and human colon cancer cells. The selection of both murine and human colon carcinoma cells was made to investigate whether the CMF effect was cell type-specific or cytotoxicity-dependent nonspecific. First, the specificity of the CMF's effect on several colon cancer cells was evaluated. CT26 murine colon carcinoma cells, SW620, COLO 205, Caco-2, and HT29 human colon carcinoma cells were treated with CMF (1-100 μg/mL) for 72 hours in cell culture. The CMF treatment dose- and time-dependently attenuated the growth of CT26, Caco-2, and HT29 colon carcinoma cells ($P < .05$), but not SW620 and COLO 205 cells (Figure 2A and B). Only very weak growth inhibition was detected at both 48 and 72 hours after treatment with a high dose of CMF (100 μg/mL) in both SW620 and COLO 205 cell growth (Figure 2B). The IC50 values for CT26 and HT29 cells were 425.0 μg/mL and 353.6 μg/mL, respectively. CMF treatment did not show any effect on normal mouse mesothelial cell growth at doses of 1 to 100 μg/mL for 24 to 72 hours. These results indicate that CMF treatment dose- and time-dependently attenuates the growth of select murine and human colon carcinoma cells, but not normal epithelial cells. These results suggest that the growth attenuation effect of CMF is not due to nonspecific cytotoxicity.

CMF Treatment Enhanced T Lymphocyte Antitumor Activities in the Spheroid Growth of CT26 Cells In Vitro

As shown in Figure 3A and B, treatment with 25 μg/mL CMF significantly attenuated spheroid growth of CT26 cells as compared with the PBS-treated group ($P < .05$). However, this CMF-dependent growth attenuation of the CT26 cell spheroid was further pronounced in the coculture with Jurkat cells ($P < .05$ as compared with all other groups). Morphological analysis of cocultured Jurkat cells in the spheroid assay revealed that CMF treatment alone or coculture with CT26 spheroids only slightly modified the morphologies of Jurkat cells (Figure 3C). However, morphologies were drastically changed by the combination treatment with CMF in the presence of a CT26 spheroid. These morphological analyses may suggest that CMF triggers the differentiation of T lymphoblasts and that the effect of CMF is significantly enhanced in the presence of cancer cells. In
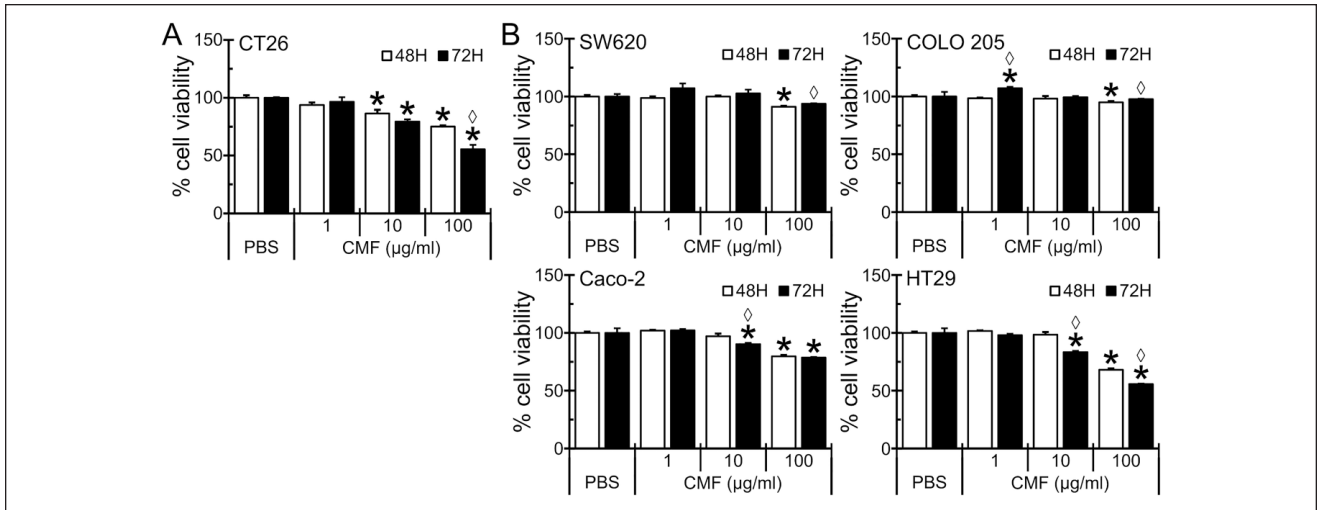
Figure 2. *Chlorella* membrane factor (CMF) dose- and time-dependently inhibited the growth of murine and human colon carcinoma cells in 2D cell culture. CMF treatment dose-dependently (1-100 μg/mL) and time-dependently (48 and 72 hours treatment) attenuated the growth of CT26 murine colon carcinoma cells (A), Caco-2 and HT29 human colon carcinoma cells (B). The cell growth was evaluated by MTT assay (n = 3). *$P <$ .05 compared with PBS-treated control. ◊$P <$ .05 compared in same group at 48 hours.
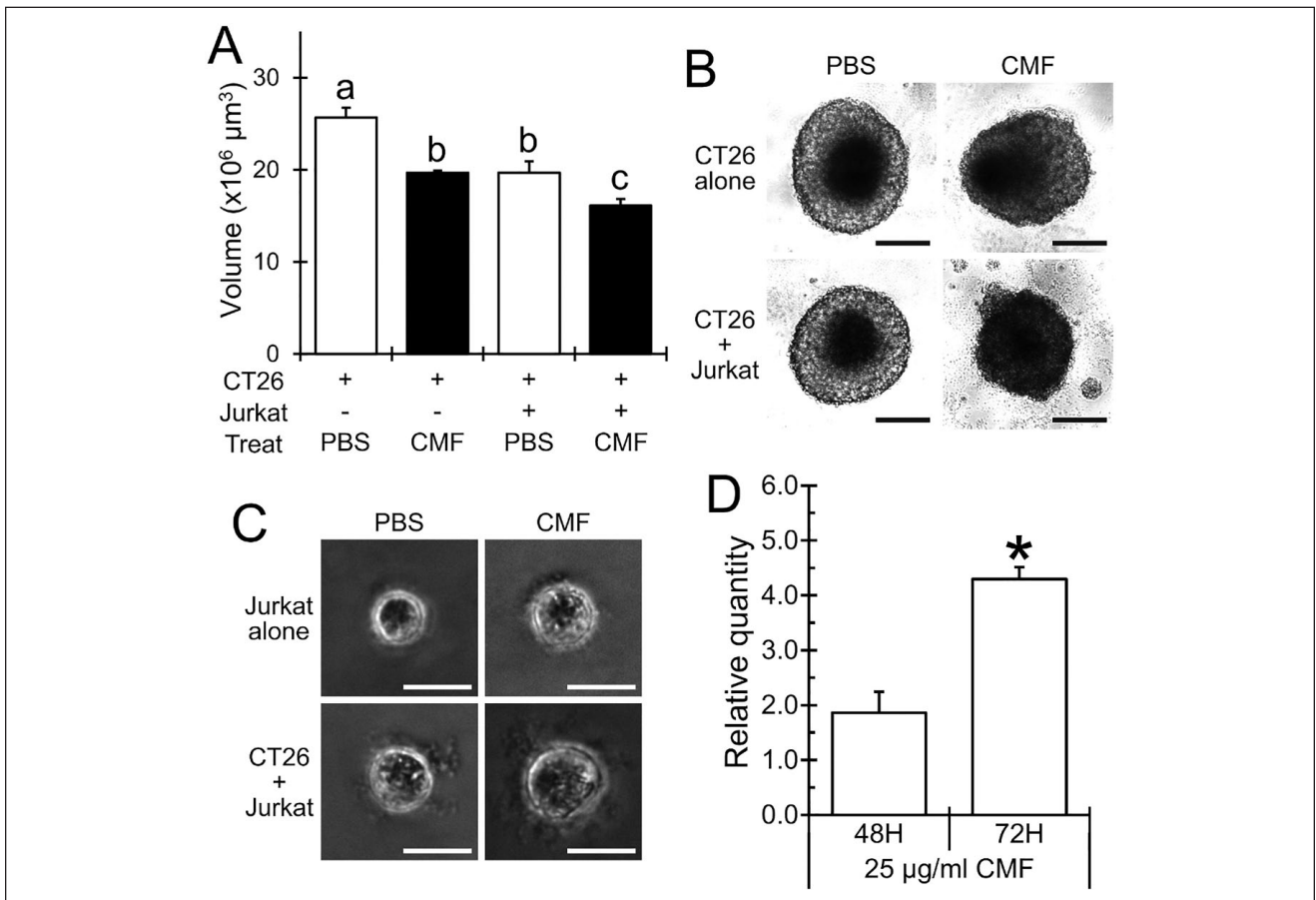

Figure 3. *Chlorella* membrane factor (CMF) treatment attenuated the growth of CT26 spheroids in coculture with T lymphoblasts. (A and B) The effect of CMF (25 μg/mL) on the growth of CT26 spheroids was evaluated in the presence or absence of lymphoblasts (Jurkat cells). (A) The volume of the spheroid was measured at Day 7 (n = 5). a–c, $P <$ .05 between different characters. (B) Typical pictures of spheroid in each group. Scale bar, 100 μm. (C) Typical morphologies of Jurkat cells in the agar matrix of each treatment group. Scale bar, 20 μm. (D) IL-2 expression in CMF-treated Jurkat cells was evaluated by RT-qPCR. *$P <$ .05 compared with PBS-treated control at each time point.
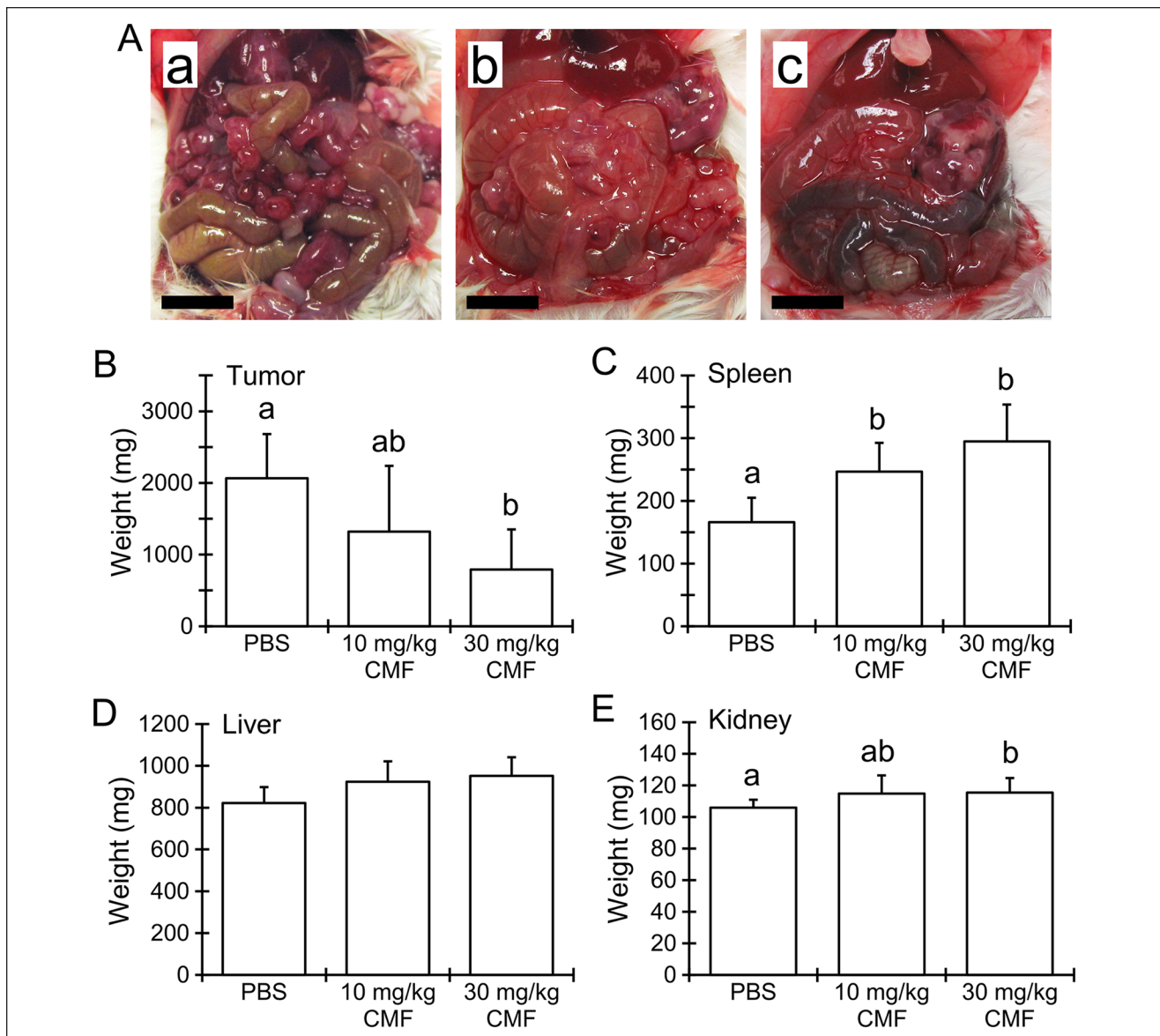
Figure 4. *Chlorella* membrane factor (CMF) treatment significantly attenuated the growth of CT26 cell tumors in mouse peritoneal cavity via an induction of tumor cell apoptosis. (A), Macroscopic views of typical peritoneal cavity in PBS, 10 mg/kg and 30 mg/kg CMF-treated mice. Scale bar in each picture represents 5 mm. (B-E), Average weight of tumor (B), spleen (C), liver (D), and kidney (E) in each treatment group was presented in the bar graphs (n = 6). a and b, $P < .05$ between different characters.

addition, IL-2 expression, which is one of the T lymphocyte activation-associated cytokines, was significantly increased by the treatment with 25 µg/mL CMF after 72 hours of treatment (Figure 3D). These results suggest that CMF assists immature T lymphocytes in differentiating into mature cells, thereby inhibiting CT26 cell tumor spheroid growth.

Intraperitoneal Injection of CMF Attenuated the Growth of CT26 Murine Colon Carcinoma in a Peritoneal Dissemination model

To evaluate the potential antitumor effect of CMF in vivo, 10 or 30 mg/kg CMF was administered into the peritoneal cavity of the CT26 cell tumor-bearing mice. As shown in Figure 4A and B, the average tumor weight (792.2 ± 558.7 mg, $P < .05$) of the group treated with 30 mg/kg CMF was significantly smaller than that of the PBS treatment group (2064.6 ± 616.9 mg). In addition, the treatment of 10 mg/kg CMF also may be associated with decreased tumor weight (1320.4 ± 917.6 mg, ns) compared with that of the PBS treatment group (Figure. 4A and B). Apoptotic cells in tumor nodules determined by a TUNEL assay and immunohistochemical analysis with anti-cleaved caspase-3 antibodies suggests that the CMF treatment significantly increased the number of apoptotic cells in CMF-treated CT26 cell tumors compared with PBS-treated tumors
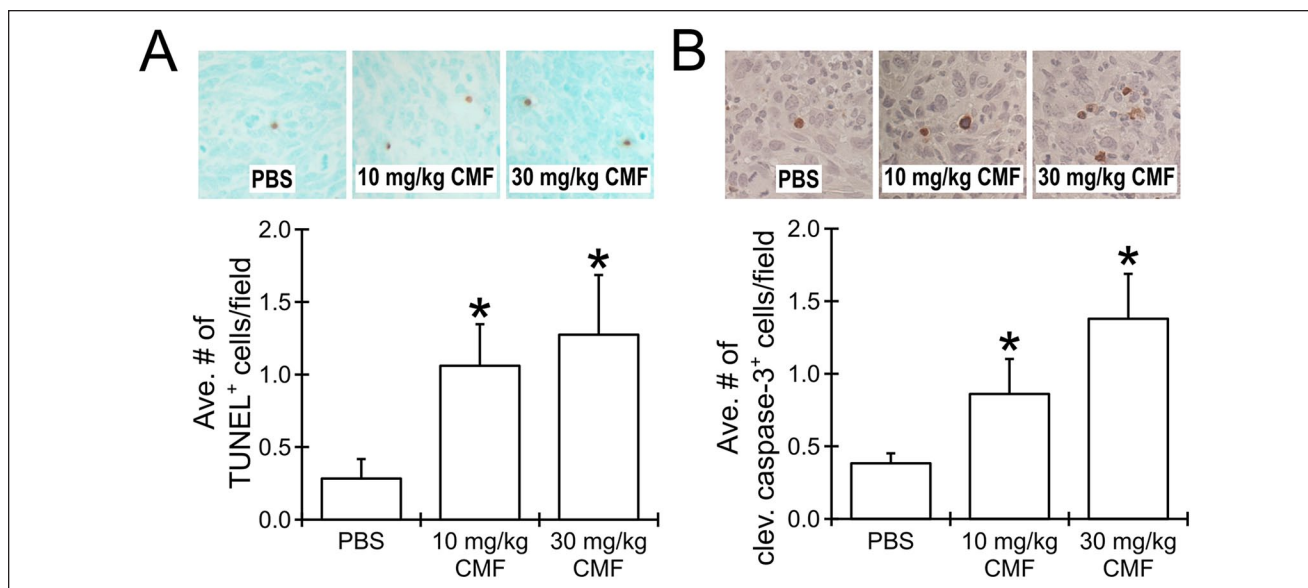
Figure 5. *Chlorella* membrane factor (CMF)-treatment induced apoptosis in tumors of CMF-treated mice. Analysis of apoptosis in tumor cells was carried out by the TUNEL assay (A, n = 6) and immunohistochemistry with anti-cleaved caspase-3 antibodies (B, n = 6). *P < .05 as compared with the level of the PBS-treated control tumors.
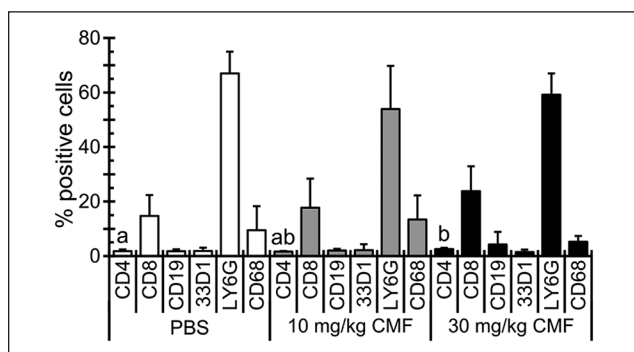

Figure 6. *Chlorella* membrane factor (CMF) treatment caused changes in leukocyte populations in ascites of mice bearing CT26 cell tumors. Leukocytes in the ascites collected from the tumor-bearing mice were analyzed by flow cytometry using anti-CD4, -CD8, -CD19 (B cells), -33D1 (DCs), -LY6G (granulocytes/neutrophils), and -CD68 (macrophages) antibodies. PBS served as a negative control treatment (n = 6). a and b, P < .05 between different characters.

(Figure 5A and B). Contrary to the decrease of the tumor weights, the spleen weights in the mice treated with 10 and 30 mg/kg CMF significantly increased compared with those of the PBS control mice (Figure 4C). This spleen weight increase may be a reflection of an effective increase of spleen function as the spleen is the major proliferation and storage site of lymphocytes and monocytes.[18,19] The liver and kidney weights also slightly increased in CMF treated mice as compared with those in PBS control mice (Figure 4E), which is presumably a result of the metabolism of CMF. However, no significant abnormality was

noted in these 2 organs by macroscopical observations. Since CMF treatment significantly increased T lymphocyte-dependent inhibition of the tumor spheroid growth in 3D culture (Figure 3), it is suggested that CMF-dependent tumor growth inhibition in mice is due at least in part to an enhancement of antitumor immunity. To test this hypothesis, immune cell populations in ascites collected from PBS- or CMF-treated tumor-bearing mice were analyzed by flow cytometry. As shown in Figure 6, CMF treatment increased CD4$^+$ and CD8$^+$ T lymphocyte populations, and CD19$^+$ B cell populations. These CD4$^+$, CD8$^+$, and CD19$^+$ cell populations were particularly increased in ascites of 30 mg/kg CMF-treated mice, with increases of 42.1% (P < .05), 61.9%, and 142.6%, respectively. In contrast, populations of CD68$^+$ macrophages (44.7% decrease) and 33D1$^+$ dendritic cells (21.2% decrease) in ascites of mice treated with 30 mg/kg CMF were decreased as compared with the PBS-treated group. The LY6G$^+$ granulocyte population in ascites was also decreased in mouse groups treated with 10 or 30 mg/kg CMF (11.6 and 19.5% decrease, respectively). This modulation of immune cell populations in CMF-treated mice suggests that CMF treatment induces antitumor immunity through an increase of anti-tumorigenic effector T cells and a decrease of pro-tumorigenic macrophages.

Discussion

A growing number of publications indicate that components in *Chlorella* extracts may be potential therapeutics against multiple diseases including cancers.[5-12,20-27] Most of these studies have used extracts from whole *Chlorella* or crushed

Chlorella powders. However, since whole *Chlorella* contains a large number of bioactive substances including cytotoxic materials, it seems likely that reported medicinal activities are due to a mixture of bioactive substances. Although it is possible that 2 or more compounds collaboratively exhibit a significant bioactivity, it is beneficial to determine the subcellular components from which the bioactive substances originate and their general chemical makeup. Accordingly, the present study was undertaken to identify the origin of the bioactivity using a water extract obtained from the purified *Chlorella* cell wall membrane fraction of *C sorokiniana*, termed CMF, and evaluating its anticancer activity in vitro and in vivo.

The effect of CMF on the growth of murine and human colon cancer cell lines was evaluated in cell culture-based studies. CMF significantly attenuated the growth of both murine (CT26) and human (HT29 and Caco-2) colon carcinoma cells in 2-dimensional (2D) cell culture in both a dose- and time-dependent manner (Figure 2A and B). However, 2 human colon carcinoma cell lines, SW620 and COLO 205, were poorly sensitive to the CMF treatment (Figure 2B). Coincidently, very similar cell line-specific growth inhibition was observed in our previous study using exopolysaccharides from *Parachlorella kessleri*.[17] The cell line specificity of CMF activity for different cell lines may be due to the different origins of the cell lines, that is, both HT29 and Caco-2 cell lines are of epithelial origin, whereas both SW620 and COLO 205 are established from colon carcinoma-derived metastatic tumors.[28,29] Nevertheless, the cell line-specific sensitivity suggests that CMF-dependent growth inhibition is not due to nonspecific cytotoxicity of the CMF.

It is known that growth of cancer cells under 3D culture mimics tumor growth in vivo more than that under 2D culture conditions.[16] For this reason, 3D culture methods such as spheroid assays[16,17,30-33] have been applied for the evaluation and screening of novel therapeutics for cancer treatments. In the present study, CMF treatment alone attenuated the growth of CT26 cells in both 2D culture (Figure 2A) and a spheroid assay (Figure 3A and B). The results of the present study suggest that CMF itself possesses a strong antitumor effect on both 2D and 3D growth of CT26 cells. In addition, the ability of CMF to attenuate the growth of CT26 spheroids was further enhanced in the presence of Jurkat cells (Figure 3A and B). These results suggest that the antitumor effect of CMF against CT26 cells in the presence of T lymphocytes is attributable to the collaborative effect of CT26 cells and lymphocytes, and that CMF stimulates this collaboration. Jurkat cells have been used as an alternative model cell for T lymphocytes collected from peripheral blood. For example, Jurkat cells were used for the evaluation of IL-2-dependent granzyme B production, which is a marker of the T lymphocyte activation.[34,35] In 3D spheroid assays, the morphology of the Jurkat cells was

significantly altered by treatment with CMF (Figure 3C). Taken together, these results imply that CMF treatment appears to induce functional differentiation in Jurkat cells.

It should be noted that the cell wall of *Chlorella* has been shown to possess lipopolysaccharide-like immunoreactivity.[36,37] CMF, on the contrary, was associated with decreased CT26 cell growth (Figure 2A), while bacterial LPS significantly increased cell growth (Figure S1; available online). It is apparent that the action of CMF is functionally different from LPS in stimulation of CT26 cells. These results suggest that the bioactive compound(s) in CMF are apparently distinct from bacterial LPS and the contribution of LPS-like immunoreactive molecules to the antitumor activities of CMF appears to be negligible. However, determination of the detailed chemical nature of such bioactive compounds in CMF awaits future study. On the other hand, this CMF-induced direct differentiation and/or activation of T lymphocytes is potentially useful in cancer therapy applicable to both primary and metastatic cancer. To the best of our knowledge, this is the first study to report that CMF stimulates functional differentiation of T lymphocytes, thereby inhibiting growth of colon carcinoma cells in vitro.

The inhibition of solid tumor growth by CMF against both murine and human colon carcinoma cells in 2D and 3D cell cultures and its significant stimulation of differentiation in T lymphoblasts compelled the in vivo efficacy study of CMF. In the mouse study, relatively small doses of CMF (10 and 30 mg/kg in PBS) were administered intraperitoneally every other day. As shown in Figure 4A and B and Figure 5A and B, these CMF treatments significantly attenuated the growth of murine colon carcinoma cell tumors in a dose-dependent manner via an induction of apoptosis in tumor cells as compared with the PBS-treated control tumors. Collectively, these results suggest that CMF inhibits the growth of colon carcinoma cells directly and indirectly through activation of T lymphocytes in the tumor microenvironment, thereby significantly attenuating colon tumor growth in mice. The major mechanism by which CMF inhibits tumor growth is likely the collaborative stimulation of antitumor immune function by bidirectional communication between tumor cells and lymphocytes, and CMF stimulates this communication. This speculation is supported by the inhibition of tumor spheroid growth in the presence of lymphoblasts in vitro (Figure 3A and B), and increases of various lymphocytes in ascites (Figure 6). However, further study will be required to detail the mechanism of tumor growth inhibition in vivo.

Conclusions

CMF from the cell membrane fraction of *C sorokiniana* inhibits the growth of murine and human colon carcinoma cells. In a 3D spheroid culture, CMF treatment significantly attenuated the spheroid growth of murine colon carcinoma

cells in the presence of lymphoblasts, that is, Jurkat cells. In a murine colon carcinoma peritoneal dissemination model with syngeneic mice, CMF treatment dose-dependently attenuated tumor growth. These data show that CMF could be a useful agent for inhibiting colon carcinoma growth in vivo by direct growth inhibition of cancer cells and, indirectly, through stimulation of anti-tumor immunity.

Acknowledgments

Declaration of Conflicting Interests

Funding

Ethical Approval

All animal experiments were carried out under strict adherence to protocols approved by Kansas State University Institutional Animal Care and Use Committee (IACUC, Protocol # 3857) and Institutional Biosafety Committee (Protocol # 1050).

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Supplemental Material

Supplemental material for this article is available online.

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