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. 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. In an animal model of HOC activation, Thy1+ HOCs express high levels of connective tissue growth factor (CTGF), which is critical for HOC activation during liver regeneration. HOCs that possess epithelial cell adhesion molecule (EpCAM), 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,
gens, and growth factors, and is frequently overexpressed in various tumor cells. 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.

During liver remodeling, including hepatic fibrosis and liver regeneration, several growth factors affect HOC proliferation and differentiation. Among them, hepatocyte growth factor (HGF) has a variety of activities in various cells, exerting mitogenic and morpho-organogenic effects by activating c-Met, a tyrosine kinase receptor. When HGF binds the c-Met receptor, signaling molecules such as extracellular signal-regulated kinase (ERK) and AKT protein kinase 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.

Hepatic stellate cells (HSCs) influence the growth and development of HOCs. HSCs in the periportal regions increase in response to liver injury 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. A positive correlation has been reported between FN concentration and COX-2 expression in human cancer cells. 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 FG 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. HOCs were cultured in I ccove'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 of normal, PHx and 2-AAF/PHx livers, as well as isolated HOCs, using an RNeasy Kit (Qiagen, Valencia, CA, USA), which were also used for PCR. The primers used for COX-2, EP1, EP2, EP3, and EP4 were: 5'-AAC CCT CGG CCA GAT GCC AT-3' forward and 5'-GTA GTA CTG TGG GAT TGA TAT C-3' reverse (COX-2, 340 bp), 5'-ACG TGG GTC ACT ACG AGC TAC-3' forward and 5'-GCT GTG GTC ATG GAA TGAT C-3' reverse (EP2, 392 bp), 5'-GGG TCT CCT TGC TCT TCT GTT-3' forward and 5'-CTT CGG GGA AGA GAG GTC TTC ATC TAC C-3' reverse (EP3, 370 bp), and 5'-GCC AAG TGT GGT GAA GAC CAT-3' forward and 5'-CCT TCT GGC TCC 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 3% 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-
ally, immunofluorescence staining of α-smooth muscle actin (SMA) (Abcam) with COX-2 was performed, using a previously described cytochemical method, to exclude myofibroblasts as the COX-2 source. Immunostaining for OV-6 (R & D Systems, Minneapolis, MN, USA), COX-2, Thy1.1, albumin (DakoCytomation, Carpinteria, CA, USA), AFP (DakoCytomation), FN, and CD45 (BD Biosciences) was performed on isolated Thy1+ HOCs. Signals were detected using a Vector ABC Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3’-diaminobenzidine tetrahydrochloride (DakoCytomation). Finally, BrdU (DakoCytomation) staining was conducted for the HOC proliferation assay on FN-coated culture dishes following NS398 treatment, as described.

**Fig. 1.** Proliferation of hepatic oval cells (HOCs) on day 9 after induction of the 2-acetylaminofluorene (2-AAF)/70% partial hepatectomy (PHx) model. Immunohistochemical (IHC) staining is conducted to detect OV-6, thymus cell antigen1 (Thy1), and cyclooxygenase (COX)-2. (A) Hema-toxylin and eosin staining (magnification, ×400). (B) OV-6 IHC staining in activated HOCs. OV-6 is a hepatic oval cell marker (magnification, ×400). (C, E) Thy1 and (D, F) COX-2 IHC staining in activated HOCs (magnification, ×100 and ×400, respectively).
previously. Cells (4.5×10⁴) in 6-well culture dishes were inoculated on FN-coated or uncoated dishes with 10% FBS supplemented with Iscove’s MDM for 24 hours, after which a medium containing 100 μM NS398 was used in some cases. After 1 hour of incubation, 100 μM BrdU cell-labeling reagent was added. The cells were fixed after 24 hours, and the percentage of BrdU-labeled nuclei was determined by counting >100 nuclei in three random fields (×400 magnification).

5. Western blot analysis

Enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Life Science (Arlington Heights, IL, USA), and a monoclonal mouse anti-COX-2 antibody was purchased from PharMingen (San Diego, CA, USA). Polyclonal rabbit anti-c-Met, -CTGF, -FN, -AKT, -p-AKT, -ERK 1/2, -p-ERK 1/2, and -β-actin antibodies were purchased from Santa Cruz Biotechnology. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6. Regulation of COX-2 expression by NS398 and HGF

HOCs were selected to evaluate the inhibitory growth effect of the COX-2 inhibitor NS398 (Cayman Chemicals, Ann Arbor, MI, USA). COX-2 expression was inhibited in HOC cultures treated with 100 μM NS398, as described previously. Cells (3×10⁴) were grown in Iscove’s MDM supplemented with 10% FBS, 100 μg/ml penicillin, 20 μM HGF, and 0.25 μg/ml streptomycin, and then stimulated with 20 μM HGF for various time periods.

7. Statistical analysis

All experiments were repeated a minimum of three times. All RT-PCR, BrdU staining, and Western blot data are expressed as the mean±SD. The data in some figures are from a representative experiment, which was qualitatively similar to replicates. Statistical significance was determined by comparing the two groups using a two-tailed Student’s t-test. Asterisks in the figures indicate significant differences (p≤0.05) between experimental groups and the corresponding control.

RESULTS

1. COX-2 gene and protein expression in activated HOCs

HOCs appeared as dark blue areas in hematoxylin and eosin-stained liver sections, due to their large nuclei and small amount of cytoplasm, with numerous HOCs in the portal region on day 9 after 2-AAF/PHx treatment (Fig. 1A). HOCs were confirmed by labeling with OV-6 (Fig. 1B). An additional immunomarker widely utilized in HOC labeling, Thy1.1, was also used (Fig. 1C and E). These results confirm that the 2-AAF/PHx model can be used effectively to activate HOCs in periportal regions during liver regeneration, consistent with previous observations. Additionally, COX-2-expressing cells were evident around the periportal region (Fig. 1D and F), and COX-2 staining in the HOCs was detected in the cytoplasm (Fig. 1F, 2D, 2G, 2J, and 2M). Double-immunofluorescence staining was performed on serial liver sections to verify COX-2 expression in the HOCs and to assess whether the HOCs co-expressed Thy1, c-Met, EpCAM, and α-SMA with COX-2 (Fig. 2). Fig. 2F and I show that in some HOCs COX-2 (Fig. 2D and G) was colocalized with the HOC markers Thy1 (Fig. 2E) and c-Met (Fig. 2H), whereas the co-localization of COX-2 (Fig. 2J) and EpCAM (Fig. 2K) did not occur in the peri-bile ductile or hepatic lobule area (Fig. 2L). Moreover, the co-localization of COX-2 (Fig. 2M) with the myofibroblast marker α-SMA (Fig. 2N) was not observed (Fig. 2O). These results indicate that Thy1+ HOCs expressed COX-2 and c-Met but not EpCAM or α-SMA.

The next experiment assessed COX-2 expression in total RNA extracted from normal liver, 2AAF/PHx-treated liver (on day 9), and PHx-treated liver (after 12 hours), and sorted Thy1+ HOCs by RT-PCR (Fig. 3A). COX-2 expression was not detected in normal liver, but was detected in the activated HOC model (2AAF/PHx), the liver regeneration model (PHx), and the sorted Thy1+ HOCs, suggesting that COX-2 participates in HOC activation during liver regeneration. Furthermore, COX-2 and c-Met (HGF receptor) expression was examined in the activated HOC model by Western blotting. Normal liver did not induce COX-2 expression, but HOC induction resulted in a progressive increase in COX-2 expression on days 3-13 after 2-AAF/PHx (Fig. 3B). The detection of COX-2 on day 3 after 2-AAF/PHx suggests that the COX-2-induced PG pathway plays a role in HOC activation. Our immunofluorescence staining results revealed that the HOCs expressed COX-2 and the HOC markers (albumin and AFP), but that they did not express CD45. Interestingly, FN was also evident in the HOCs, indicating that it might play a role in HOC activation or proliferation in the 2AAF/PHx liver model (Fig. 3C).

2. Detection of PG receptors in HOCs

The finding that COX-2 was expressed in HOCs indicated that a COX-2-mediated PG pathway might be involved in the HOC proliferation mediated by PG receptors. Indeed, PGE2 binds its receptors, designated EP1, EP2, EP3, and EP4, which have been implicated in carcinoma cell proliferation. RT-PCR was performed for each EP receptor subtype to determine their expression profiles in HOCs (Fig. 4). Tissues from normal rat liver and rat liver after PHx, as well as sorted Thy1+ HOCs expressed all four PG receptors (though faint EP3 expression was noted in the Thy1+ HOCs), indicating the existence of these receptors in Thy1+ HOCs and their possible role in proliferation.

3. Role of COX-2 and ECM in activated HOCs

To explore the downstream events in the PG pathway, HOCs were cultured with 100 μM NS398, which suppressed HOC proliferation. CTGF is a downstream mediator of the fibrogenic properties of transforming growth factor (TGF)-β1, which
stimulates the expression of ECM genes in many biological processes. Western blotting of HOCs cultured with NS398 showed increased levels of the ECM proteins CTGF, FN, and COX-2 (Fig. 5). Moreover, AKT phosphorylation was inhibited by COX-2 signaling, whereas ERK 1/2 phosphorylation was not. These results are consistent with the expression of CTGF, FN, and phosphoryl-}

serine-specific AKT in activated HOCs via the PG pathway, and they indicate that ERK functions independently of PG signaling. FN is an ECM molecule that induces cell proliferation by inducing COX-2 signaling and subsequent PGE2 production. Furthermore, we examined the HOC proliferation rate in FN-coated cultured dishes using BrdU staining. When HOCs were cultured on non-FN-coated dishes as a control and FN-coated dishes in the absence of NS398, dramatic proliferation was evident in the FN-coated dishes (Fig. 6A). However, the proliferation on non-FN-coated dishes was suppressed significantly when HOCs were treated with NS398. Interestingly, HOC proliferation was not inhibited significantly on FN-coated dishes, despite NS398 treatment (Fig. 6B). These observations are evidence of a positive interaction between HOCs and FN, which is critical for HOC proliferation.

COX-2 expression increased in a time-dependent manner following a change to HGF-containing medium after NS398 exposure for 24 hours (Fig. 7), suggesting a role for HGF in increased COX-2 expression during HOC proliferation.

**DISCUSSION**

We demonstrated that COX-2 is expressed and that ECM proteins were produced in activated HOCs through COX-2-induced
PG signaling during liver regeneration. The relationship between COX-2 and HOC proliferation-related signals involved two ECM proteins (CTGF and FN) and AKT activity. The COX-2 inhibitor NS398 decreased HOC proliferation and significantly suppressed CTGF and FN expression and AKT activation, but not ERK 1/2 activation. Moreover, the observation that NS398 did not affect HOC proliferation on FN-coated dishes indicates that the FN-induced proliferation of HOCs occurs through COX-2-induced PG signaling. The observation that HOCs treated with NS398 were shifted to an HGF-containing medium for HOC regulation is entirely consistent with a role for HGF in HOC proliferation.

In the liver regeneration model, HOCs participate in liver regeneration by activating a complex mixture of compounds, including cytokines and growth factors. Several growth factors (e.g., HGF and epidermal growth factor) and intracellular signaling pathways (e.g., ERK and Akt) are important regulators of HOC-mediated liver regeneration.
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Population of ECM proteins is important for HOC proliferation during liver regeneration induced by 2-AAF/PHx. However, the underlying mechanism linking the COX-2-induced PG signaling pathway with HOC-mediated liver regeneration is unclear.

We determined that COX-2 expression occurred in the HOC activation model, in which 2AAF pellets were implanted into animals to inhibit hepatocyte proliferation. Normal liver tissue did not express COX-2, and COX-2 and EP1-4 were expressed and upregulated in liver tissue obtained from the 2AAF/PHx model and from isolated HOCs.

EpCAM is a cell surface marker for HOCs. Immunofluorescence microscopy showed that COX-2-expressing cells were stained with the HOC markers albumin (albeit weakly), AFP, FN, and c-Met, but not with EpCAM or α-SMA. Therefore, the sorted Thy1+ cells were HOCs, contrary to the opinion that EpCAM+ cells are representative of the HOC population.

An increase in c-Met and COX-2 expression was observed over the course of regeneration, suggesting that the PG signaling pathway plays a role in HOC activation and proliferation. Previous studies reported that selective COX-2 inhibitors reduced cancer growth in carcinogen-treated rodents, inhibited various cancer cell lines, and delayed wound healing by reducing inflammation-induced epithelial cell proliferation. We found that NS398 suppressed HOC proliferation and decreased CTGF, FN, and AKT expression during liver regeneration. In contrast, the proliferation of HOCs cultured on FN-coated dishes was not inhibited by COX-2 inhibitor treatment. These results indicate that COX-2 and ECM proteins might be involved in HOC activation through PG-dependent and independent signaling pathways.

The physical condition of the normal subendothelial ECM is essential for maintaining different functions in all liver cells. We found that NS398 decreased COX-2, CTGF, FN, and AKT expression, but not ERK expression, and that it suppressed HOC proliferation. However, NS398-mediated inhibition was obliterated by the presence of FN on the culture dishes. These results indicate that COX-2 and ECM proteins might be involved in HOC activation through PG-dependent and independent signaling pathways.

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broad spectrum of biological activities, HGF is most likely the physiological hepatotrophic factor that triggers or modulates liver regeneration. HGF induces HOC proliferation in a 2-AAF/PHx rat model, and PGs induce HGF expression in skin fibroblasts. In contrast, the inhibition of PG synthesis by dexamethasone reduces HGF production in these cells. In the present study, c-Met and COX-2 protein levels increased during liver regeneration in the rat 2-AAF/PHx model, and COX-2 expression decreased gradually following NS398 treatment in a time-dependent manner, suggesting that the effects of NS398 on HOC proliferation are mediated by a COX-2-dependent signaling pathway (data not shown). Our findings are consistent with those of a previous report, although a reduction in the number of oval cells is associated with a reduced incidence of liver cancer in mice. In the context of human chronic liver disease, these results are of considerable relevance, as liver regeneration is paralleled by changes in the number of hepatic progenitor cells, which can differentiate into hepatocytes and bile ductular cells.

COX-2 expression in HOCs was upregulated following exposure to an HGF-containing medium after NS398 treatment. These results suggest that HGF has a proliferative effect on HOCs, and that COX-2 mediates the effects of HGF on HOC proliferation due to binding of the growth factor to the c-Met receptor on the HOC surface. Thus, COX-2 may be used with ECM and HGF to investigate the regulation of HOC proliferation during liver regeneration.

There are some limitations to this study. First, the identification of the PGE2 receptors was preliminary; additional functional assays are needed. Second, a clear organized analysis of the signal transduction mechanisms involved in HOC proliferation, including total and phosphorylated ERK and AKT, is required.

In conclusion, we showed that COX-2 is expressed in activated HOCs, which proliferate through COX-2, extracellular FN, and AKT signaling, during liver regeneration after acute liver injury. HGF also stimulated HOC proliferation by increasing COX-2 expression. Therefore, COX-2 plays an important role in HOC proliferation after liver injury.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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