**Proliferation of Hepatic Oval Cells via Cyclooxygenase-2 and Extracellular Matrix Protein Signaling during Liver Regeneration Following 2-AAF/Partial Hepatectomy in Rats**

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**Background/Aims:** In the 2-acetylaminofluorene (2-AAF)/70% partial hepatectomy (PHx) model, the mechanism underlying the differentiation of activated hepatic oval cells (HOCs) into hepatocytes and bile ductile cells is unclear. We investigated the role of cyclooxygenase-2 (COX-2) in HOCs and the relationship between COX-2 and extracellular matrix proteins in cellular proliferation. **Methods:** Reverse transcription-polymerase chain reaction, immunohistochemical staining, and Western blotting were used to assess COX-2 expression. The co-localization of COX-2 with Thy1, c-Met, epithelial cell adhesion molecule, and α-smooth muscle actin was also examined. Additionally, we investigated whether connective tissue growth factor (CTGF), fibronectin (FN), extracellular signal-regulated kinase 1/2 (P-ERK1/2), and AKT were expressed in HOCs. **Results:** The expression of COX-2, prostaglandin E2 receptors, and c-Met was upregulated in HOCs. However, HOCs treated with the COX-2 inhibitor NS398 showed decreased COX-2, CTGF, FN, and AKT expression, whereas P-ERK1/2 was unaffected. Additionally, NS398 inhibited HOC proliferation, but not the proliferation of HOCs cultured on FN-coated dishes. Furthermore, the proliferative response of HOCs treated with NS398 was reversed by hepatic growth factor treatment. **Conclusions:** These results suggest that HOC proliferation is mediated through COX-2, extracellular FN expression, and AKT activation. Thus, COX-2 plays an important role in HOC proliferation following acute injury. *Gut Liver 2011;5:367-376*

**Key Words:** Hepatic oval cells; Cyclooxygenase-2; Liver generation

**INTRODUCTION**

The proliferative liver responds to traumatic, chemical, metabolic, infectious, and other injuries by activating a complex mixture of cytokines and chemokines from remnant tissue. In hepatic injury animal models, the administration of 2-acetylaminofluorene (2-AAF) before and during hepatic injury is associated with increased numbers of hepatic oval cells (HOCs) and increased differentiation to hepatocytes and bile ductular cells during liver regeneration, as the impaired hepatocyte response-to-growth signal suppresses hepatocyte proliferation. HOCs display distinct phenotypic markers, including CD34, c-kit, c-Met, alpha-fetoprotein (AFP), and Thy1 surface antigen. Among them, Thy1, which is expressed on bone marrow cells and stem cells in the fetal liver, plays an important role in hematopoiesis and hepatic development. HOCs are potential adult hepatic epithelial progenitors, but they do not express the previously reported hematopoietic stem cell markers CD34, c-kit, or Thy1.

The conversion of arachidonic acid to prostaglandins (PGs), which is the irreversible step in prostanoid biosynthesis, is mediated by two cyclooxygenases (COXs), COX-1 and COX-2, which are encoded by unique genes located on different chromosomes. Between them, COX-2, which was initially identified as an immediate early growth-response gene, is induced by a wide variety of stimuli such as cytokines, hormones, mito-
gens, and growth factors,16-20 and is frequently overexpressed in various tumor cells.21 The effects of COX-2 and PG-dependent signaling on HOC activation have been reported in liver cancer. The COX-2 inhibitor-induced reduction in HOC number may be due to the COX-2-dependent inhibition of Akt phosphorylation and the induction of apoptosis.22

During liver remodeling, including hepatic fibrosis and liver regeneration, several growth factors affect HOC proliferation and differentiation.13,23 Among them, hepatocyte growth factor (HGF) has a variety of activities in various cells, exerting mitogenic24 and morpho-organogenetic25 effects by activating c-Met, a tyrosine kinase receptor.26 When HGF binds the c-Met receptor, signaling molecules such as extracellular signal-regulated kinase (ERK)27 and AKT protein kinase28 are activated. Among them, the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is dedicated to a variety of system-related biological effects of HGF, including protection against apoptosis in primary hepatocytes.29

Hepatic stellate cells (HSCs) influence the growth and development of HOCs. HSCs in the peripoortal regions increase in response to liver injury30 and produce fibronectin (FN), a component of the extracellular matrix (ECM), which is crucial for liver regeneration. CTGF on an FN-concentrated provisional matrix facilitates HOC activation, and FN is critical for HOC proliferative functions.31 A positive correlation has been reported between FN concentration and COX-2 expression in human cancer cells.32-34 However, the precise mechanism linking FN and COX-2 expression during liver regeneration is unclear.

Herein, we explored COX-2 expression during HOC activation and liver regeneration following 2-AAF/70% partial hepatectomy (PHx), and the nature of the signals that modulate COX-2 expression. The co-localization of c-Met and EpCAM with COX-2 clearly demonstrated that COX-2 is expressed in HOCs. Our results show that an increasing level of COX-2 and c-Met expression in HOCs may occur through PG signaling during liver regeneration in the 2-AAF/PHx model. Moreover, FN was expressed in HOCs. The relationships between COX-2 and proliferation-related signals during liver regeneration were also investigated. The COX-2 inhibitor NS398 decreased HOC proliferation and significantly suppressed CTGF, FN, and AKT signaling, but did not affect phosphorylated ERK 1/2 (P-ERK 1/2). Interestingly, NS398 did not impair HOC proliferation in FN-coated dishes, indicating that FN induces HOC proliferation through COX-2 signaling. COX-2 expression increased when HOCs were challenged with a COX-2 inhibitor, followed by a change to an HGF-containing medium.

MATERIALS AND METHODS

1. Animals and experimental groups

Male Fischer rats weighing 120 to 150 g were divided into 3 equal groups (20 rats each). All animal experiments were conducted according to protocols approved by the Animal Care and Use Committee of the Catholic University of Korea. Time-released 2-AAF (35 mg/pellet over 14 days) treatment was achieved using a product supplied by Innovative Research (Sarasota, FL, USA). The pellets were inserted subcutaneously 7 days before PHx. Three rats from each group were killed, and their livers were collected. All time points are indicated as days after PHx treatment.

2. HOC culture

A Thy1-specific antibody in conjunction with magnetic-activated cell sorting (MACS) was used to isolate HOCs, with yields averaging 3×10⁶ cells per animal on day 9. Thy1⁺ cells were isolated by MACS, as described previously.3 HOCs were cultured in Iscove’s MDM solution containing 10% fetal bovine serum (FBS), 5 μg/ml insulin, 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Grand Island, NY, USA).

3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of COX-2 and prostaglandin E2 receptor mRNA expression

RT-PCR analysis was conducted on total RNA isolated from tissues from normal, PHx and 2-AAF/PHx livers, as well as isolated HOCs, using an RNeasy Kit (Qiagen, Valencia, CA, USA). Two micrograms of RNA were used for each round of cDNA synthesis. RT was performed using a GeneAmp RNA PCR Kit and a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA), which were also used for PCR. The primers used for COX-2, EP1, EP2, EP3, and EP4 were: 5’-AAG CCT CGG CCA GAT GCC AT-3’ forward and 5’-GTA GAT TGC ACT ACG AGC TAC-3’ reverse (COX-2, 340 bp), 5’-ACG TGG TGC ACT AGC AGC TAC-3’ forward and 5’-GCT GTG GTT GAA GTG ATG CTC GAT C-3’ reverse (EP1, 301 bp), 5’-GGG TCT CCT TGC TCT TCT GAT-3’ forward and 5’-CCG TCG GGA AGA GGT TTC ATC C-3’ reverse (EP2, 392 bp), 5’-GTA GTC CAG CCA GAT GAA GAC-3’ forward and 5’-GAT GTG CCC CAT AAG CTG GAT AGC-3’ reverse (EP3, 370 bp), and 5’-GAG TGT GGT GAA AGA CAT-3’ forward and 5’-GTC TCT GGG TGC TCC CAC TAA CCT -3’ reverse (EP4, 482 bp). Briefly, mRNA was reverse-transcribed and the cDNA was subjected to 30 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and an extension period of 72°C for 1 minute. The amplified products were subjected to 1% agarose gel electrophoresis and stained with ethidium bromide. The mRNA levels were normalized using GAPDH as a housekeeping gene.

4. Immunohistochemical staining

Double-immunofluorescence staining for Thy1.1 (BD Biosciences, San Jose, CA, USA), c-Met (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and EpCAM (Abcam, Cambridge, MA, USA) with COX-2 (Transduction Lab, Franklin Lakes, NJ, USA) was performed to verify the identity of the HOCs. Addition-