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Anti-apoptotic phenotypes of cholestan-3β,5α,6β-triol-resistant human cholangiocytes: Characteristics contributing to the genesis of cholangiocarcinoma

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ABSTRACT

The oxysterols cholestan-3β,5α,6β-triol (Triol) and 3-keto-cholest-4-ene (3K4) are increased in *Opisthorchis viverrini*-associated hamster cholangiocarcinoma and induce DNA damage and apoptosis via a mitochondria-dependent mechanism in MMNK-1 human cholangiocytes. Based on these observations, we hypothesized that chronic exposure of cholangiocytes to these pathogenic oxysterols may allow a growth advantage to a subset of these cells through selection for resistance to apoptosis, thereby contributing to cholangiocarcinogenesis. To test this hypothesis, we cultured MMNK-1 cells long-term in the presence of Triol. Alteration in survival and apoptotic factors of Triol-exposed cells were examined. Cells cultured long-term in the presence of Triol were resistant to H_2O_2-induced apoptosis, and demonstrated an increase in the phosphorylation of p38-α, CREB, ERK1/2 and c-Jun. Elevations in the ratio of Bcl-2/Bax and in the protein levels of anti-apoptotic factors including cIAP2, clusterin, and survivin were detected. These results show that long-term exposure of MNNK-1 cells to low doses of Triol selects for kinase-signaling molecules which regulate resistance to apoptosis and thereby enhance cell survival. Clonal expansion of such apoptosis-resistant cells may contribute to the genesis of cholangiocarcinoma.

1. Introduction

Oxysterols are oxygenated derivatives of cholesterol derived from enzymatic pathways mediated by CYP450 enzymes or from non-enzymatic mechanisms via reactive oxygen and/or nitrogen species (ROS/RNS) [1,2]. Numerous studies have demonstrated pathological effects of oxysterols such as 7-ketocholesterol, 24-hydroxycholesterol and Triol in a variety of diseases including cardiovascular diseases, neurological diseases and cancer [1–3]. Oxysterols also appear to play critical roles in multiple stages of carcinogenesis [3], such as in tumor initiation via enhancement of oxidative stress. Oxysterols increase intracellular ROS levels by inducing NADPH oxidase [4,5]. Persistent production of pro-oxidative molecules including ROS/RNS induced by oxysterols may perturb cellular stress responses [6,7] that can impair the DNA repair system and inhibit apoptosis [8,9]. Apoptosis induction by oxysterols may select a subset of cells that are resistant to apoptosis and thereby enhance carcinogenesis [10,11]. Infection with the liver fluke *Opisthorchis viverrini* (Ov) induces the generation of ROS/RNS in bile duct epithelia [12,13]. We have previously reported a possible role for oxysterols in the genesis of Ov-associated cholangiocarcinoma (CCA), a malignant tumor of bile duct epithelia [14]. Two oxysterols, cholestan-3β,5α,6β-triol (Triol) and 3-keto-cholest-4-ene (3K4), were increased in Ov-induced hamster CCA. Triol and 3K4 induced DNA damage and apoptosis in the human cholangiocyte MMNK-1 cell line [14]. Moreover, the expression of cytosolic oxysterol-binding proteins was increased in Ov-induced hamster CCA [15].

Mechanisms that explain the role of oxysterols in the initiation and progression of CCA remain undefined. Certain oxysterols can stabilize cyclooxygenase-2 (COX-2) mRNA via a p38

Abbreviations: CCA, cholangiocarcinoma; Triol, cholestan-3β,5α,6β-triol; 3K4, 3-keto-cholest-4-ene; p38-α, p38 mitogen-activated protein kinase α; CREB, cAMP response element-binding; ERK 1/2, extracellular signal-regulated kinase 1/2; cIAP2, cellular inhibitor of apoptosis 2.

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MAPK-dependent mechanism resulting in COX-2 protein accumulation in a CCA cell line [16]. Previous experiments, however, have examined the effects of high doses of oxysterols over short time courses, a situation that is diametrically opposed to that encountered in clinical Ov-induced CCA development where long term exposure to the liver fluke is present, and low concentrations of oxysterols are present in the biliary tract [14,17]. Indeed, culturing cholangiocytes with high concentration of oxysterols is expected to enhance their cytotoxicity. Interestingly, Gregorio-King and colleagues demonstrated that long-term culture of hematopoietic HL60 cells in the presence of low doses of the oxysterol 25-hydroxycholesterol (25-OHC) led to increased survival but it did not show resistance to apoptosis [18]. On the other hand, repeated exposure to oxysterols induced resistance to apoptosis in rat colon crypt cells and in a colon cell line [19,20]. Such resistance, was stable after at least 4 weeks growth in the absence of the oxysterol [20], and thus must have been due to somatically inherited changes in the cells, such as epigenetic or mutational alterations. Based on these observations, we hypothesized that chronic exposure of cholangiocytes to low doses of oxysterols may allow a growth advantage to a subset of these cells through selection for resistance to apoptosis, thereby contributing to cholangiocarcinogenesis. To test this hypothesis, we cultured MMNK-1 cells long-term in the presence of low doses of Triol, and investigated the molecular signaling pathways that have been associated with apoptosis resistance.

2. Materials and methods

2.1. Cell culture

The immortalized human cholangiocyte MMNK-1 cell line, transduced with SV40T and hTERT, was generated and supplied by Professor Naoya Kobayashi (Okayama University, Japan). Cells were cultured in Hams F12 (Invitrogen, CA, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

2.2. Establishment of a cholangiocyte cell line chronically exposed to low doses of Triol

MMNK-1 cells were cultured by step-wise exposure to cholestan-3β,5α,6β-triol (Triol) (Steraloids Inc., USA). In brief, MMNK-1 cells were cultured repeatedly in the presence of 15 μM Triol. When the viability of Triol-treated cells was similar to that observed in untreated control cells, the concentration of Triol in the culture media was increased to 18 μM and 20 μM consecutively. Cell viability was determined using sulforhodamine B as described below.

2.3. Cell viability assay

MMNK-1 cells (2×10^3/100 μl) were seeded into 96-well plates and incubated overnight at 37 °C and 5% CO_2. Cells were treated with Triol at designated concentrations for 48 h. Triol was dissolved in 100% ethanol and added to culture media at different concentrations; the final concentration of ethanol was 0.5%. The number of viable cells was determined using sulforhodamine B (SRB, Sigma–Aldrich, MO, USA) as described previously [21]. Briefly, cells were fixed with 10% cold trichloroacetic acid for 1 h at 4 °C and stained with 0.4% SRB in 1% acetic acid for 30 min. Excess dye was washed with 1% acetic acid and stained cells were solubilized with 200 μl of 10 mM unbuffered Tris-base. The absorbance was measured with a microplate reader (Sunrise, TECAN Trading, Switzerland) at 540 nm. The results were expressed as a percentage of cell viability relative to control (cells treated with 0.5% ethanol).

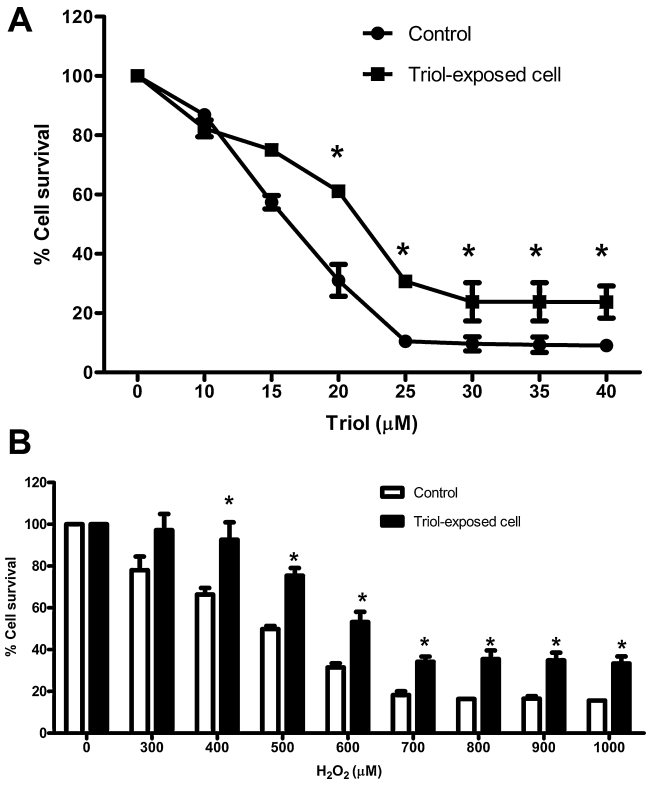

Fig. 1. Triol-exposed MMNK-1 cells are resistant to Triol and H_2O_2 treatment. (A) Both control and Triol-exposed cells were treated with Triol at designated concentrations for 48 h. After repeated exposure to Triol, cells were more resistant to Triol than control. (B) Control and Triol-exposed cells were treated with H_2O_2 at designated concentrations for 24 h. Increased numbers of viable cells were observed in Triol-exposed cells compared to control cells. Results are expressed as percent of control. Two individual experiments were performed. * indicates $P < 0.05$.

The concentration that inhibited cell growth to 50% of control (IC_{50}) was calculated by plotting the percentage of cell growth inhibition against oxysterol concentration.

2.4. Determination of apoptosis resistance

To determine resistance to apoptosis in MMNK-1 cells cultured in the presence of Triol, cells were treated with 300–1000 μM of hydrogen peroxide (H_2O_2), an apoptosis inducer [22], for 24 h. The number of viable cells was determined using sulforhodamine B as described above. Parental MMNK-1 cells were used as controls.

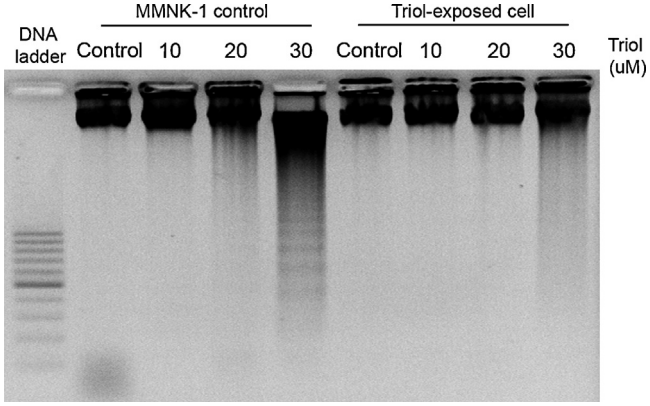

Fig. 2. Triol-exposed cells are resistant to apoptosis induction by Triol. Cells were treated with 10–30 μM Triol for 48 h. First lane was 100 bp DNA ladder. 0.5% ethanol-treated cells were used as control.