Oxidation-modified CT26 Cell Vaccine by HOCl Inhibits Colon Tumor Growth Significantly in Mouse Model

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Abstract

Despite progress in elucidating mechanisms associated with colorectal cancer and improvement of treatment methods, it remains a frequent cause of death worldwide. New and more effective therapies are therefore urgently needed. Recent studies have shown that immunogenicity of whole ovarian tumor cells and subsequent T cell response were potentiated by oxidation modification with hypochlorous acid (HOCl) in vitro and ex vivo. These results prompted us to investigate the protective antitumor response with an HOCl treated CT26 colorectal cancer cell vaccine in an in vivo mouse model. Administration of HOCl modified vaccine triggered robust antitumor immunity to autologous tumor cells in mice and prolonged survival period significantly. In addition, increased necrosis and apoptosis were found in tumor tissue from the oxidation group. Interestingly, ELISPOT assays showed that specific T cell responses were not elicited in response to the immunizing cellular antigen, in contrast to raising sera antibody titer and antibody binding activity shown by ELISA assay and flow cytometry. Further evaluation of the mechanisms underlying HOCl modified vaccine mediated humoral immunity highlighted the role of antibody-dependent cell-mediated cytotoxicity. These results combined with previous studies suggest that HOCl oxidation modified whole cell vaccine has wide applicability as a cancer vaccine because it can target both T cell- and B cell-specific responses. It may thus represent a promising approach for the immunotherapy of colorectal cancer.

Keywords: HOCl - colorectal cancer - ADCC - antibody - dependent cell-mediated cytotoxicity

Introduction

Colorectal cancer (CRC), a major health problem internationally, is the third most common type of cancer and the second major cause of cancer death in the developed world (Jemal et al., 2008). Typical treatments such as surgery are generally used to treat early-stage colorectal cancer, but 30%-40% of patients cannot be resected with curative intent due to local regionally advanced or metastatic disease on presentation (Penland and Goldberg, 2004). For patients with metastatic colorectal cancer (mCRC), oxaliplatin-based first line chemotherapy has been demonstrated to extend median overall survival to 2 years and beyond. However, adverse effects such as neurotoxicity brings the poor quality of life for patients receiving chemotherapy (Grothey, 2010).

Obviously, new and more effective therapies with fewer side effects are urgently needed.

Cancer vaccines stimulating host-generating antitumor immune response for tumor treatment and prevention, is one of the effective ways of tumor immunotherapy. Which conclude whole tumor cell vaccines, tumor-extracted protein vaccines, tumor antigen vaccines, tumor antigen-encoding virus vaccines and dendritic cell (DC)-based vaccines. In spite of that significant advances in the identification of tumor antigens and in their production in recombinant and synthetic forms as protein or peptide vaccines, whole tumor cell vaccine remains the most widely studied vaccine strategy because they can potentially express a whole array of tumor associated antigens (TAAs) and has strong immunogenicity, simple procedure and low cost (Van Der Bruggen., 2002; Ward et al., 2002; Lysaght et al., 2003).

During the last 25 years, attempts at whole tumor cell vaccines against colorectal cancer have been made. Whole tumor cell vaccines combined with bacilli Calmette–Guerin (BCG), an attenuated strain of mycobacterium bovis, was the first colorectal tumor cell vaccine. Then three randomized trials of this tumor cell BCG vaccine have been carried out (Hoover et al., 1993; Vermorken et al., 1999; Harris et al., 2000). Regrettably, a meta-analysis of these three trials showed that overall
survival trended towards improvement but failed to reach statistical significance. To enhance the immunogenicity, genetic modification of whole tumor cell vaccines emerged. Suh KW et al (Suh et al., 1999), using a hepatic metastases model of the CT26 murine colorectal cell line, demonstrated that GM-CSF autologous tumor vaccination was effective for the treatment of hepatic colorectal metastases, providing support for further development of whole-cell-based adjuvant vaccine therapy for colorectal cancer. In addition to genetic modification, oxidation-modification has been showed to be an effective approach to enhance the immunogenicity. Hypochlorous acid (HOCl), a potent oxidant produced by neutrophil enzyme myeloperoxidase (MPO) during acute inflammation has been showed to enhance the immunogenicity of protein antigens by several folds via oxidation modification (Marcinkiewicz, 1991; Marcinkiewicz, 1992; Allison and Fearon, 2000). Because HOCl, typically produced by inflammatory cells exerts augment of immunogenic activity, it acts as links between innate and adaptive immunity (Marcinkiewicz, 1997). Recently, use of HOCl to potentiate the immunogenicity of whole ovarian tumor cells in vitro and ex vivo has been previously described. Chiang CL et al. showed that ovarian tumor cells, SK-OV-3, oxidized by HOCl became potent immunogens that are efficiently taken up and cross-presented by Dendritic cells (DCs) to stimulate autologous tumor specific T cell responses (Chiang et al., 2006). Moreover, using autologous DCs loaded with oxidized SKO3V and autologous T cells, they further found that in the ovarian cancer setting, oxidation-modified whole tumor cells by HOCl enhanced their immunogenicity for uptake by myeloid DCs and priming autologous tumor specific CD4+ and CD8+ T cell responses (Chiang et al., 2006). However, the antitumor effect of oxidative modification in vivo has not been reported yet.

In the present study, we report that vaccination with HOCl modified CT26 cells rejected live CT26 tumor cell challenge and prolonged survival period in vivo mice model. Further analysis of the mechanisms underlying this antitumor activity by HOCl modified CT26 cells vaccine suggest humoral immunity mainly contribute to efficient antitumor effect. We have thus documented that HOCl modified whole tumor cell vaccine, in addition to its potential to stimulate efficient antitumor T cell responses showed by recent reports, is also capable of generating potent humoral responses that can lead to delay in growth of colorectal tumors.

### Materials and Methods

#### Cell lines and animals

The murine colon adenocarcinoma cell lines Balb/c-derived CT26 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained in RPMI1640 medium (Gibco, Eggenstein, Germany) were supplemented with 10% fetal bovine serum (Gibco, Eggenstein, Germany). Female Balb/c mice aged 6-8 weeks were purchased from the West China Hospital Experiment Animal Center of Sichuan University (Chengdu, China). All mice used in the experiments were housed in a specific pathogen-free environment and treated humanely in accordance with Institution Animal Care and Use Committee guidelines.

#### Preparation of HOCl modified tumor vaccine

Treatment CT26 cell with HOCl solution was conducted as previously described with minor modification.15 NaOCl reagent (Sigma-Aldrich, Sigma, Saint Louis, MO, USA) was diluted by HBSS (Invitrogen, paisley, UK) to generate HOCl solutions with different concentrations (10-110 μM). Different concentrations of HOCl solution was added to the CT26 cells to get a final cell density of 8x10^6 per ml. The cell suspensions were immediately incubation at 37 °C in a humidified atmosphere of 5% CO2 for 1 h with gentle shaking every 20 min. After the incubation, the cells were washed twice with sufficient HBSS solution for further use. An equal volume of 4% trypan blue solution was added dropwise to the cells and counts the death cell and the live cells under microscope. Then, a suitable concentration for HOCl modification was determined. After inactivation with formalin in 4 °C for 72 h, HOCl modified CT26 cells or CT26 cells alone were washed twice with PBS solution for further use.

#### Vaccination and tumor challenge

Mice were randomly divided into three groups, with 8 mice each group, and were subcutaneously immunized with CT26-HOCl vaccine, CT26 alone and PBS (tumor cell population 1x10^6 for each group) in the left groin, respectively. Both CT26 and PBS served as control. The vaccines were delivered by subcutaneous (s.c.) injections on days -21, -7, 0. One week after the last vaccination (on day 7), each mouse was inoculated with 2x10^6 fresh CT26 cells on the opposite groin. Tumor volume was then monitored regularly and calculated by the following formula: tumor volume (mm3) = π/6 × length (mm) × width (mm) × width (mm). After 5 weeks of tumor inoculation, mice were anesthetized euthanized and the tumors were dissected in each group randomly, followed by tumor weight/body weight calculation.

#### Histology and Immunohistochemistry

Tumors were removed for studies by Immunohistochemistry from each groups. Tissue fixed in 10% buffered formalin and paraffin-embedded was sectioned (5 μm), and mounted on microscope. After reaction of CD4 or CD8 antibodies(Zhongshan Tech, Shanghai, China), The sections were incubated for 30 min at 37 °C with HRP-labeled goat anti-mouse IgG antibody, and positive signals were visualized by development in diaminobenzidine tetrahydrochloride (DAB) solution. Images were acquired using an Olympus Biological Microscope: IX50 (Olympus, Tokyo, Japan). The paraffin-embedded tumors above were also stained with hematoxylin and eosin.

#### In situ terminal deoxyribonucleotide transferase-mediated nick end labeling (TUNEL)

Cell apoptosis of CT26 xenograft tumors was detected by a TUNEL assay following the manufacture’s instructions (Promega, San Luis Obispo, CA, USA).
Five tumors per group were analyzed. The number of TUNEL-positive cells was quantified under fluorescence microscopy. The apoptotic index was determined in five random fields per group. The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of cells.

**Dual-Color ELISPOT assay**

IFN-γ and IL-4 secreting were detected by Dual-Color ELISPOT Mouse IFN-γ/IL-4 Kit (R&D, Minneapolis, MN, USA) following the manufacturer’s instruction with minor modification. Briefly, splenocytes (5 x 10^5/well) from different treated mice were added into the Microplate IFN-γ/IL-4 and a polyclonal antibody specific for mouse IL-4 and co-cultured with inactivated (treated with 100 µg/ml MMC for 1 h) CT26 cells (4.5 x 10^5/well). After culture for 36 h with no disturbance at 37°C in a humidified atmosphere of 5% CO₂, cells were completely removed with a Detection Antibody Mix (the antibody solution was composed of biotinylated polyclonal antibody specific for mouse IL-4 and horseradish peroxidase-conjugated polyclonal antibody specific for mouse IFN-γ) was added and incubate at 4 °C overnight. After extensive wash with PBS/T and PBS, streptavidin–alkaline phosphatase solution was added and incubated with the plates for 2 h at room temperature. Unbound enzyme was subsequently removed by washing and substrate solution 5-Bromo-4-Chloro-3-Indolyolphosphate p-Toluidine Salt (BCIP) /Nitro Blue Tetrazolium Chloride (NBT) was added for blue spots visualization. After washing out the BCIP/NBT from the cells with deionized water, red spots were visualized by adding the substrate 2%3-Amino-9-Ethyl-Carbazole (AEC). each individual blue spot or red spot represents an individual IL-4 secreting cell or IFN-γ secreting cell respectively. The number of spots were counted using Immunospot Analyzer (S5 Versa, Cellular Technology Ltd. Cleveland ohio, USA) in a blinded manner.

**Antiserum titer determination by ELISA**

The titer of antiserum was detected by an indirect ELISA. Seven days after the first vaccination, mice were bled from the lateral tail vein on days -14, -7, 0, 7. For each well of the 96-well ELISA plate, 1 x 10^5 were seeded and incubated overnight. After extensive wash with PBS, 125 µL/well 10% neutral formalin solution was added for fixing. After five washes with deionized water, the wells were blocked using 3% bovine serum albumin, CT26 cells were stained with 2%3-Amino-9-Ethyl-Carbazole (AEC). each individual blue spot or red spot represents an individual IL-4 secreting cell or IFN-γ secreting cell respectively. The number of spots were counted using Immunospot Analyzer (S5 Versa, Cellular Technology Ltd. Cleveland ohio, USA) in a blinded manner.

**Statistical analysis**

The statistical significance of results in the experiments was analyzed using Student’s t-test and ANOVA. Differences were considered significant where P<0.05.

**Antibody-dependent cellular cytotoxicity assay by LDH**

CT26 cells were incubated with different dilution of pooled sera as target cells for 1 h at 37 °C. After unbound antibodies were removed, splenocytes harvested from naive mice were added as effectors and incubated for 4 h at 37 °C. The ratio of effector cells to target cells was 100:1. Cytotoxicity of the effectors was determined by using CytoTox96 (Promega, USA) according to the manufacturer’s manual. The Cytotoxicity activity of the effectors against tumor cells was calculated.

**Results**

**HOCl induces dose dependent death of CT26 cells**

To determine a suitable concentration of HOCl to induce oxidation-dependent tumor cell death, CT26 cells were treated with different concentrations of HOCl solutions (10–110 µM). As showed in Figure 1, increasing concentration of HOCl solution caused a dose dependent increase death of CT26 cells. Approximately 100% of tumor cell death was observed at 100µM HOCl, and extensive cell fragmentation occurred when 110 µM HOCl or higher was used. So 100 µM HOCl was chosen
**HOCI modified tumor cell vaccine rejects live CT26 tumor challenge and prolongs survival times**

To evaluate the efficacy of HOCI modified vaccine in mouse colorectal cancer model, after vaccination with PBS, CT26 or CT26+HOCI, groups of 8 BALB/c mice were injected with $2 \times 10^5$ CT26 cells s.c. and were monitored for tumor growth. The results showed that vaccination with HOCI modified cells significantly delayed tumor growth when compared with non-immunized mice (PBS) or mice immunized with CT26 alone (Figure 2A, B and C). In addition, the preventative effect was further confirmed by tumor weight to body weight calculation as showed in Figure 2D ($p<0.05$). Moreover, survival time for the CT26+HOCI immunized group significantly prolonged compared to other groups in Figure 2E ($p<0.05$). Interestingly, despite some preventative effect in CT26 group compared with PBS, both groups had bad similar survival times. No adverse reactions such as weight of loss (data not shown), anorexia, bleeding, diarrhea and toxic deaths were observed in each group.

**HOCI modified tumor cell vaccine induces more necrosis and apoptosis in vivo**

The effects of HOCI modified CT26 cell vaccine on histology and apoptosis were analyzed by H&E and TUNEL staining. As shown in Figure 3A, in contrast to obvious necrosis in tumors from HOCI oxidation group, no obvious necrosis was observed in PBS and CT26 groups. In addition, TUNEL staining in Figure 3B showed that the rate of apoptosis in tumors from mice treated with HOCI modified CT26 cell vaccine was much higher than that of other modified groups.

**HOCI modified tumor cell vaccine induces more CD4+ T cells infiltrated in tumor tissue**

Since CD4+ and CD8+ play an important role in immune response mediated by whole cell vaccine, immunostaining of CD4 and CD8 was carried out. As showed in in Fig.4A, more CD4+ T cells infiltration was found in the tumor region from CT26+HOCI group than that of PBS group and CT26 group. Interestingly, infiltration of CD8+ T cells in CT26+HOCI group tumor tissues had no obvious difference compared to other groups, suggesting that CD4+ T cells exert a major function in antitumor effects of HOCI modified tumor cell vaccine.

**HOCI modified tumor cell vaccine increases IL-4-producing frequency of splenic lymphocytes from the mice**

To further explore the mechanism by which HOCI oxidation enhanced the efficacy of the vaccine, tumort-specific T cells from various groups were assayed after treatment by IFN-γ/IL-4 ELISPOT. As shown in Fig. 4B, IL-4-producing frequency of T cells from mice vaccinated with HOCI modification was the highest among all other groups ($p<0.05$). The IFN-γ-producing frequency of lymphocytes in the mice treated with CT26 cells alone increased slightly without significant differences, which typically associated with the Th1 response and is linked to the cytotoxic capacity of T cells. The increased secretion of IL-4 suggested that HOCI modified tumor cell vaccine could enhance Th2 cell function.

**HOCI modified CT26 cell vaccine elicits higher antibody production that could bind to CT26 cell surface**

Since that T cells mediated cellular immunity may not play a major role indicated by ELISIPOT assay. We therefore measured serum antibody titers in tumor-bearing mice vaccinated with oxidized CT26. Sera from PBS and CT26 alone-immunized animals were used as controls. As showed in Fig. 5A, IgG antibody titer in oxidized CT26 group displayed a time-dependent increase and was higher than other groups. Western Blot Analysis of the antibody were then conducted to detect whether...
there was any specific antibody against CT26 tumor cell in the vaccinated mice. Two specific bands in the sera from oxidation group but not in other groups were found as showed in Figure 5B. In order to determine whether elicited serum antibody can binding to antigen molecule on the CT26 cell surface, Flow cytometry analysis was carried out. A positive staining only in the cells incubated with serum from oxidiaed CT26 group was showed in Figure 5C. This indicates that the sera of immunized mice with oxidized CT26 contain antibodies (IgG) that can specifically bind to CT26 cells.

**ADCC was mediated by the elicited antibodies from HOCI modified CT26 cells vaccinated mouse sera**

We used effector splenocytes from mice of each group to test whether the CT26 tumor cell-specific IgGs could mediate ADCC to kill CT26 cells. It was shown that at 1:5 dilution of the sera about 30% cytotoxicity was detected from HOCl oxidized CT26-vaccinated mice sera when compared with the 5% killing with control sera (Fig. 6), suggesting that antibodies elicited by HOCI modified vaccine mediated important role in humoral immunity. This effect was specific as we were able to abrogate lysis of tumor cells with increasing dilutions of the sera obtained by oxidized CT26 immunization.

**Discussion**

Despite the amazing advances in the knowledge of mechanisms associated with colorectal cancer and significant improvement of treatment, colorectal cancer remains one of most common types of cancer and frequent
cause of death due to cancer worldwide. Clearly, new and more effective therapies with fewer side effects are urgently needed. For the first time we investigated effect of oxidation modification by HOCl on immunogenic activity in vivo animal model in the present study.

The first important observation in this study is that the immunogenic activity of the CT26 tumor cells was enhanced by treatment with HOCl, a potent oxidant. As demonstrated by Figure 2, although the CT26 vaccine alone showed some protection in mice from a live CT26 tumor cell challenge compared with PBS group, there were similar and bad survival times in both groups. However, when the HOCl-modified vaccine was given, nearly one hundred percent of the vaccinated mice rejected live CT26 tumor challenge and had longer survival times. This protective effect was also demonstrated by increased necrosis and apoptosis in tumor tissue from HOCl modification group.

Enhanced immunogenicity of protein antigens modified by HOCl may partially explain the antitumor activity in vivo animal model. In addition, proteins oxidized by HOCl are more readily taken up and processed by APCs, resulting in enhanced activation of antigen-specific T cells in vitro (Stark, 1998; Tata., 1999; Callahan et al., 2002; Reth, 2002). Recently, the mechanism by which HOCl improves antigen immunogenic activity has been well reviewed (Chiang et al., 2010). Firstly, tagging protein antigens with such aldehydes by HOCl′ serine deamination results in significant improvement in the responses directed against the antigen (Hazan et al., 1996; Anderson et al., 1997; Anderson et al., 1999). Secondly, Oxidation of protein antigens allows protein unfolding and increases both proteolytic processing by DCs and exposure of peptide epitopes to specific T cells (Carrasco-Marin., 1998). Thirdly, scavenger receptors expressed on the surface of dendritic cells, macrophage might be involved in antigen cross-presentation in DCs (Delneste et al., 2002). Moreover, oxidized tumor cells could induce a partial activation of DCs by upregulating the maturation associated markers CD86 and CD40 (Chiang et al., 2006). It is worth of note that such mechanisms mentioned above mainly account for improvement of T cell mediated cellular immunity. Future research focused on the precise mechanism by which HOCl mediated enhanced immunogenicity has jet to be needed.

A second intriguing observation is that in addition to enhanced T cell response elicited by oxidation modification using HOCl (Chiang et al., 2006, 2008), our results showed that HOCl-modified tumor cell vaccine is also capable of generating potent humoral responses that can lead to delay in growth of tumors. Facilitated ADCC by serum from HOCl modified CR26 cells—vaccinated mice indicates that natural killer cells may play an important role in enhancing anti tumor activity, which remains to be investigated. Moreover, more CD4+ T cells infiltration into tumor region caused by HOCl-modified vaccine may also participate immunogenicity improvement, since that tumor-reactive CD4+ T lymphocytes are crucial for the induction of antitumor immunity (Rovero et al., 2002; Park et al., 2005).

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