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Free radicals enzymatically triggered by *Clonorchis sinensis* excretory–secretory products cause NF-κB-mediated inflammation in human cholangiocarcinoma cells

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ABSTRACT

Chronic clonorchiasis, caused by direct and continuous contact with *Clonorchis sinensis* worms and their excretory–secretory products, is associated with hepatobiliary damage, inflammation, periductal fibrosis and even development of cholangiocarcinoma. Our previous report revealed that intracellular reactive oxygen species were generated in *C. sinensis* excretory–secretory product-treated human cholangiocarcinoma cells; however, their endogenous sources and pathophysiological roles in host cells were not determined. In the present study, we found that treatment of human cholangiocarcinoma cells with excretory–secretory products triggered increases in free radicals via a time-dependent activation of NADPH oxidase, xanthine oxidase and inducible nitric oxide synthase. This increase in free radicals substantially promoted the degradation of cytosolic IκB-α, nuclear translocation of nuclear factor-κB subunits (RelA and p50), and increased κB consensus DNA-binding activity. Excretory–secretory product-induced nuclear factor-κB activation was markedly attenuated by preincubation with specific inhibitors of each free radical-producing enzyme or the antioxidant, *N*-acetylcysteine. Moreover, excretory–secretory products induced an increase in the mRNA and protein expression of the proinflammatory cytokines, IL-1β and IL-6, in an nuclear factor-κB-dependent manner, indicating that enzymatic production of free radicals in ESP-treated cells participates in nuclear factor-κB-mediated inflammation. These findings provide new insights into the pathophysiological role of *C. sinensis* excretory–secretory products in host chronic inflammatory processes, which are initial events in hepatobiliary diseases.

1. Introduction

Clonorchiasis is food-borne trematodiasis caused by chronic infection with *Clonorchis sinensis*, also known as the Chinese or oriental liver fluke. *Clonorchis sinensis* infects an estimated 35 million people in eastern Asian countries, where it constitutes a significant public health concern (Lun et al., 2005). Human infection is acquired by the intake of raw or undercooked freshwater fish harbouring *C. sinensis* metacercariae. These flukes reside in the peripheral small bile ducts and provoke pathological changes in the bile duct and surrounding liver tissue such as inflammation, dilatation, hyperplasia, fibrosis and bile duct wall thickening (Rim, 2005; Lim et al., 2008). Severe and chronic infections may be complicated by cholangitis, cholelithiasis, cholangiectasis and even the development of cholangiocarcinoma. Carcinogenesis associated with clonorchiasis, in particular, may result from chronic irritation and prolonged inflammation of the bile duct epithelium and bile contamination as a result of direct contact with *C. sinensis* worms and their secretion products (Watanapa and Watanapa, 2002). The correlation of *C. sinensis* infection with a high incidence of cholangiocarcinoma in endemic areas, as well as several case-control studies, have recently led the International Agency for Research on Cancer to classify *C. sinensis* as a group I biological human carcinogen (Bouvard et al., 2009).

Similar to other parasitic helminths, C. *sinensis* continuously releases excretory–secretory products (ESPs) into its extracellular surroundings during infection, products that play pivotal roles in host–parasite interactions. A proteomic analysis of *Fasciola hepatica* and *C. sinensis* ESPs revealed that these products predominantly consist of various proteins, the most abundant of which might be involved in protecting the parasite from host immune responses

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(Jefferies et al., 2001; Morphew et al., 2007; Ju et al., 2009; Robinson et al., 2009). In addition, some ESP proteins immunoreact with the sera of *C. sinensis*-infected patients, suggesting that they might serve as effective serodiagnostic antigens for clonorchiasis (Hong et al., 2002; Kang et al., 2004; Li et al., 2004; Ju et al., 2009; Pak et al., 2009a). Cells exposed to ESPs from liver flukes (*C. sinensis, F. hepatica* and *Opisthorchis viverrini*) display diverse pathophysiological responses including proliferation (Thuwajit et al., 2004; Kim et al., 2008), apoptotic cell death (Serradell et al., 2007), and inflammation (Pinlaor et al., 2005). We recently profiled changes in the transcriptosomes and proteomes of human cholangiocarcinoma cells (HuCCT1) caused by exposure to *C. sinensis* ESPs (Pak et al., 2009a,b). The identified genes/proteins shown to be differentially regulated by ESPs were identified as those that participate in apoptotic modulation, carcinogenesis, metabolism, redox homeostasis and signal transduction, implying that ESPs contribute to multiple biological processes in host cells. However, the pathological functions of these proteins and the mechanisms responsible for their induction by ESPs remain to be elucidated.

Endogenous free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are produced through multiple sources, including the electron transport chain in mitochondria and as by-products of enzymatic reactions involving NADPH oxidases (NOX), xanthine oxidase (XO), lipoxygenases (LO), cyclooxygenases (COX) and nitric oxide synthases (NOS). Excess intracellular free radical generation promotes serious cellular injuries and contributes to several human diseases. However, at low or intermediate (non-toxic) levels, free radicals act as second messengers that participate in signal transduction, differentiation, proliferation, apoptosis, migration and modulation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Thannickal and Fanburg, 2000; Droge, 2002). In particular, free radical-induced NF-κB activation plays an essential role in controlling a variety of physiological aspects of immune and inflammatory responses. NF-κB is a dimer of Rel family proteins, the most prevalent form of which consists of a heterodimer of RelA (p65) and p50. In the resting state, NF-κB is sequestered in the cytoplasm through covalent binding with inhibitory proteins called IκBs. Exposure of cells to external stimuli, including H_2O_2, ionising radiation, cytokines or bacterial endotoxins, results in phosphorylation of IκB and its subsequent proteolytic degradation. This allows the free NF-κB dimer to translocate to the nucleus, where it binds to the cis-acting κB element of target genes such as inflammatory cytokines, proinflammatory mediators and cell adhesion molecules, to regulate their expression (Gloire et al., 2006; Pantano et al., 2006). NF-κB expression has been reported to be up-regulated in mouse RAW 264.7 macrophage cells treated with *O. viverrini* crude protein extracts and in the nucleus of bile duct epithelial cells of *O. viverrini*-infected hamsters, suggesting the involvement of NF-κB activation in liver fluke infection (Pinlaor et al., 2005, 2006). We and others have recently demonstrated that ESPs from liver flukes mediate ROS generation, showing that ROS generated in response to *C. sinensis* ESPs lead to elevated expression of such antioxidant proteins as thioredoxin and peroxiredoxin isoforms in HuCCT1 cells, whereas those from *F. hepatica* cause apoptosis in rat eosinophils via mitochondrial membrane depolarisation (Pak et al., 2009b; Serradell et al., 2009). Collectively, these findings imply that ESPs from liver flukes trigger a generation of free radicals that is associated with the initial pathophysiological responses in host cells; however, the molecular details of these events and the host enzymatic sources responsible for free radical production are poorly defined.

In the present study, we examined the activation of potential free radical-generating enzymes in HuCCT1 cells treated with *C. sinensis* ESPs and investigated the involvement of ESP-induced enzyme activation in NF-κB-mediated inflammatory processes. Our results indicate that free radical-mediated NF-κB signalling in host cells is an important factor in the chronic inflammation caused by *C. sinensis* infection.

2. Materials and methods

2.1. Materials

Cell culture medium components were purchased from Life Technologies (Grand Island, NY, USA) unless otherwise stated. *N*-acetylcysteine (NAC), allopurinol (ALP; XO inhibitor), N^G-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor), Bay 11-7082 (IκB-α phosphorylation inhibitor), and a complete protease inhibitor cocktail were obtained from Sigma–Aldrich (St. Louis, MO, USA). Diphenyleneiodonium chloride (DPI; NOX inhibitor) was purchased from Calbiochem (La Jolla, CA, USA). Polyclonal antibodies against the following proteins were obtained from the indicated sources: NF-κB p65 and IκB-α (Cell Signaling Technology, Beverly, MA, USA); $p47^{phox}$, $p67^{phox}$ and inducible NOS (iNOS) (BD Biosciences, San Joes, CA, USA); NF-κB p50, lamin B and calnexin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and glyceradehyde-3-phosphate dehydrogenase (GAPDH; AbFrontier Co., Seoul, Korea). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratory (West Grove, PA, USA). All other chemicals (biotechnology grade) were purchased from Amresco, Inc. (Solon, OH, USA).

2.2. Preparation of C. sinensis ESPs

Metacercariae from *C. sinensis* were collected from naturally infected freshwater fish (*Pseudorasbora parva*) in an endemic area of Korea. Whole flesh of the fish was digested with artificial gastric juice (0.6% pepsin in 0.7% HCl, pH 2.0) for 1 h at 37 °C and then filtered through a 0.147-mm diameter sieve. After thorough washing with cold physiological saline, metacercariae were collected under a stereoscopic microscope. New Zealand albino rabbits (1.5–2 kg) were orally infected with ~500 metacercariae and sacrificed 8 weeks later. Adult worms were collected from the bile ducts and washed three times with cold physiological saline to remove any host contaminants. ESPs were prepared by incubating five live worms in 1 ml of sterile PBS (pH 7.2) for 3 h at 37 °C under 5% CO_2. The supernatants were pooled, centrifuged and passed through a polymyxin B-agarose column (Sigma–Aldrich) to remove any residual lipopolysaccharide (LPS). Subsequently, ESPs were concentrated with a Centriprep YM-10 (Millipore, Bedford, NA, USA) and filtered through a sterile 0.2 μm syringe filter. The protein concentration was measured using DC Protein Assays (Bio-Rad, Hercules, CA, USA), and ESP aliquots were stored at −80 °C until use.

Animal care and experimental procedures were in accordance with institutional guidelines and the protocols were approved by the Animal Care and Use Committee of the Korea National Institute of Health.

2.3. Cell culture and ESP treatment

The HuCCT1 human cholangiocarcinoma cell line (originally developed by Miyagiwa et al., 1989) was cultured in RPMI 1640 medium supplemented with 10% FBS and an antibiotic mixture at 37 °C in a humidified 5% CO_2 atmosphere. For ESP treatment, cells were seeded at ~70% confluence on 35 or 100 mm culture dishes and grown for 24 h under standard culture conditions. Cells were gradually deprived of serum by incubation in 1% FBS overnight, followed by incubation in serum-free medium for 3 h. These