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Clonorchis sinensis excretory–secretory products regulate migration and invasion in cholangiocarcinoma cells via extracellular signal-regulated kinase 1/2/nuclear factor-κB-dependent matrix metalloproteinase-9 expression

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ABSTRACT

Matrix metalloproteinase-9 plays an important role in the invasion and metastasis of various types of cancer cells. We have previously reported that excretory–secretory products from *Clonorchis sinensis* increases matrix metalloproteinase-9 expression. However, the regulatory mechanisms through which matrix metalloproteinase-9 expression affects cholangiocarcinoma development remain unclear. In the current study, we examined the potential role of excretory–secretory products in regulating the migration and invasion of various cholangiocarcinoma cell lines. We demonstrated that excretory–secretory products significantly induced matrix metalloproteinase-9 expression and activity in a concentration-dependent manner. Reporter gene and chromatin immunoprecipitation assays showed that excretory–secretory products induced matrix metalloproteinase-9 expression by enhancing the activity of nuclear factor-kappa B. Moreover, excretory–secretory products induced the degradation and phosphorylation of IκBα and stimulated nuclear factor-kappa B p65 nuclear translocation, which was regulated by extracellular signal-regulated kinase 1/2. Taken together, our findings indicated that the excretory–secretory product-dependent enhancement of matrix metalloproteinase-9 activity and subsequent induction of IκBα and nuclear factor-kappa B activities may contribute to the progression of cholangiocarcinoma.

1. Introduction

Cholangiocarcinoma is a rare type of liver cancer; however, the incidence of cholangiocarcinoma is now increasing worldwide. The cancer is highly metastatic, and metastasis is the main cause of death in patients with cholangiocarcinoma (Cardinale et al., 2010; Khuntikeo et al., 2016). Based on their anatomical location, cholangiocarcinomas are classified as either intrahepatic or ductal cholangiocarcinoma. Several well-established risk factors for cholangiocarcinoma have been identified, including bile duct cysts, primary sclerosing cholangitis, hepatolithiasis, chronic viral hepatitis, and infection with liver flukes such as *Opisthorchis viverrini* and *Clonorchis sinensis* (IARC, 2012; Patel, 2014). Surgery is the main treatment for resectable, localised cholangiocarcinoma. However, it is extremely easy for cholangiocarcinoma cells to metastasize and cause relapse (Nathan et al., 2009; Sun et al., 2014).

Carcinogenesis associated with liver fluke infection results from mechanical damage to biliary epithelia caused by the feeding and migratory activities of worms, immunopathology due to infection-related inflammation, and a direct effect of their excretory–secretory products (ESPs) (Sripa et al., 2012). In particular, ESPs continuously released from the tegument and excretory openings of *C. sinenis* worms play pivotal roles in host-parasite interactions. A proteomic analysis of *C. sinenis* ESPs revealed that these products predominantly consist of cysteine proteases, detoxifying enzymes, myoglobin, and metabolic enzymes; some of which immunoreact with the sera from clonorchiasis patients, thus affecting the human immune system (Ju et al., 2009; Pak et al., 2009a). Cells exposed to *C. sinensis* ESPs display diverse pathological responses including proliferation and inflammation (Kim et al., 2008; Nam et al., 2012). We have recently profiled differentially regulated-transcriptomes and proteomes in ESP-treated

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cholangiocarcinoma cells (HuCCT-1), which participate in apoptotic modulation, carcinogenesis, metabolism, redox homeostasis, and signal transduction (Pak et al., 2009a,b). These findings suggest that ESPs contribute to multiple pathophysiological processes in the host.

Metastasis is a complex, multistep process that involves detachment of tumour cells from the original tumour site, intravasation, extravasation, and settlement of the cells at a secondary site (Geiger and Peeper, 2009). The invasiveness of tumour cells is critical throughout the metastatic process. Several proteases, including matrix metalloproteinases (MMPs), cysteine proteases, and serine proteases, are required for invasion, which requires altering the extracellular matrix to permit the egress and ingress of cancer cells (Denhardt et al., 1987; Sreenath et al., 1992;Achbarou et al., 1994).

MMPs, a family of zinc-dependent endopeptidases, are primary enzymes involved in extracellular matrix (ECM) degradation, which is essential for the invasive growth, metastasis and angiogenesis of cancer (Nabeshima et al., 2002; Rao, 2003). Among the various MMP isoforms, MMP-9, which is abundantly expressed in various malignant tumours, plays critical roles in tumour invasion and metastasis (Kang et al., 2012). MMP-9 degrades type IV collagen, which is the major structural component of the basement membrane and ECM, and its activity increases with the degree of malignancy. Moreover, elevated serum MMP-9 or immunohistochemical MMP-9 expression is significantly correlated with poor disease-free and overall survival and may be of prognostic value in some cancers, including cholangiocarcinoma (Tanioka et al., 2003; Ruokolainen et al., 2004; Sun et al., 2014). MMP-9 activity in various tumour cells is tightly controlled, mainly at the transcriptional level (Stamenkovic, 2000). Moreover, the MMP-9 promoter is highly conserved and contains multiple functional elements, including nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1)-binding sites, and previous studies have suggested that NF-κB is one of the most important transcription factors regulating MMP-9 expression (Kim et al., 2011; Kang et al., 2012). We have recently reported that exposure of HuCCT-1 cells to *C. sinensis* ESPs triggers enzymatic free radical generation, leading to NF-κB-mediated inflammatory processes (Nam et al., 2012). Moreover, ESP treatment causes three-dimensional invasion into neighbouring ECM with concomitant elevation of focal and cell–cell adhesion protein expression and MMP secretion (Won et al., 2014). These findings provide potential links among NF-κB, MMP-9, and invasion in ESP-exposed cholnagiocarcinoma cells, although the molecular mechanisms are not yet fully understood.

Accordingly, in the present study, we examined the role of ESPs in the invasion and migration of human cholangiocarcinoma cells. Our results provided important insights into the effects of ESPs on MMP-9 expression and the extracellular signal-regulated kinase 1/2 (ERK1/2)/NF-κB signalling pathway.

2. Materials and methods

2.1. Cells and reagents

The human cholangiocarcinoma cell lines HuCCT-1, Cho-CK and Choi-CK (generously gifted by Dr. Dae Ghon Kim, Department of Internal Medicine, Chonbuk National University Medical School, Jeonju, Korea) were maintained in RPMI 1640 or DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C with 5% CO_2 atmosphere in a humidified incubator. The human H69 cholangiocyte cells between passages 25 and 30 were grown in DMEM/F12 (1:1) containing 10% FBS, an antibiotic mixture, 1.8×10^{-4} M adenine, 5 μg/ml of insulin, 5.5×10^{-6} M epinephrine, 2×10^{-9} M triiodothyronine, 5 μg/ml of transferrin, 1.64×10^{-6} M epidermal growth factor (EGF), and 1.1×10^{-6} M hydrocortisone. Cells were cultured at 37 °C in a humidified 5% CO_2 incubator. Gelatin was obtained from DIFCO (Lexington, KY, USA). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA, USA). Anti-MMP-9, anti-p65, anti-tubulin, anti-proliferating cell nuclear antigen (PCNA) and anti-IκBα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-IκBα antibody was from Cell Signaling Technology (Beverly, MA, USA). PD98059 (PD; ERK inhibitor), SB203580 (SB; p38 MAPK inhibitor), and SP600125 (SP; JNK inhibitor) were obtained from Calbiochem (La Jolla, CA, USA). Bay 11-7082 (Bay; NF-κB inhibitor) was purchased from Sigma-Aldrich (St Louis, MO, USA). All chemicals not mentioned above were from Sigma-Aldrich.

2.2. Preparation of C. sinensis ESPs

Preparation of *C. sinensis* ESPs was as described in Nam et al. (2012) with minor changes. Briefly, whole flesh of a cyprinid fish, *Pseudorasbora parva*, was digested with artificial gastric solution (0.6% pepsin in 0.7% HCl, pH 2.0) for 2 h at 37 °C. The digested content was then filtered through a 0.147-mm diameter sieve and washed thoroughly with 0.85% saline. *Clonorchis sinensis* metacercariae were collected under a stereoscopic microscope and stored at 4 °C until required for infection. Three male New Zealand albino rabbits (12 weeks old) were infected with ~500 metacercariae via intragastric intubation and housed in individual cages at a constant temperature of 23 °C in a 12:12 h light:dark cycle. The animals freely accessed a standard pellet diet and filtered tap water. Animal care and protocols were performed in accordance with institutional guidelines and were approved by the Animal care and Use Committee of the University of Ulsan, Korea. Animal care and maintenance was conducted under the supervision of the Laboratory Animal Unit of the Asan Institute for Life Sciences. After 12 weeks, the rabbits were euthanized with a single i.v. injection of pentobarbital sodium (50 mg/kg). Adult worms were recovered from the bile ducts and washed several times with cold PBS to remove any host contaminants. Five fresh worms were cultured in 1 ml of prewarmed PBS containing antibiotic mixture and protease inhibitor cocktail (Sigma-Aldrich) for 3 h at 37 °C in a 5% CO_2 environment. The culture fluid was then pooled, centrifuged, concentrated with a Centriprep YM-10 (Merck Millipore, Billerica, MA, USA), and filtered through a sterile 0.2-mm syringe membrane. The protein concentration of the ESPs was measured using DC Protein Assays (Bio-Rad, Hercules, CA, USA), and ESP aliquots were stored at −80 °C until use.

2.3. In vitro invasion/migration assay

Cells (4×10^4 cells/well) were cultured in 10% FBS RPMI-1640 or DMEM in 24-well plates for overnight. Cells at 80–90% confluence were serum-starved and treated with ESPs or various pharmacological inhibitors. Cells were then resuspended in 30 μl of serum-free medium and added to the upper chamber of the QCM™ 24-well Colorimetric Cell Migration assay kit (EMD Milipore, Billerica, MA, USA) following the manufacturer's instructions (Hou et al., 2007; Amini et al., 2013). Cells that migrated through the polycarbonate membrane were incubated with "Cell Stain Solution" and then subsequently extracted and detected on a standard microplate at 560 nm. Cell invasion was assessed using the Chemicon cell invasion assay kit. This assay was performed in an invasion chamber, which is a 24-well tissue plate with 12 cell culture inserts. The inserts contain an 8-μm pore size polycarbonate membrane over which a thin layer of dried ECMatrix™ is coated. The ECM layer occludes the membrane pores, blocking non-invasive cells from migrating through. Invaded cells migrate through the

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