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Cytokine Profiles in *Opisthorchis viverrini* Stimulated Peripheral Blood Mononuclear Cells from Cholangiocarcinoma Patients

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Abstract

The carcinogenic liver fluke *Opisthorchis viverrini* causes chronic inflammation in the bile duct and resulting in unremitting tissue damage that lead to hepatobiliary diseases, including cholangiocarcinoma (CCA). Despite inflammatory cytokine expression having been studied in the animal model, so far no studies have been carried out on cytokines in human CCA cases. Here we report the profile of cytokine production in peripheral blood mononuclear cells (PBMCs) collected from *O. viverrini*-associated human CCA and uninfected normal controls after stimulation with *O. viverrini* excretory-secretory (ES) product. Eleven cytokine profiles including IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF- α and LT- α measured by flow cytometry revealed both pro-inflammatory and anti-inflammatory cytokines were increased in the *O. viverrini*-associated CCA compared to uninfected normal controls. Specifically, net production levels of IFN- γ , IL-10, and LT- α were 40 to > 320 times higher in CCA than those of controls. These results suggest dysregulation of the immune response in the liver fluke associated CCA.

Graphical Abstract

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Keywords

Cholangiocarcinoma; Liver fluke; Cytokines; *Opisthorchis viverrini*; PBMCs

1. Introduction

Cholangiocarcinoma (CCA) is a relatively rare cancer worldwide but it is highly prevalent in Thailand where the liver fluke, *Opisthorchis viverrini* (*Ov*) is endemic. The cholangiocarcinogenesis related to chronic *Ov* infection is proposed to be multi-factorial process [1]; one of which is immunopathological mechanism [2, 3]. The host's cellular response to *Opisthorchis* antigens have been observed in infected hamsters as shown by periductal inflammatory cell infiltration [4–7]. Oxidative tissue damages caused by free radicals released from effectors cells have also been demonstrated surrounding the infected bile ducts [8, 9].

Cytokines are a group of small soluble or cell membrane bound proteins or glycoprotein messenger molecules with high potential in the regulation of inflammatory responses [10, 11]. It can apply opposing effects on the parasitic infection, to either control infection, or, promote disease. Moreover, clinical outcomes of many infectious, autoimmune, or malignant diseases also appear to be influenced by the overall balance of production (profiles) of pro- and anti-inflammatory cytokines [12].

Cytokine detection can be done in serum, plasma and isolated peripheral blood mononuclear cells (PBMC). Serum and plasma cytokine profiles reflect the overall systemic cytokine production from many cell types [13]. Cytokine detection in the supernatant of cultured PBMC can provide more specific cytokine expression from immune cell especially the mononuclear cells, which plays a central role in immune response. Different stimuli can result in differential expression of cytokine profiles [14]. Certain interleukins and chemokines, specifically soluble interleukin-6 receptor alpha (sIL-6Ra) was identified as putative markers of the progression of hepatitis C virus (HCV) to liver cirrhosis (LC) by increasing fibrosis and its inverse index may associated with hepatocellular carcinoma development [15].

In *Ov* infection, elevated total serum IL-6 and *Ov* stimulated IL-6 production in PBMC have been reported in infected individuals with advanced periductal fibrosis [16, 17]. IL-6 was also increased in the cholangiocyte cell line (H69) co-cultured with *Ov* excretory-secretory products (*Ov*-ES) [18]. This pro-inflammatory cytokine may potentiate the proliferation and survival of cholangiocytes within the local microenvironment leading to cancer development

[17]. We hypothesize that individual with the liver fluke-associated CCA displays different pro- and anti-inflammatory cytokines that may lead to cancer development.

However, to our knowledge there is no report on specific cytokine profiles in the liver fluke-associated CCA. Therefore, the objective of this study was to assess the production of pro-inflammatory/anti-inflammatory cytokines by PBMC from CCA patients upon stimulation with *Ov*-ES. The result from this study will advance our knowledge on *Ov* stimulated immune response and its potential effect on CCA development.

2. Materials and Methods

2.1 Study subjects

All subjects lived in *Ov* endemic areas of Khon Kaen or neighboring provinces. The 21 normal control subjects were uninfected with *O. viverrini* as determined by fecal examination and by absence of advanced periductal fibrosis on abdominal ultrasonography [19]. Sixty-one histologic proven CCA cases were patients who underwent hepatectomy at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. All samples were obtained from the Liver Fluke and Cholangiocarcinoma Research Center (HE571283) and Tropical Disease Research Laboratory (TDR) (HE 480528), Faculty of Medicine, Khon Kaen University. The study protocol was approved by the Khon Kaen University Human Ethics Committee (HE581272).

2.2 *Opisthorchis viverrini*-excretory secretory (*Ov*-ES) product preparation

Ov-ES was prepared as described previously with minor modifications [6, 7]. Briefly, fresh worms collected from infected hamster were cultured in RPMI-1640 containing antibiotics and the protease inhibitors (0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM leupeptin and 0.1 mM N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucine]-agmatine, E-64). Worms were maintained *in vitro* at 37°C and supernatants containing the *Ov*ES were collected twice each day for up to 7 days and centrifuged at 3,000 *g* for 10 min to remove the eggs. The clarified supernatants were pooled, dialyzed against PBS, and concentrated. Lipopolysaccharide (LPS) was removed using Triton-X114 extraction [20], followed by passage through Bio-Beads SM2 (Bio-Rad, (USA) to remove Triton-X114. Finally, *Ov*ES was filtered through a 0.2 µm membrane and then aliquoted and stored at -80°C. The LPS concentration was determined using Limulus amoebocyte assay (less than 100 ng/ml had no effect on cell proliferation or cytokine production (unpublished data).

2.3 Peripheral blood mononuclear cells (PBMCs) culture

The PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Sigma Diagnostic, St. Louis, MO). The cells were counted on a hemacytometer and viability assessed by 0.5% trypan blue dye test. The PBMC were cultured (2×10^4 cells/ml) in RPMI-1640 medium, supplemented with antibiotics and 5% fetal bovine serum under the presence or absence of *Ov*-ES product (20 µg/ml) and phytohemagglutinin (PHA)-L (Difco Laboratories, Detroit, MI) 10 µg/ml as control stimulation. The cells were cultured at 37°C in a humidified 5% CO₂ incubator for 48 hr. The supernatants were harvested and stored at -80°C until used in test.

2.4 Cytokine detection

Twenty-five to 50 μ l of supernatant were used for IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF- α and LT- α cytokine assays. The secreted cytokines were quantified using commercial FlowCytomix bead-based multiplexing assays kit, human Th1/Th2 11 plex kit (BMS810FF, ebioscience) according to the manufacturer's instruction and then assessed by flow cytometry (Beckman-Coulter). The amounts of cytokines were quantified from standard curves using human recombinant cytokines and the results were expressed as pg/ml, based on comparisons with respective standards.

2.5 Total IgG antibody by ELISA technique

Ov-ES antigen against total IgG antibody measurements were performed by indirect ELISA in microtiter plates (MaxiSorp; Nunc, Denmark) following the procedure by Elkins et al. (1991) with some modification. A pool of positive control sera was derived from 30 *Ov* positive individuals. A pool of negative control sera was sourced from 30 age and sex matched subjects who were *Ov* egg negative and without any other apparent infection. MaxiSorp™ (Nunc, Denmark) 96-well microtiter plates were coated with 100 μ l/well of 1 μ g/ml of *Ov*-ES antigen in carbonate-bicarbonate buffer (Coating buffer), pH 9.6, sealed, and incubated overnight at 4°C. Plates were washed three times with PBS (pH 7.2) and blocked with 200 μ l/well of 5% skim milk (Sigma, St. Louis, MO) diluted in coating buffer and then incubated for 2 hours at 37°C. Control and experimental serum samples were diluted 1:1,000 of 2% skim milk in incubation buffer and 100 μ l was added to each well of the microtiter plate in duplicates. The plates were sealed and incubated at 37°C for 2 hours and then washed three times with PBS with 0.05% Tween 20 (PBST) at pH 7.2. The horseradish peroxidase-conjugate goat anti-human Ig (Zymed, USA) at 1:10,000 dilution in PBS was applied at 100 μ l/well and then incubated for 1 hour at 37°C in the dark. After washing, the plates were incubated in the dark at 37°C for 30 minutes with 3,3',5,5'-Tetramethylbenzidine or TMB (Zymed, USA). Sulfuric acid (50 μ l) was added to each well to stop the reaction. The optical density (OD) was measured at 450 nm using a microplate ELISA reader (VersaMax, USA) and analysis performed by SOFTmax Pro software (Molecular Devices). The data were expressed as the OD level.

2.6 Statistical analysis

The data are presented as a mean $\pm$ SD. Statistical analysis was performed with non-parametric Mann-Whitney U test and Wilcoxon Match-paired sign ranks test using IBM SPSS Statistics for Windows version 19 (SPSS, USA). P value < 0.05 was considered statistically significant.

3. Results

3.1 Demographic and clinicopathological characteristics

The clinicopathological characteristics of CCA and normal control subjects are summarized in Table 1. The 21 normal controls consisted of 6 men and 15 women with a mean age of 52.76 years (range: 36–59 years). The 61 CCA patients were 40 men and 21 women, whose mean age was 55.19 years (range: 33–73). The distribution of mass-forming, periductal

infiltration and intraductal infiltration of gross appearance of the CCA cases were 41.0%, 6.6% and 23.0%, respectively.

3.2 Total serum IgG antibody against *Ov*ES and cytokine production in *Ov*ES stimulated PBMCs

The mean serum IgG antibody against *Ov*ES antigen was significantly higher in CCA patients (0.683 ± 0.733) than in uninfected normal controls (0.096 ± 0.074) ($p < 0.05$). The cytokine profiles produced from unstimulated and stimulated PBMCs with *Ov*ES in the normal control subjects are shown in Fig. 1A. The cytokine level of IL-1 β , IL-4, IL-6, IL-8, IFN- γ and TNF- α were significantly increased after *Ov*ES stimulation ($p < 0.05$). However, the levels of IL-12p70 and LT- α in supernatants of both conditions were undetectable. Fig. 1B shows the cytokine profiles of PBMCs from CCA patients. Levels of all cytokines were significantly higher in *Ov*ES stimulated condition ($p < 0.05$). Net production of all cytokines in *Ov*ES stimulated PBMCs were significantly higher in CCA group compared with the normal control group (Fig. 2). For example, net production levels of IFN- γ , IL-10, and LT- α upon stimulation with *Ov*-ES were 40 to > 320 times higher in CCA than those of controls.

4. Discussion

Inflammation around infected hamster bile ducts caused by *Ov* infection is a consequence of host responses to *Ov* antigens. *Ov*ES induced TLR4 receptor leading to NF- κ B nuclear translocation, and then activated the expression and secretion of IL-6 and IL-8 from cholangiocytes [18]. Our previous study examined the level of proinflammatory/anti-inflammatory cytokines in the PBMCs collected from *Ov*-infected individuals with advanced periductal fibrosis, a precancerous condition. After stimulation by *Ov*ES, only the proinflammatory cytokine IL-6 was significantly elevated in the infected individuals with fibrosis when compared with the individuals without fibrosis [16]. Moreover, elevated IL-6 to parasite ES was associated with increased risk of advanced periductal fibrosis [17].

In this study, we further investigated cytokine expression profile in the PBMCs collected from *Ov* associated CCA patients. Only IL-4 and proinflammatory cytokines were increased in uninfected normal controls after *Ov*ES stimulation indicating a normal inflammatory response in the control group. Interestingly, all cytokines were increased in the CCA patients when compared with the normal control. The presence of both proinflammatory and anti-inflammatory cytokines in CCA is similar to other cancers, where it suggests that simultaneous immunostimulation and immunosuppression occur in patients with cancer [13].

The difference of cytokine profiles among different stages of *Ov* infection may reflect different immune responses. These differences might explain why not all individuals who are chronically infected with *Ov* develop advanced hepatobiliary abnormalities and only few of those go on to develop CCA. It is proposed that individuals with proinflammatory phenotypes in the advanced periductal fibrosis group have a dysregulation of inflammatory cytokine production in response to chronic fluke infection [17]. In this study, we present supportive data for a marked immune dysregulation process in CCA patients.

It is generally accepted that chronic inflammation is important in generating malignancy through the exposure of proinflammatory cytokines and sustained activation of signaling pathways. Inflammation induces the production reactive oxygen species (ROS) and reactive nitrogen species (RNS) from epithelial cells and immune cells, which results in cellular stress and oxidative tissue damages. Proinflammatory cytokines IL-6, IFN- γ LT- α and TNF- α stimulate cell to cell communication, proliferation and reduce apoptosis, while anti-inflammatory cytokines, such as IL-10 and TGF- β , contribute to tumor immune evasion [21]. Suppression of inflammation by anti-inflammatory drug prednisolone enhanced cholangiocarcinogenesis in *Ov* infected hamster [22].

In conclusion, we have uncovered the cytokine profiles of *Ov* induced CCA and revealed a marked immune deregulation process in CCA. Studies to further elucidate the mechanism underlying this immunopathologic phenomenon should lead to better understanding of the carcinogenesis of CCA.

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Highlights

- Cytokine profiles of liver fluke *O. viverrini*-associated bile duct cancer.
- Marked increased of pro- and anti-inflammatory cytokines in the cholangiocarcinoma
- Dysregulation of immune response in the liver fluke associated cancer

Fig. 1. Cytokine profile of OvES stimulated and unstimulated PBMCs in uninfected control (A) and *O. viverrini* infected CCA patients (B). Levels of 11 cytokines were measured by FlowCytomix assay and flow cytometry in the PBMCs of individual from normal control (n=21) and CCA patients (n=61). The statistical significance was analyzed using Wilcoxon Matched-paired Signed rank test. * $p < 0.05$.

Fig. 2.

Comparison of net cytokine production between PBMCs from CCA patients and control group. Levels of 11 cytokines were quantified by FlowCytomix assay and flow cytometry in the PBMCs of individual from normal control (n=21) and CCA patients (n=61). The statistical significance was analyzed using a Mann-Whitney U test. * $p < 0.05$. The net cytokine production was calculated by cytokine level of stimulated with *OVES* minus cytokine level of unstimulated.

Table 1

Clinicopathological characteristics of cholangiocarcinoma patients and normal controls.

Demographics	Normal control (n=21)	CCA patients(n=61)
Sex		
Male	6 (28.57%)	40 (65.57%)
Female	15 (71.43%)	21 (34.43%)
Age (years)		
Mean±SD	52.76 ±5.89	55.19±11.87
Range	36–59	33–73
Age (in year)		
30	0 (0%)	0 (0%)
31–40	1 (4.76%)	3 (4.91%)
41–50	5 (23.80%)	14 (22.95%)
50	15 (71.42%)	44 (72.13%)
Gross type		
MF	-	25 (40.98%)
PD	-	4 (6.56%)
ID	-	14 (22.95%)
MF + PD	-	8 (13.11%)
MF + ID	-	0 (0.0%)
NA	-	10 (16.40 %)

MF = Mass forming, PD = Periductal infiltrating, ID = Intraductal infiltrating, NA= Non-available