CHAPTER I
INTRODUCTION

1.1 Background and rationale of the study

Chronic inflammation is known to be one of the diving forces of transformation that together with other determinants, including the intrinsic properties of pre-malignant cells, support initiation of cancer. In general, inflammatory conditions are characterized by recruitment of inflammatory cells. Neutrophil (and some eosinophil) are the first recruited effectors of the acute inflammatory response, whereas monocytes, which differentiate into macrophage in tissue injury, are key effectors in chronic inflammation. This is because the large variety of bioactive products including cytokines, chemokines, prostaglandins and reactive oxygen/nitrogen species released by macrophages, provide an important defense mechanism against invading organisms. However, these processes are supposed to be self-limiting, at time they are inappropriate regulated, it can lead to continuous stimulation and eventually to pathogenesis (Ben-Baruch, 2006). Therefore, The pro-tumor actions of inflammatory cells are proposed, such as releasing growth and survival factors, promoting angiogenesis and lymphagiogenesis, stimulating DNA damage, remodeling the extracellular matrix to facilitate invasion, coating tumor cells to make available receptors for disseminating cells via lymphatics and capillaries and evading host defense mechanisms (Coussens, Werb, 2002). In the setting of tumors, the major infiltrating leukocyte is macrophage or tumor-associated macrophages (TAMs). They have a range of functions which affect diverse aspects of neoplastic tissues including angiogenesis, stromal formation and dissolution, as well as modulation of tumor cell growth (Mantovani et al., 2004; Mantovani et al., 2008; Mantovani et al., 2003). Nowadays, there is a subset of circulating $CD14^{+}CD16^{+}$ monocytes that exhibit feature of tissue macrophages and potentially migrate into tumor area with proangiogenic properties. Thus, they may develop to be TAM subpopulation in tumor microenvironments (De Palma et al., 2007). In addition, the

expansion of $CD14^+CD16^+$ monocytes was found in blood circulation of patients with chronic inflammation syndromes and several diseases including cancers.

Cholangiocarcinoma (CCA) generally is believed to arise as a result of malignant transformation of epithelial cells (cholangiocytes) lining the intrahepatic and extrahepatic bile duct (Gores, 2003; Sirica et al., 2002). It is devastating cancers that is increasing both worldwide incidence and mortality rate (Sirica, 2005). CCA found frequently in Southeast Asia and is highly prevalent in Northeast Thailand (Sripa, Pairojkul, 2008). Both epidemiology and experimental evidence implicate chronic inflammation from the carcinogenic liver fluke: *Opisthorchis viverrini* (OV), which is endemic in this region, as a major risk factor of CCA (Thamavit et al., 1978).

In animal studies, high accumulation of infiltrating leukocytes were shown in both OV infected and OV associated CCA mediate oxidative and nitrative DNA damage through chronic inflammation (Pinlaor et al., 2004; Pinlaor et al., 2003). In addition, local tumor immunology studied by Krungmee et al. (Krungmee et al., 2003) revealed that MHC antigen, Fas and FasL were aberrantly expressed in CCA tissues. Patients who showed intense infiltration of T, B, and NK cells tended to have longer survival. In contrast, high infiltrating macrophage was found to correlate with poor prognosis. The association of macrophages to poor prognosis of CCA patients was further supported by Miwa, et al (2005). They found that macrophages at the invasive margin of CCA may contribute to tumor angiogenesis through PD-ECGF secretion (Miwa et al., 2005).

Therefore, we hypothesize that the inflammatory cells especially monocytes/macrophages in circulation and tumor area of CCA patients have significant effect on tumor development. Early in the neoplastic process, these cells are powerful tumor promoter, producing an alternative environment for tumor growth, facilitating genomic instability and promoting angiogenesis. Later in tumorigenic process, neoplastic cells also get supports from inflammatory mechanism such as protease production and chemokine function to favor CCA spread and metastasis.

Up to our search, there is no any report on the $CD14^+CD16^+$ monocytes in CCA. The aims of this study are: to identify and characterize the $CD14^+CD16^+$monocytes in CCA patients and, its association to clinico-pathological features and survival of CCA patients, the expression profile of peripheral blood

leukocytes (PBLs) and the possible role in pathogenesis of CCA. The outcome of this study may lead to a new prognostic marker and new anti-inflammatory therapeutic approaches to cancer development.

1.2 Research questions

1.2.1 Is the level of $CD14^+CD16^+$ monocyte increased in CCA patients comparing to healthy subjects?

1.2.2 Is the level of $CD14^+CD16^+$ monocyte related to clinico-pathological features and survival of CCA patients and can it be used as a marker of CCA?

1.2.3 Is the gene expression profile of the peripheral blood leukocytes from CCA patients associate to clinico-pathological features and survival of CCA patients?

1.2.4 Are there candidate genes in peripheral blood leukocytes expressed in macrophages in CCA tissues and related to CCA progression?

1.3 Scope of the study

The study was performed using specimen from three groups of subjects 1) healthy persons, 2) benign biliary tract disease patients (BBD) and 3) CCA patients. Healthy subjects were defined as the persons who had normal clinically health with normal blood picture determined by complete blood count (CBC), normal liver function test and no appeared chronic inflammatory diseases such as diabetic mellitus and hepatitis. BBD subjects were persons who had abnormal biliary tract or gall bladder such as cholangitis, cholecystitis, etc., as shown by ultrasonography or histological examination. CCA subjects were patients who underwent surgical resection of hepatogastrointestinal cancer and the histology proved to be CCA. Informed consents were signed by each subject. Heparinized blood (10 ml) were collected from each subjects, and CCA tissues samples were obtained from CCA patients and analyzed as follows:

(i) Heparinized blood from all 3 groups was determined the level of $CD14^+CD16^+$ monocytes and their surface adhesion molecules and scavenger receptors.

(ii) Heparinized blood from all 3 groups was performed genes expression

profiles of PBLs to identify profile of differentially expressed genes in leukocytes associated with CCA.

(iii) CCA tissue was taken to verify the expression of candidate gene in infiltrating leukocytes especially macrophages.

According to the research questions, this study was divided in to three parts as follow:

Part I: EXPANSION AND CHARACTERIZATION OF CIRCULATING $CD16^+$ MONOCYTE IN CHOLANGIOCARCINOMA PATIENTS

Levels of $CD14^+CD16^+$ monocytes in peripheral blood from CCA patients were compared to those of BBD and healthy subjects. The correlations between level of $CD14^+CD16^+$ monocytes and clinicopathological features of CCA patients were analyzed. The expression of surface adhesion molecules and scavenger receptors on $CD14^+$ and $CD14^+CD16^+$ monocytes from CCA patients and healthy subjects were compared by four color immunofluorescent staining and analyzed by flow cytometer.

Part II: DIFFERETIAL EXPRESSION PROFILES OF PERIPHERAL BLOOD LEUKOCYTES ASSOCIATED WITH CHOLANGIOCARCINOMA AND POSSIBILITY FOR USING AS PROGNOSTC MARKER

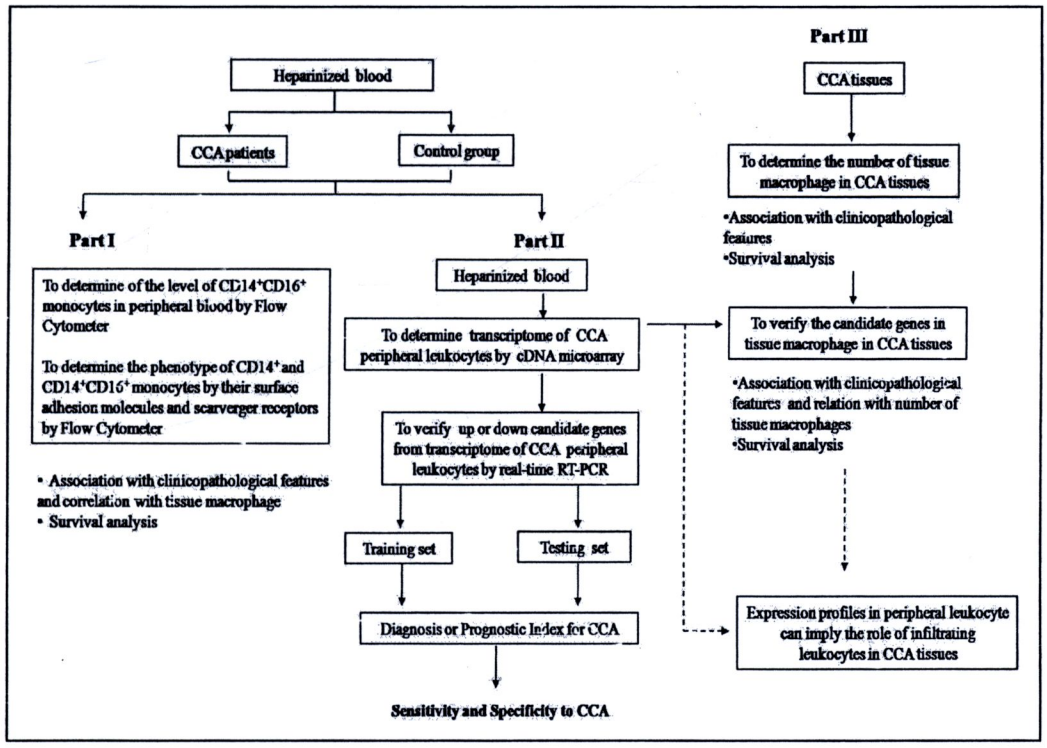
To determine the expression profile of PBLs associated to CCA, gene expression profile of PBLs from CCA patients, BBD subjects and healthy persons were performed using cDNA microarray technique. The data were analyzed using Gene Chip Operating Software and Patek suite Software. The candidate genes were verified in peripheral leukocytes from CCA patients and healthy subjects by Real time RT-PCR. Finally, a set of tumor- invasion promoting gene was selected and formulated as prognostic index or risk score relating to patient survivals.

Part III: TISSUE INVASIVE MACROPHAGE DENSITY CORRELATES WITH PROGNOSIS IN CHOLANGIOCARCINOMA

The expression of candidate genes in infiltrating macrophages was determined by immunohistochemistry in serial paraffin sections of CCA patients' tissues. The double immunofluorescent staining was used to demonstrate the co-expression of macrophages marker (MAC387) and the candidate proteins-namely uPA and MMP9.

The possible associations between the density of MAC387, uPA and MMP9 positive cells and the clinicopathological features as well as patients' survivals were evaluated.

1.4 Conceptual protocol



1.5 Objectives of the study

1.5.1 To investigate the levels, and phenotypes (adhesion molecules and scavenger receptors) of $CD14^+CD16^+$ monocytes in peripheral blood of CCA patients and healthy controls.

1.5.2 To investigate the association of $CD14^+CD16^+$ monocyte levels and the clinico-pathological features as well as survival of CCA patients.

1.5.3 To determine the expression profile of peripheral blood leukocytes of CCA patients, BBD subjects and controls and elucidate the candidate genes which may serve as prognostic index or risk score for CCA patients.

1.5.4 Using CCA associated genes described in 1.5.3 to verify the relation between peripheral leukocytes and infiltrating leukocytes, especially macrophages,in CCA tissues.

1.6 Location of research conducting

Most of the experiments and analytical processes were performed at Departments of Biochemistry and Microbiology, Faculty of Medicine, Khon Kaen University, Thailand. Characterizations of $CD14^+CD16^+$ monocytes by FACS analysis was trained under the supervision of Prof. Seiji Okada, at the Division of Hematopoiesis, Center for AIDS Research, Kumamoto University. Gene expression profiles using cDNA microarray, data filtering and analysis were performed under supervision of Prof. Michael M McGrath, at Department of pathology, Faculty of Medicine, University of California, San Francisco (UCSF), and Pathologica Company, Balingame, California, United State of America.

1.7 Anticipated outcomes

The anticipated outcome of this study was the thorough understanding in the role of blood and tissue leukocytes in progression of CCA. Because microenvironment of CCA tissue is largely orchestrated by inflammatory cells (especially $CD14^+CD16^+$ monocytes in blood and infiltrating macrophages in CCA tissues), it is indispensable to co-opt some signaling molecules of innate immune system for invasion, migration and metastasis. The molecular predictors of candidate genes from PBLs can use as prognostic marker for CCA patients. In addition, the $CD14^+CD16^+$ monocyte levels may be used to monitor disease condition for CCA patients after surgical treatment.

Beside the poster and oral presentations in several scientific meetings, three publications in the international index journals are expected.

(1) Enhanced frequencies of circulating CD16 monocytes in cholangiocarcinoma *(Will be submitted to Clinical Exp Immunology)*

(2) Transcription profiles of peripheral blood leukocytes and prognostic of clinical outcome in patients with cholangiocarcinoma *(Will be submitted to Cancer Research)*

(3) Tissue invasion macrophage density correlates with prognosis in cholangiocarcinoma *(Submitted to World Journal of Gastroenterology)*