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Antitumor immunity to cholangiocarcinoma from DC-tumor cell fusion

Part 1 Abstract

Dendritic cells (DCs) are potent antigen-presenting cells and play a central role in the initiation and regulation of primary immune responses. Therefore, their use for the active immunotherapy against cancers has been studied with considerable interest. The fusion of DCs with whole tumor cells represents in many ways an ideal approach to deliver, process, and subsequently present a broad array of tumor-associated antigens, including not identified antigens, in the context of DCs-derived costimulatory molecules. DCs/tumor fusion vaccines have shown promising antitumor responses in mice as well as in humans in many works. The majority of studies reported to date have used autologous or syngeneic tumor cells. Unfortunately, the clinical application of this strategy is limited because in many patients, the access to sufficient amount of tumor tissue is difficult. Therefore the establishment of allogeneic cancer cell line may address this problem as in some published works already demonstrated it is likely that the antitumor specificity also comprises an allogeneic component and offer the possibility to vaccinate patients where the autologous tumor is not surgically accessibility or there is no prior knowledge of the exact antigenic repertoire of the tumor.

Cholangiocarcinoma (CCA), a cancer of bile ducts that primarily associated with the liver fluke *Opisthorchis viverrini* in northeast Thailand. The disease is associated with late presentation, difficult to diagnose and also show high rate of patient mortality. Unfortunately, the therapeutic approaches, such as surgical resection, liver transplantation, and other alternative therapies, have little impact on this malignancy, especially in the last stage of cancer.

For this application, we plan to investigate the possibility of hybridizing allogeneic human bile duct epithelial carcinoma cell line with human-monocyte-derived DC and thereby investigate their antitumor response using *in vitro* assay. All of these findings from this proposal will certainly provide the opportunity for future therapeutic immunotherapy approach in cholangiocarcinoma cancer.

Part 2 Introduction to the research problem and its significance

Cholangiocarcinoma (CCA), or cancer of bile ducts, is primarily associated with the liver fluke *Opisthorchis viverrini* in northeast Thailand [1]. Worldwide, CCA accounts for 3 % of all gastrointestinal cancers and is the second commonest primary hepatic tumor [2]. The disease is associated with late presentation, difficult to diagnose and also show high rate of patient mortality. The therapeutic approaches, such as surgical resection, liver transplantation, and other alternative therapies, have little impact on this malignancy [3]. These highlight the urgent need for new therapies for treatment. Recently, immunotherapy has become a critically important component of clinical cancer therapies and has the potential to prevent tumor recurrence and to prolong survival of patients receiving conventional treatment for malignant disease. The major concept of these approaches is to artificially enhance the immunogenicity of tumor cells.

Dendritic cells (DC) are potent inducers of anti-tumor responses and are being used as vehicles for the delivery of tumor associated antigen (TAA) in immunotherapy protocols. Although numerous approaches have been employed for the loading of DC with TAA, attention has focussed on the use DC–tumor cell hybrids, which in theory co-express all TAAs in concert with the func- tional attributes of DC [4-6]. Studies in a diverse range of animal tumor models have shown that vaccination with hybrid preparations not only stimulates protective immune responses [6-9], but is also one of the few immunotherapeutic approaches, which is effective in eliminating established metastatic disease [6, 10, 11]. Promising results have also been obtained with human DC–tumor hybrid preparations in both in vitro studies [12-14] and clinical trials [15, 16] although it is clear that further optimisation of this approach is needed.

Therefore the fusion of allogeneic human bile duct epithelial carcinoma cell line with dendritic cell will provide some useful pre-clinical information on antitumor immunity, which may develop to a promising DC-based cancer vaccine.

Part 3 Literature review

Cholangiocarcinoma (CCA) is first reported by Durand Fardel in 1840 [17], is extremely invasive, develops rapidly, often metastasizes, and has a very poor prognosis [18]. It is the second most common primary liver cancer in the world associated with a high mortality [19]. The tumor arises from the ductular epithelium of the biliary tree, generally divided into within the liver (intrahepatic cholangiocarcinoma) or more commonly from the extrahepatic bile ducts (extrahepatic cholangicarcinoma). CCA has a worldwide distribution, account for about 3 % of all gastrointestinal malignancies [20] and approximately 10 to15% of all cases of primary hepatobiliary malignancy [21, 22].

There are well-recognized risk factors for CCA, such as congenital biliary anomalies, primary sclerosing cholangitis (PSC), hepatolithiasis, parasitic infections, chronic typhoid carriage, bile duct adenoma, biliary papillomatosis, drug exposure and genetic risks [2, 20]. Throughout Southeast Asia there is a high incidence of CCA particularly in people from rural area in Laos, Vietnam, Cambodia and Northeast Thailand who are infected with the liver fluke, especially Opisthorchis viverrini [1, 23]. Humans are infected by eating undercooked fish. Adult worms inhabit the biliary system and lay eggs there. The mechanisms by which causes cancer are involving mechanical irritation from the activities and movements of the flukes, immunopathology, dietary nitrosamines and the secretion of parasite proteins that act as a co factor promote cholangiocarcinogenesis [24] and approximately one-sixth of people with opisthorchiasis will develop CCA [25]. In recent years, the advance diagnosis of CCA rely on many approaches such as clinical presentation, serum tumor markers, ultrasonography, computed termography, MRI and Magnetic Resonance Cholangiopancreatography, cholangioscopy and endoscopic ultrasound which provide better treatment [13]. However the lack of effective medical treatment makes a radical surgical resection or hepatectomy the only therapeutic option. Most of the CCA are unresectable at presentation and the prognosis for these patients is poor.

The failure of conventional treatment for many forms of cancer has opened the doors for novel, experimental therapies. Cancer vaccine is an optional treatment enhances the patient's own immune system and a major area of investigation in cancer vaccine involves the design of DC-based cancer vaccines [26]. The first clinical trial with dendritic cell (DCs) was started in the early 1990s though Steinman and Cohn discovered this cell in 1973 [27]. DCs are specialized to capture and process tumor associated antigens (TAAs), converting the proteins to peptides that are presented on major histocompatibility complex (MHC) class I and class II molecules to CD4+ T helper 1 (T_H1) cell and cytolytic CD8+ cells, respectively. DCs then migrate to T-cell areas of secondary lymphoid organs and become competent to present antigens to T cells, thus initiating antigen-specific immune responses [26, 28]. The first step in the induction of an effective anti-tumor therapy/vaccine is to identify a specific tumor antigen from a list of tumor antigens. If a tumor antigen is presented to an antigen-presenting cell (APC), a more efficient and effective anti-tumor response is generated. Thus the APC most suitable for cancer vaccine are DCs, which can be distinguished from B cells and macrophages by their abundant expression of costimulatory molecules and ability to initiate a strong primary immune response [28-30]. DCs represent a heterogenous cell population, mainly localised in tissues and represent only a small proportion of less than 0.5 per cent of peripheral blood leukocytes [31, 32]. DC develop from $CD34^+$ bone

marrow-derived hematopoietic progenitor cells and are thought to undergo sequential differentiation through a number of intermediate precursor states, prior to populating the tissues as immature DC [33]. Usually, $CD34^+$ precursors are mobilized from bone marrow by G-CSF or GM-CSF and isolated by leucopheresis to obtain large number of peripheral cells for therapeutic purposes. These cells seem to be more efficient in the activation of tumor-specific cytotoxic T lymphocytes (CTLs) than $CD14^+$ derived DCs [34]. Briefly, the most common way of generation of DCs from $CD14^+$ cells (monocytes) includes isolation of peripheral blood lymphocytes from buffy coats or from blood by Ficoll density centrifugation [35, 36]. The isolated adherent cells are cultured in RPMI 1640 with serum, GM-CSF and IL-4. Maturation of DCs has been achieved by using different cytokine cocktails [37, 38]. Mature DCs are more potent in inducing Th1 and CTL responses in vitro. They differentiate themselves to macrophages if the medium lacks GM-CSF and IL-4 [39].

Different strategies have been developed to load DCs with TAAs, including synthetic peptides derived from the known antigens [40], tumor lysates [41], tumor RNA [42] and dying tumor cells [43] to induce antigen-specific immune responses. Pulsing of DCs with small peptides is the easiest method of delivering antigen to immune cells. The first clinical study involved injecting monocyte derived DCs pulsed with idiotype protein derived from B cell lymphoma [44]. Since then there have been many clinical studies using peptide pulsed DCs [45]. In some cancers like pancreatic carcinoma, allogeneic tumor cells have been used as a source of antigens [46] and even more than 200 tumor-associated epitopes recognise T cells but most of them do not elicit a strong immune response [47]. A major drawback of this strategy comes from a limited number of known tumor peptides available in many HLA contexts and the potential evasion of immunological targeting through downregulation of their antigens. To solve this problem, an alternative approach has been developed by fusing DCs with tumor cells [6].

The fusion of syngeneic DCs and tumor cells creates a heterokaryon with both tumor-derived antigens and DCs-derived MHC class II costimulatory molecules, intracellular adhesion molecule, lymphocyte function-associated antigen and CD40, all of which are efficient antigen-processing and presentation machinery [48, 49]. Ex vivo generated DCs can be fused with whole tumor cells and reinfused to the patients [50], or they can be used for ex vivo induction and expansion of cytotoxic T lymphocytes (CTLs) [51, 52]. Indeed, DCs/tumor fusion cell vaccines have been shown to possess the elements essential for processing and presenting tumor antigens to host immune cells for inducing effective antitumor immune response and for breaking T- cell tolerance to tumor-associated antigens in animal models [52, 53].

As a fusion partner, autologous tumor cells are an obvious source of TAA for vaccination purposes, since, by definition, all relevant candidate TAA should be contained within them [54]. However, in the clinical setting of the patients with cancer, a major difficulty for the DCs/tumor fusion vaccine is the preparation of sufficient amounts of autologous tumor cells because of both the availability of limited tumor samples and the difficulty in culturing tumor cells. It has been reported that hybrid cells generated by fusing DC from healthy donor with allogeneic tumor cell line have induced CTL responses against the allogeneic tumor cells used for fusion [13, 14]. On the other hand, the rationale for using allogeneic DCs as a fusion partner is based on the finding that a high frequency of unprimed T cells from an individual react against the foreign MHC antigens of another individual. Additional potential benefit of using allogeneic DCs is that DCs from healthy donors are readily available in unlimited amounts. DCs from cancer patients may be defective in APC function, owing to cancer treatment, such as chemotherapy and irradiation. It has been demonstrated that fusions of both autologous and allogeneic DCs are effective in inducing antitumor immunity in human and animal model [55, 56]. The allogeneic DCs/autologous tumor fusions express DCs-derived allogeneic HLA class II molecules and HLA class I molecules derived from both DCs and tumor cells [57, 58].

In approach to develop anti-tumor DC based vaccine, several studies demonstrate the successful from many kinds of cancer. Previous reports in mouse tumor models have demonstrated that vaccination with heterokaryons prepared by fusing tumor cells and DC induces the regression of established carcinomas, lymphomas, and melanomas [6, 10, 59, 60]. In animal models, antitumor vaccines have been developed by pulsing DC with peptides derived from tumor antigens [40, 61]. With regard to ovarian cancer, human DC loaded with HER2/neu peptides have been shown to stimulate proliferation of autologous T cells that induce lysis of peptide-pulsed targets [62, 63]. Other studies have demonstrated that fusions of DC and tumor cells have also

been developed to induce a polyclonal antitumor immune response [6] and vaccination with fusions of murine tumor cells and syngeneic DC have been shown to eliminate established metastatic disease [6, 10, 59, 60]. Another group demonstrated that autologous DCs fused with allogeneic hepatocellular carcinoma (HCC) cell line can activae T-cells from patients to differentiate into antigen specific CTL able to kill autologous HCC cells [64]. Recently the DC vaccines plus activated T-cell transfer in an adjuvant setting for patients with intrahepatic cholangiocarcinoma (ICC) showed the potential efficacy of the clinical utilization [65]. Therefore, the results of anti-tumor response obtain from dendritic cells fuse with CCA cell line derived from Thai patients is essential that may develop to DCs/tumor fusions-based cancer vaccination.

Part 4 Objectives

To investigate the specific antitumor responses against human cholangiocarcinoma cell line of dendritic cell (DCs) fused with allogeneic human bile duct epithelial carcinoma cell line.

Part 5 Methodology

1. Generation of DCs from human peripheral blood monocytes

Mononuclear cells are isolated from the peripheral blood of healthy donors by Ficoll-density gradient centrifugation. The PBMC are cultured in RPMI 1640 medium containing 10% FBS serum for 1-2 h. The non adherent cells are removed, and the adherent cells are cultured for 1 wk in RPMI 1640 medium containing 10% FBS, 1000 U/ml GM-CSF, 500 U/ml IL-4 and TNF-alpha (ImmunoTools), DCs are harvested and undergo immunohistochemistry and/or flow cytometric analysis to assess expression of major histocompatibility complex class II, costimulatory (CD86, CD40, and or CD80) and maturation (CD83) (ImmunoTools),

2. Cancer cell line preparation

The human bile duct epithelial carcinoma cell line, derived from bile duct tumor mass of patient, are grown in Ham's F12 culture medium containing 15 mM HEPES and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/ml streptomycin and 125 ng/ml amphotericin B.solution.

3. Preparation of DC/Tumor fusions

DC/tumor fusions are generated by mixing tumor cells and DCs at a 1:3 to 1:10ratio (depending on cell yields). The cells are then washed extensively, and the cell pellet is resuspended in a 50% solution of polyethylene glycol in phosphate-buffered saline. After a short incubation, the polyethylene glycol is slowly diluted by the addition of media, and after several washing steps, the cells are placed in media that contained 10% FBS and GM-CSF 500 U/mL and incubated at 37°C. DC/tumor fusions are quantified by determining the percentage of cells that co-expressed unique DC and tumor-associated antigens by immunohistochemical and/or flow cytometry analysis.

4. Immunocytochemical analysis

Cells are stained with general marker for biliary epithelial cells; cytokeratin 7 (monoclonal mouse Anti-Human Cytokeratin 7); cytokeratin 19 (monoclonal mouse Anti-Human Cytokeratin 10); major histocopatilibity class II (CD86, CD40, CD80 or CD83) (ImmunoTools), and an isotype-matched negative control for 60 min. The cells are incubated with a biotinylated F(ab)2 fragment of horse anti-mouse IgG for 45 min, washed twice with PBS and incubated for 30 min with avidin biotin complex reagent solutions followed by 3 amino-9-ethyl carbazole solution. Detection of DC and tumor associated antigen with the avidin biotin complex reagents is followed by staining for other markers with the avidin biotin complex-alkaline phosphatase kit. Membrane staining intensity is evaluated using a scale ranging from 0 to 4 (0, completely negative; 1□, faint positivity; 2□, moderate positivity; 3□, strong positivity; 4□ very strong positivity). For a grade 2□to 4□, staining had to be present in a majority (~50%) of the cells.

5. Flow cytometry analysis

Cells are incubated with the indicated primary monoclonal antibody or a matching isotype control as described in 10.3.1 for 30

min at 4°C. Bound primary monoclonal antibodies are detected with FITC-conjugated and/or phycoerythrin secondary antibody followed by fixation in 2% paraformaldehyde. Analysis is performed on flow cytometer.

6. T-cell proliferarion assays

The $CD4^+$ T cells isolate from PBMCs by using $CD4^+$ T cell isolation kit are coupled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated at 37°C in dark for 10 min. Cells are then washed with cold HBSS including 5% FBS and resuspended in completed RPMI media. The labeled $CD4^+$ T cells are subsequently co-cultured with DCs/Cholangiocarcinoma cell lineA-1 fusion cells, DCs mixed with tumor cells, DCs alone, cell line alone for 4-7 days at 37 °C in a humidified CO_2 incubator. Cells associated fluorescence intensity are determined by flow cytometer with the excitation at 488 nm and emission filter used for fluorescein, usually the FL1 channel of a cytometer.

7. Interferon- gamma (IFN-gamma) release ELISA assay

The $CD8^+$ T cells isolate from PBMC by using $CD8^+$ T cell isolation kit are used as effector cells in CTL assays. DCs/ cancer cell line fusion cells, DCs mixed with tumor cells, DCs alone, Cholangiocarcinoma cell lineA-1 alone are plated with the effector T-cells at a ratio of 1:20. IFN-gamma release in the supernatants of effector cells is evaluated by ELISA assay using IFN-gamma ELISA detection set (ImmunoTools).