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Vaccination with mitoxantrone-treated primary colon cancer cells enhances tumor-infiltrating lymphocytes and clinical responses in colorectal liver metastases

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ABSTRACT

Background: Colorectal cancer remains a leading cause of cancer-related mortality worldwide. Metastases to the liver are often present at initial presentation and will form in most patients during their course of disease. We have previously demonstrated that enhanced trafficking and activation of tumor-infiltrating lymphocytes in colorectal liver metastases (CRLM) may improve antitumor immune responses. Thus, development of novel mechanisms to increase lymphocyte infiltration and activation are needed to improve patient outcomes.

Methods: CT26 murine colorectal cancer cells were treated with physiologic levels of the potent inducer of immunogenic cell death mitoxantrone (MTX). An *in situ* vaccine was created with treated cells in an established model of CRLM. Cells were evaluated by flow cytometry for cell cycle evaluation and calreticulin expression. Splenic and tumor-infiltrating lymphocytes were isolated for phenotypic studies.

Results: MTX-treatment of colon cancer cells resulted in a sub-G1 peak, inhibition of G1 cell cycle progression, and increased G2/M cell fractions while simultaneously increasing dynamic exposure of calreticulin on the cell surface ($P < 0.05$). Vaccination with MTX-treated cells resulted in significant decreases in CRLM formation associated with increased tumor-infiltrating leukocytes that displayed increased expression of the T cell surface activation marker CD69.

Conclusions: Vaccination with MTX-treated primary colon cancer cells enhances tumor-infiltrating lymphocytes and clinical responses in CRLM.

Introduction

Colorectal cancer remains one of the leading causes of cancer-related mortality worldwide. Colorectal liver metastases (CRLM) are often present at the time of diagnosis and remain the most common site of metastatic disease.[1] In selected patients, surgical resection of CRLM has improved long-term survival, but unfortunately only 10%-20% of patients are

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surgical candidates. Most stage IV patients with CRLM will be managed with palliative intent utilizing multidrug chemotherapy regimens. However, systemic chemotherapy has limited efficacy with many treatment-related toxicities.[2] New therapeutic strategies are focusing on enhancing the antitumor immune response by attempting to drive increased numbers of tumor-infiltrating lymphocytes into the tumor microenvironment. Although this strategy has increased response rates in microsatellite instable colon cancers, which represent less than 5% of colon cancers, to date current immunotherapeutic strategies have been unsuccessful in achieving this goal for most colon cancers.[3-6]

Previous studies have documented that the presence of activated and proliferating T cells within primary colorectal tumors is associated with improved survival.[7,8] In addition, we have previously demonstrated an association between increased T cell infiltrates and improved outcomes in patients with CRLM.[9,10] Thus, enhancing the antitumor immune response may play a viable role in treating patients with advanced gastrointestinal malignancies, including colon cancer and CRLM.

Although chemotherapy is traditionally linked to its immunosuppressive effects in treating cancer patients, emerging studies have demonstrated that selected chemotherapeutics may enhance tumor immunogenicity.[11] Chemotherapy-induced immunogenic cell death (ICD) is characterized by the release and/or increased expression of defined damage-associated molecular patterns, including calreticulin (CRT),[12-14] and we have previously demonstrated that induction of ICD in colon cancer cells can generate specific antitumor immunity in colorectal cancer.[15] Therefore, chemotherapy agents not traditionally used in colon cancer but that may induce ICD could have potential for enhancing antitumor immunity in colon cancer patients.

Mitoxantrone (MTX) is a well-established anthracenedione antineoplastic agent used in the treatment of multiple sclerosis, lymphoma, leukemia, hormone-refractory prostate cancer, and advanced stage breast cancer.[16,17] MTX has recently been demonstrated to induce ICD and may elicit a tumor-specific immune response in murine colon cancer models.[14,18-20] Therefore, the aim of the present study is to investigate whether MTX-treated murine colon cancer cells can be used as a tumor vaccine to increase tumor-infiltrating lymphocytes and thus impact the growth of CRLM.

Materials and methods

Cell culture

The murine colorectal carcinoma cell line, CT26, was obtained from the American Type Culture Collection (ATCC, Manassas, VA), tested for mycoplasma, and utilized at low passage numbers. Cells were cultivated with RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 1% penicillin/streptomycin in 37°C incubator containing 5% CO2. All cell culture reagents were purchased from Invitrogen (Grand Island, NY) and cells were passaged at 1 to 10 dilutions on confluence.

Cell cycle analysis and CRT expression

CT26 cells were seeded in a 6-well plate at 3×10^5 cells per well and treated with 1 μM MTX (Cayman Chemical Company, Ann Arbor, MI) or 1:1000 DMSO as vehicle control for 24 h. Cells were harvested with 0.05% trypsin and fixed with 80% ethanol on ice for 30 min and then incubated with 1 mL of DNA staining solution (1 μg/mL DAPI, 0.1% NP-40 in phosphate buffered saline [PBS], all from Sigma Aldrich, St. Louis, MO) at room temperature for 30 min followed by flow cytometry analysis.

To detect cell surface expression of CRT, cells were suspended in 100 μL PBS containing 3% FBS without fixation and incubated with fluorochrome-conjugated rabbit anti-CRT antibody (1:250, from Abcam, Cambridge, MA) at 4°C for 30 min. The cells were washed and incubated subsequently with a 1:500 dilution of Fluro-488 labeled goat anti-rabbit IgG (Thermo Scientific, Waltham, MA) for 30 min. The stained cells were analyzed using a CyAn ADP analyzer (Beckman Coulter, Brea, CA). Events were collected and analyzed using FlowJo software (Tree Star Incorporated, Ashland, OR).

In vivo studies

Female, 6- to 12-wk-old CT26 cell Balb/c mice, weighing 18-20g, were obtained from Charles River Labs (Wilmington, MA). All mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago and performed in the animal facility as per the approved protocol. Five animals were utilized per group based on a biological difference in tumor formation of at least 30% at an alpha of 0.05 and power of 80%.

Vaccination with MTX-treated colon cancer cells and generation of CRLM

CT26 cells were treated with 1 μM of MTX or DMSO (1:1000) as vehicle control for 24 h. The cells were then washed and resuspended in PBS. Mice were randomized to separate cages assigned to receive MTX-treated or control cells subcutaneously into the right flank (1×10^6 in 100 μL PBS) on days 0 and 7. On day 14, each mouse underwent intrasplenic wild-type CT26 cell injection to form reliable CRLM as we have previously reported in this well-established model.[21,22] In this model, 1×10^6 CT26 cells are introduced into an isolated and divided hemispleen that is excised after injection, leaving half of the untreated spleen for immunologic studies.

Preparation of spleen mononuclear cells and tumor single-cell suspension

Mice were euthanized on day 28. Spleen tissue was placed into a cell strainer and gently homogenized using a syringe plunger. Cells were pelleted and red blood cells lysed with ACK lysis buffer.

After being weighed and photographed, liver tumors were manually dissected and minced, and incubated in 10 mL RPMI containing 5% FBS, collagenase IV (1 mg/mL, Sigma), with DNase I (50 μg/mL, Sigma) at 37°C for 30 min, and strained to obtain a single-cell suspension.