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Development and characterization of a hydrogen peroxide-resistant cholangiocyte cell line: A novel model of oxidative stress-related cholangiocarcinoma genesis

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ABSTRACT

Oxidative stress is a cause of inflammation–related diseases, including cancers. Cholangiocarcinoma is a liver cancer with bile duct epithelial cell phenotypes. Our previous studies in animal and human models indicated that oxidative stress is a major cause of cholangiocarcinoma development. Hydrogen peroxide (H_2O_2) can generate hydroxyl radicals, which damage lipids, proteins, and nucleic acids, leading to cell death. However, some cells can survive by adapting to oxidative stress conditions, and selective clonal expansion of these resistant cells would be involved in oxidative stress-related carcinogenesis. The present study aimed to establish H_2O_2-resistant cell line from an immortal cholangiocyte cell line (MMNK1) by chronic treatment with low-concentration H_2O_2 (25 μM). After 72 days of induction, H_2O_2-resistant cell lines (ox-MMNK1-L) were obtained. The ox-MMNK1-L cell line showed H_2O_2-resistant properties, increasing the expression of the anti-oxidant genes catalase (CAT), superoxide dismutase-1 (SOD1), superoxide dismutase-2 (SOD2), and superoxide dismutase-3 (SOD3) and the enzyme activities of CAT and intracellular SODs. Furthermore, the resistant cells showed increased expression levels of an epigenetics-related gene, DNA methyltransferase-1 (DNMT1), when compared to the parental cells. Interestingly, the ox-MMNK1-L cell line had a significantly higher cell proliferation rate than the MMNK1 normal cell line. Moreover, ox-MMNK1-L cells showed pseudopodia formation and the loss of cell-to-cell adhesion (multi-layers) under additional oxidative stress (100 μM H_2O_2). These findings suggest that H_2O_2-resistant cells can be used as a model of oxidative stress-related cholangiocarcinoma genesis through molecular changes such as alteration of gene expression and epigenetic changes.

1. Introduction

Oxidative stress is a major cause of inflammation-related diseases, including cancer [1]. Overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can damage lipids, proteins, and nucleic acids [1]. ROS damage lipids, leading to chain reactions of lipid peroxidation and formation of oxysterols, and consequently to carbonyl protein formation and etheno-DNA adducts [2,3]. RNS and ROS can also directly oxidize DNA, causing oxidation products such as the 8-nitroguanine and 8-hydrohy-2′-deoxyguanosine (also known as 8-oxodG) [4], which are mutagenic lesions that can induce point mutations [1]. Recently, many literature reviews have suggested that oxidative stress is also involved in genetic instability, epigenetic changes, and alteration of gene expression [1,5–7].

The etiologies of cholangiocarcinoma have been identified as diseases that damage the bile duct and liver, such as primary sclerosing cholangitis, Caroli's disease, congenital choledochal cysts, hepatitis C virus infection, and liver fluke infections [8,9]. The highest incidence of cholangiocarcinoma is found in northeastern

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Thailand, where liver fluke (*Opisthrochis viverrini*) infection is endemic [10]. Oxidative damage to biomolecules is key events in opisthorchiasis–driven cholangiocarcinoma genesis [11]. Our previous studies demonstrated that the etheno-DNA adducts, 8-nitroguanine and 8-oxodG were formed in the epithelial cells of normal and hyperplastic bile ducts of *O. viverrini*–infected hamster liver tissues [12,13]. Moreover, 8-oxodG and other etheno-DNA adducts were also detected high levels in urine from *O. viverrini*–infected subjects and cholangiocarcinoma patients [12,14]. Significant formation of carbonylated proteins was also found in cholangiocarcinoma tissues [15]. Oxysterols, a kind of oxidized cholesterol, were also detected in cholangiocarcinoma tissues. Our recent study demonstrated that anti-apoptosis was significantly increased in an immortal cholangiocyte cell line (MMNK1) that was exposed long-term to the oxysterols cholestan-3-beta,5-alpha,6-beta-triol (Triol) and 3-keto-cholest-4-ene (3K4) [16]. In general, oxidative stress-damaged cells eventually undergo cell apoptosis or necrosis, whereas damaged cells that can survive under oxidative stress conditions may play important roles in the pathological effect of inflammation–related cancer development.

Hydrogen peroxide (H_2O_2) can generate highly reactive hydroxyl radicals in the presence of metal ions via Fenton-like reactions, resulting in oxidative damage to cellular biomolecules. Additionally, unsaturated fatty acids can be oxidized and consequently produce superoxide anions and hydroxyl radicals in the presence of Fe^{2+} [17]. Chronic treatment of mammalian cells with H_2O_2 has shown that cells are able to adapt to oxidative stress [18–22]. The most common resistance mechanisms are based on an increase in the activity of anti-oxidative enzymes such as catalase (CAT) and superoxide dismutases (SODs), as well as glutathione (GSH) content [18–22]. CAT is a H_2O_2 specific antioxidant enzyme that converts H_2O_2 to water and oxygen [23]. SODs are antioxidant enzymes that catalyze superoxide anion radicals to H_2O_2. SOD1 (CuZn-SOD) localizes at the cytoplasm whereas SOD2 (Mn-SOD) and SOD3 (EC-SOD) localize at the mitochondrial matrix and extracellularly, respectively [24]. Identification of novel defense mechanisms against oxidative stress could lead to the development of new drug targets for the treatment of cholangiocarcinoma.

In this study, we aimed to develop and characterize a H_2O_2-resistant cell line from an immortal normal cholangiocyte cell line (MMNK1). MMNK1 parental cells were treated daily with low-concentration H_2O_2 (25 μM, one-third of the 50% inhibitory concentration (IC50) value by 48-hr cytotoxicity assay) for 72 days. We analyzed the anti-oxidant properties (GSH content and gene expression levels of CAT, SOD1, SOD2, and SOD3 as well as the enzyme activities of CAT and SODs) and epigenetic regulation by DNA methytransferase-1 (DNMT1) gene expression. Cell proliferation and wound healing assays were performed to characterize the resistant cell line compared with their parental cells.

2. Materials and methods

2.1. Cell culture

The immortalized human cholangiocyte MMNK1 (also known as MMNK-1) cell line was supplied by Professor Naoya Kobayashi (Okayama University, Japan). MMNK1 was established from SV40T-transduced human liver OUMS-21 cells using serial transfection of simian virus 40 large T (SV40T) followed by human telomerase reverse transcriptase (hTERT) [25]. Cells were cultured in Hams F12 (Invitrogen, CA, USA) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin or 0.1 mg/mL kanamycin at 37 °C in 5% CO_2. The culture medium was changed every 48 h.

2.2. Induction of a cholangiocyte cell line exposed daily to low doses of hydrogen peroxide

MMNK1 cells were treated daily with 25 μM H_2O_2 for 15 passages. Briefly, MMNK1 cells (5×10^5 cells) were seeded overnight in a 100-mm^2 petri dish with 10 mL Ham F12 complete medium. After that, H_2O_2 was added to the culture dish to a final concentration of 25 μM. Every 24 h, a new aliquot of H_2O_2 was added and the culture medium was changed every 48 h until cells reached about 80–90% confluence; this entire phase was treated as the 1st passages. The cells were then harvested by trypsinization and seeded in a new culture dish for the 2nd passages of treatment with 25 μM H_2O_2 every 24 h. A total of 15 passages were performed over 72 days. The H_2O_2-resistant cells were named ox-MMNK1-L. The established cells were maintained by culture in Ham F12 complete medium without H_2O_2, and aliquots of living cells were stored in 10% DMSO containing fetal calf serum in liquid nitrogen.

2.3. Hydrogen peroxide cytotoxicity assay

Cytotoxicity in Ham F12 complete medium containing various H_2O_2 concentrations for 48 h was analyzed using a tetrazolium (MTT)-based assay. Briefly, suspensions of MMNK1 and ox-MMNK1-L cells in Ham F12 complete medium were seeded in 96-well plates at 1.5×10^3 cells per well. The culture medium was removed after 24-h incubation. Ham F12 complete medium containing various concentrations of H_2O_2 (0, 12.5, 25, 50, 100, 200, 300, 400 μM) was added and the cells were continuously cultured for 24 h. After that, the culture medium was removed and new complete medium containing various concentrations of H_2O_2 was added and the culture was continued for another 24 h. Finally, relative cell numbers (%) were analyzed by the standard MTT assay protocol. The IC50 of daily treatment with H_2O_2 at 48 h for each cell type was also calculated.

An additional 5×10^5 MMNK1 and ox-MMNK1-L cells suspended in Ham F12 complete medium were seeded in100-mm^2 culture dishes and incubated at 37 °C in 5% CO_2 overnight. After that, H_2O_2 was added to the culture dish at a final concentration of 100 μM. Every 24 h, a new aliquot of H_2O_2 was added and the culture medium was changed every 48 h. Cell growth and morphology were observed under an inverted microscope.

2.4. Collection of cell pellets

MMNK1 and ox-MMNK1-L cell lines were cultured in 10-mm^2 petridishes containing Ham F12 complete medium, the medium was changed every 48 h until 80–90% confluence was achieved, then cells were harvested by trypsinization. Cell pellets were washed twice with cold PBS and stored at −80 °C until used.

2.5. Measurement of CAT, SOD1, SOD2, SOD3, and DNMT1 mRNA expression levels by real-time PCR

CAT, SOD1, SOD2, SOD3, and DNMT1 mRNA expression levels were analyzed with the ABI Step One Plus Real-time PCR System (Applied Biosystems, Singapore) using TaqMan Probes (CAT,Hs00156308_m1; SOD1, Hs00916176_m1; SOD2, Hs01553554_m1; SOD3, Hs00162090_m1; DMNT1, Hs00945899_m1 and β-actin, Hs99999903_m1, Applied Biosystems, Foster City, CA, USA). Briefly, total RNA was isolated from MMNK1 and ox-MMNK1-L cell pellets with Trizol®reagent (Invitrogen, CA, USA) following the manufacturer's protocol. For the reverse transcription reaction, 1 μg total RNA was converted to