**Gut Microbiota Promotes Obesity-Associated Liver Cancer through PGE2-Mediated Suppression of Antitumor Immunity**

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**ABSTRACT**

Obesity increases the risk of cancers, including hepatocellular carcinomas (HCC). However, the precise molecular mechanisms through which obesity promotes HCC development are still unclear. Recent studies have shown that gut microbiota may influence liver diseases by transferring its metabolites and components. Here, we show that the hepatic translocation of obesity-induced lipoteichoic acid (LTA), a Gram-positive gut microbial component, promotes HCC development by creating a tumor-promoting microenvironment. LTA enhances the senescence-associated secretory phenotype (SASP) of hepatic stellate cells (HSC) collaboratively with an obesity-induced gut microbial metabolite, deoxycholic acid, to upregulate the expression of SASP factors and COX2 through Toll-like receptor 2. Interestingly, COX2-mediated prostaglandin E2 (PGE2) production suppresses the antitumor immunity through a PTGER4 receptor, thereby contributing to HCC progression. Moreover, COX2 overexpression and excess PGE2 production were detected in HSCs in human HCCs with noncirrhotic, nonalcoholic steatohepatitis (NASH), indicating that a similar mechanism could function in humans.

**SIGNIFICANCE:** We showed the importance of the gut–liver axis in obesity-associated HCC. The gut microbiota–driven COX2 pathway produced the lipid mediator PGE2 in senescent HSCs in the tumor microenvironment, which plays a pivotal role in suppressing antitumor immunity, suggesting that PGE2 and its receptor may be novel therapeutic targets for noncirrhotic NASH-associated HCC.

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INTRODUCTION

Obesity has become a worldwide health problem and is known to increase the risk of diabetes, cardiovascular disease, and several types of cancer (1). Although hypernutrition-related systemic alterations are thought to be involved in cancer development (2–4), the molecular mechanisms that integrate these events still remain largely unclear. Among obesity-associated cancers, liver cancer has been shown to have a strong relationship with obesity, based on epidemiological studies (1). The most common risk factor for hepatocellular carcinoma (HCC) is long-term infection by hepatitis B virus (HBV) or hepatitis C virus (HCV; ref. 5). However, obesity-associated nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) have recently emerged as important risk factors for liver cancer (6). Therefore, elucidation of the precise molecular mechanisms mediating the development of obesity-induced NASH-associated HCC is urgently needed.

We previously reported that increased enterohepatic circulation of the obesity-induced Gram-positive gut microbial metabolite deoxycholic acid (DCA) facilitates HCC development by inducing cellular senescence and the senescence-associated secretory phenotype (SASP) in hepatic stellate cells (HSC) in the tumor microenvironment. This recently identified phenotype of senescent cells involves secretion of a series of inflammatory cytokines, chemokines, matrix-remodeling factors, and growth factors (7), suggesting that the gut–liver axis plays a pivotal role in inducing cellular senescence of HSCs and liver tumorigenesis (4). The importance of the SASP phenotype has also been recognized in vivo in a variety of pathophysiologic contexts, giving rise to not only deleterious effects such as chronic inflammation and tumorigenesis (4, 8, 9), but also beneficial effects such as embryonal development (10, 11), immunosurveillance (12–14), and tissue repair (15, 16), depending on the biological context. Indeed, the paracrine effects of SASP have been reported to be dependent on the surrounding cells (17), particularly on the p53 status of these cells (18). Accumulating evidence has indicated that SASP is regulated by a combination of several transcription factors, epigenetic regulators, and metabolic pathways, in response to DNA damage (19–23). DCA can create DNA
damage by elevating the levels of reactive oxygen species (ROS), thereby inducing cellular senescence and the SASP to promote obesity-associated HCC (4). However, the trigger that initiates the SASP signals may vary depending on physiologic status, and thus the precise mechanisms regulating the expression of SASP factors need to be elucidated in each pathophysiologic setting (24).

One of the mechanisms that may trigger the SASP could be Toll-like receptor (TLR) signaling. High mobility box group 1 (HMGBox1) activates TLR4-mediated cytokine signals in an autocrine manner in senescent cells (25). In addition, previous in vivo studies have demonstrated that TLR4-mediated inflammatory signals induced by the Gram-negative bacterial component lipopolysaccharide (LPS) are important for promoting liver fibrosis and fibrosis-associated liver tumorogenesis (26). However, in our obesity-associated HCC model, Gram-positive bacteria, but not Gram-negative bacteria, were found to be dramatically increased in obese mice fed a high-fat diet (HFD; ref. 4), consistent with previous reports (27). Moreover, we did not observe the reduction of obesity-associated liver tumor formation in TLR4-deficient mice as compared with those in wild-type mice when we performed the same protocol of neonatal 7,12-dimethylbenz(a)anthracene (DMBA) treatment with HFD (4), suggesting that TLR4-mediated signals are unlikely to be involved in the acceleration of obesity-associated HCC in our experimental setting.

In this study, we investigated the effects of the signals by lipoteichoic acid (LTA), a cell wall component from Gram-positive bacteria, on the induction of the SASP factors in DCA-induced senescent HSCs and on obesity-associated liver tumor development. We newly identified that a gut microbiota–driven COX2 pathway generated the lipid mediator prostaglandin E2 (PGE2), which functioned as a key SASP factor in the tumor microenvironment. Our current study provides important mechanistic insights into the relevance of the gut–liver axis in obesity-associated liver carcinogenesis.

**RESULTS**

**A Normal Diet Delays Liver Carcinogenesis Compared with an HFD**

We previously reported that neonatal DMBA treatment in HFD-fed mice resulted in development of HCC within 30 weeks, and demonstrated that DCA, an obesity-associated gut microbial metabolite, is a critical factor promoting obesity-associated HCC development (4). To further define the role of DCA itself, DMBA-treated mice were fed DCA with a normal diet (ND; Fig. 1A). DCA administration in ND-fed mice did not induce HCC development at 30 weeks, at which point HFD-fed mice exhibited HCC with sufficient body-weight gain (Fig. 1B–D). Senescence markers, such as upregulation of p21 and the DNA damage response marker 53BP1, were detected in HSCs in the livers of mice fed DCA and an ND, reaching levels similar to those in the livers of HFD-fed mice at 30 weeks (Fig. 1E). However, at this time point, the frequency of SASP induction in HSCs was much lower in mice fed DCA and an ND as compared with that in mice fed an HFD (Fig. 1E, IL1β, Groα, and IL6). We found that mice fed DCA and an ND required approximately six months for complete SASP induction and HCC development at the 55-week time point (Fig. 1B–E). Therefore, we speculated that other factors underlying the HFD-fed obese liver could be necessary to accelerate the effects of DCA in aggressively promoting HCC development. Accordingly, we searched for factors that promote obesity-associated HCC progression, based on (i) the obesity-induced gut microbial component(s) and (ii) the increased lipid storage in the liver tumor areas of HFD-fed obese mice.

**LTA- and TLR2-Mediated Signaling Promotes Obesity-Associated HCC Development**

When we reanalyzed the gut microbial profiles of DMBA-treated mice, we again noted that HFD-fed mice exhibited a dramatic increase in Gram-positive gut microbiota in their feces (Supplementary Fig. S1; refs. 4, 27). The gut microbial overgrowth and increased hepatic translocation of the gut microbial components and metabolites from the intestine, the so-called gut–liver axis, are known to play a role in the pathogenesis of liver disorders (26, 28). We therefore examined whether LTA, a major cell wall component in Gram-positive bacteria, accumulated in the livers of DMBA-treated and HFD-fed mice. We found relatively high amounts of LTA accumulation in the areas of the liver tumors (Fig. 2A). Because LTA is a major ligand of the innate immunity receptor TLR2, we next investigated the involvement of TLR2-mediated signaling in liver tumor development. Interestingly, both the numbers and sizes of HFD-induced liver tumors were strikingly reduced in TLR2-deficient mice, although body weights were not different between the two groups (Fig. 2B–E). Accordingly, in livers from TLR2-deficient mice, the expression of SASP factors, such as IL1β, Groα, and IL6, was significantly reduced in HSCs, suggesting that TLR2-mediated signaling induced the expression of SASP factors (Fig. 2F). Notably, however, the presence of p21 and 53BP1 foci was still observed in activated HSCs from TLR2-deficient mice (Fig. 2F). The majority of senescent signals are detected in the HSCs but not in HCC cells in the tumor region (Supplementary Fig. S2). Taken together, these results suggest that TLR2-mediated signaling is important for the upregulation of SASP factors in senescent HSCs but not for the induction of senescent cell-cycle arrest.

**Figure 1.** DCA administration with ND induced a long delay in liver carcinogenesis as compared with HFD. A, Timeline of the experimental procedure (ND, n = 3; ND + DCA 30 weeks, n = 3; ND + DCA 55 weeks, n = 5; HFD, n = 15). Eut, euthanasia. B, The average body weight of each group at the indicated age. Data, mean ± SD. ***, P < 0.01. C, Average liver tumor numbers and the relative size distribution (classified as <6 mm, 2 mm–6 mm, ≤2 mm). **, P < 0.01. D, Macroscopic photograph of livers of male wild-type mice kept with ND (left), with ND and DCA 30 weeks (the second from left), with ND and DCA 55 weeks (the second from right), with HFD (right) for 30 weeks after the administration of DMBA. The arrowheads indicate HCCs. E, Immunofluorescence analysis of liver section. HSCs were visualized by smooth muscle actin staining (α-SMA; green) and the cell nuclei were stained by 4,6-diamidino-2-phenylindole (DAPI; blue). Arrowheads indicate α-SMA expressing cells that were positive for indicated markers (red). The histograms indicate the percentages of α-SMA expressing cells that were positive for indicated markers. Data of three to four individual mice in each group are represented as means ± SD. More than 100 cells in total were counted for statistical analysis. Data, means ± SD. *, P < 0.05; **, P < 0.01.
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**A**

ND or HFD ±DCA

1x DMBA

Eut Eut

0 8 30 55

Weeks

**B**

Body weight (g)

| (30 w) | (30 w) |
| D-ND | D-HFD |

**C**

Average tumor number

| (30 w) | (30 w) |
| D-ND | D-HFD |

>6 mm 2–6 mm ≤2 mm

**D**

D-ND

D-HFD

DCA– (30 w) DCA+ (30 w) DCA+ (55 w)

Eut

Eut

1 cm 1 cm 1 cm

**E**

DCA– (30 w) Nontumor

DCA– (30 w) Tumor

DCA+ (55 w) Tumor

ND

HFD

α-SMA

p21

DAPI

53BP1

DAPI

IL1β

DAPI

Groα

DAPI

IL6

DAPI

Body weight (g)

Average tumor number

α-SMA p21

53BP1

IL1β

Groα

IL6

**Statistical Significance**

* p < 0.05

** p < 0.01

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Figure 2. LTA and TLR2-mediated signaling promote obesity-associated HCC development. A, Immunohistochemical staining of LTA (red), α-SMA (green), and DAPI (blue) in 30-week-old male mouse liver sections. Scale bars, 50 μm. Data, means ± SD. **, P < 0.01. B, Timeline of the experimental procedure [wild-type (WT) ND, n = 23; WT HFD, n = 15; Tlr2−/−, n = 12]. Eut, euthanasia; D, DMBA-treated. C, The average body weight of each group at the age of 30 weeks. Data, means ± SD. **, P < 0.01. D, DMBA-treated. D, The average liver tumor numbers and the relative size distribution (classified as >6 mm, 2–6 mm, ≤2 mm). **, P < 0.01. E, Representative macroscopic photographs of liver. Arrowheads indicate HCCs. F, Immunofluorescence analysis of liver sections. HSCs were visualized by α-smooth muscle actin staining (α-SMA; green) and the cell nuclei were stained by DAPI. Arrowheads indicate α-SMA expressing cells that were positive for indicated markers (red). The histograms indicate the percentages of α-SMA expressing cells that were positive for indicated markers. Scale bars, 25 μm. Data of three to four individual mice in each group are represented as means ± SD. More than 100 cells in total were counted for statistical analysis. Data, means ± SD. NS, not significant; **, P < 0.01.
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In particular, TXB2, PGD2, PGE2, and PGF2α, important for promoting obesity-associated HCC. Although these prostaglandins (PG), COX-mediated metabolites of arachidonic acid, were detected in liver tumor tissues as compared with the levels in nontumor areas (Supplementary Fig. S7A). In particular, TXB2, PGD2, PGE2, and PGF2α were abundant in the tumor region (Supplementary Fig. S7A). Interestingly, the levels of PGs in livers from vancomycin-treated, HFD-fed mice, where Gram-positive gut microbiota (phylum Firmicutes) are primarily targeted (Supplementary Fig. S7B), were significantly lower than those in livers from untreated mice (Supplementary Fig. S7A). These results suggest that PGs in the tumor region are produced through a pathway where Gram-positive bacteria are involved.

We found that excess production of PGE2 was detected in α-SMA-positive HSCs and that the frequency of PGE2-producing HSCs was significantly decreased in the livers from TLR2-deficient mice or vancomycin-treated mice with decreased accumulation of LTA in the liver (Fig. 4A). COXs are rate-limiting enzymes involved in PG biosynthesis. The mRNA level of inducible Cox2, but not that of constitutive Cox1, was highly induced in the liver tumor tissues of wild-type mice (Fig. 4B). However, COX2 expression was significantly reduced in livers of TLR2-deficient or vancomycin-treated mice (Fig. 4A and B). Moreover, the level of PG inactivating enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) was significantly reduced in the liver tumor region, further facilitating the elevation of PG levels in the obesity-associated liver tumor region (Supplementary Fig. S7C and S7D). We found that COX2 was detected predominantly in the activated HSCs in the liver tumor region (Fig. 4A; Supplementary Fig. S7E), but at lower levels in CD45+ or F4/80+ immune cells (Supplementary Fig. S7E). The expression of COX2, but not COX1, in DCA-treated primary HSCs was significantly induced by increasing amounts of LTA (Fig. 4C), and the COX2 expression was dependent on TLR2 (Supplementary Fig. S3). Indeed, we confirmed that TLR2 was expressed in HSCs and this expression was enhanced by LTA alone or DCA plus LTA treatment (Supplementary Fig. S8A). On the other hand, hepatocytes did not express TLR2 (Supplementary Fig. S8B). Notably, phosphorylation of the inhibitor of κB (IκB), an indicator of the activation of NF-κB, was detected in HSCs in the liver tumor tissue from HFD-fed wild-type mice but not in HSCs from TLR2-deficient or vancomycin-treated mice (Fig. 4A). These results suggest that LTA may activate NF-κB through TLR2, and could induce the expression of SASP factors as well as COX2 to promote local production of PGs in DCA-induced senescent HSCs, thereby promoting HCC development in HFD-fed mice.

**PGE2 Suppresses Antitumor Immunity of the Host to Promote the HFD-Induced HCC Development**

We next focused on determining which PG was most important for promoting obesity-associated HCC. Although PGs including PGE2 have been shown to directly activate growth signals in epithelial cells of the gastrointestinal tract (33–35), we did not observe activation by PGs in hepatocytes (data not shown). On the other hand, because PGs have been reported to affect immune cells (36), we examined the effects of PGs on immune cells in the tumor microenvironment. We treated the immune cells isolated from liver tissues (Supplementary Fig. S8C) with PGE2 in the presence of LTA for 24 hours and measured cytokine production using a bead-based flow cytometric multiplex assay (Fig. 5A; Supplementary Fig. S8D and S8E). The production of IFNγ and TNFα, which are known to activate antitumor immune responses and inhibit cancer cell growth, was significantly and specifically
Figure 3. DCA and LTA cooperatively induce the expression of TLR2 and SASP factors in HSCs. A, Schematic representation of the experimental design. Murine primary HSCs were treated with medium containing DCA for 8 days to induce cellular senescence. Senescent HSCs were then incubated with LTA for 6 hours until collected.

B, Murine primary HSCs were treated with medium containing DCA for 8 days to induce cellular senescence with following doses: lane 1 (0 mmol/L), lane 2 (100 mmol/L), lane 3 (200 mmol/L), and lane 4 (300 mmol/L). mRNA and cDNA were prepared from each sample and were subjected to RT-qPCR analysis for $\text{Cdkn1a}$ (encoding p21) and $\text{Cdkn2a}$ (encoding p16) gene expression. Data, means $\pm$ SD (n = 3). *, $P < 0.05$; **, $P < 0.01$.

C, LTA was added to senescent murine primary HSCs as following doses: lane 1 (DCA, 0 mmol/L; LTA, 0 mg/mL), lane 2 (DCA, 0 mmol/L; LTA, 10 mg/mL), lane 3 (DCA, 0 mmol/L; LTA, 25 mg/mL), lane 4 (DCA, 300 mmol/L; LTA, 0 mg/mL), lane 5 (DCA, 300 mmol/L; LTA, 10 mg/mL), and lane 6 (DCA, 300 mmol/L; LTA, 25 mg/mL). mRNA and cDNA were prepared from each sample and were subjected to RT-qPCR analysis for $\text{Tlr2}$, $\text{Il6}$, $\text{Gro}\alpha$, or $\text{Il1}\beta$ gene expression. Data, means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$.

D, Immunofluorescence analysis of serial liver sections. HSCs were stained by $\alpha$-SMA and cell nuclei were stained by DAPI. Arrowheads indicate $\alpha$-SMA expressing cells that were positive for indicated markers. The histograms indicate the percentages of $\alpha$-SMA expressing cells that were positive for indicated markers. Scale bars, 25 $\mu$m. Data of three to four individual mice in each group are represented as means $\pm$ SD. More than 100 cells in total were counted for statistical analysis. Data, means $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$. 

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Figure 4. Prostaglandins, cyclooxygenase-mediated metabolites of arachidonic acid, are overproduced in the HFD-induced liver cancer. A, Immunofluorescence analysis of serial liver sections. HSCs were visualized by α-smooth muscle actin staining (α-SMA; green) and the cell nuclei were stained by DAPI. Arrowheads indicate α-SMA expressing cells that were positive for indicated markers (red). The histograms indicate the percentages of α-SMA-expressing cells that were positive for indicated markers. Scale bars, 25 μm. Number of mice analyzed, wild-type (WT), n = 15; Tlr2−/−, n = 12; vancomycin (Vcm)-treated, n = 9. Data of three to four individual mice in each group are represented as means ± SD. More than 100 cells in total were counted for statistical analysis. Data, means ± SD. NS, not significant. *, P < 0.05; **, P < 0.01. D, DMBA-treated.

B, The relative levels of Cox1 or Cox2 mRNA in non-tumor or tumor region of mice developed obesity-associated HCC analyzed by RT-qPCR. Representative results of three individual male mice are shown. Data of three individual mice are represented as means ± SD (n = 3). *, P < 0.05.

C, LTA was added to senescent murine primary HSCs with following doses: lane 1 (DCA, 0 mmol/L; LTA, 0 mg/mL), lane 2 (DCA, 0 mmol/L; LTA, 10 mg/mL), lane 3 (DCA, 0 mmol/L; LTA, 25 mg/mL), lane 4 (DCA, 300 mmol/L; LTA, 0 mg/mL), lane 5 (DCA, 300 mmol/L; LTA, 10 mg/mL), and lane 6 (DCA, 300 mmol/L; LTA, 25 mg/mL). mRNA and cDNA were prepared from each sample and were subjected to RT-qPCR analysis for Cox1 or Cox2 gene expression. Data, means ± SD (n = 3). NS, not significant; *, P < 0.05; **, P < 0.01.
Figure 5. Prostaglandin E₂ attenuates the host antitumor immunity to promote the HFD-induced HCC development. A, Schematic representation of the experimental design. Immune cells isolated from DMBA-treated, HFD-fed livers were incubated with 1 µmol/L PGD₂, PGE₂, or PGF₂α in the presence of 10 µg/mL LTA for 24 hours and culture supernatants were harvested to determine cytokine production. Veh, vehicle (DMSO). B, IFNγ, TNFα, and IL5 levels in culture supernatant measured by multiplex assay. Data, means ± SD (n = 3). NS, not significant; *, P < 0.05; **, P < 0.01. C, qPCR analysis of relative mRNA levels of Ptger1, Ptger2, Ptger3, and Ptger4 in nontumor (NT) or tumor (T) region of mouse livers. Data of three individual mice were shown as means ± SD. NS, not significant; *, P < 0.05. D, qPCR analysis of relative mRNA levels of Ptger4 in each cell type isolated from the liver. Data, means ± SD (n = 3).
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COX2 Overexpression and Excess PGE\textsubscript{2} Production Are Detected in Noncirrhotic NASH-Associated HCC in Humans

Finally, we sought to determine whether PGE\textsubscript{2} was indeed a biomarker mediating the development of NASH-associated HCC in humans. Approximately 20\% of NASH-associated HCC is nonfibrotic and arises directly from NAFLD-based NASH (41, 42), and this is categorized as noncirrhotic NASH-associated HCC. We previously found that the histology of HFD-induced HCC in our mouse model is less fibrotic and that this mouse model could be used as an animal model for noncirrhotic NASH-associated HCC in humans (4). Therefore, we histologically classified NASH-associated HCC into four groups: (i) HCCs with low lipid accumulation in fibrotic NASH, (ii) HCCs with high lipid accumulation in fibrotic NASH, (iii) HCCs with low lipid accumulation in nonfibrotic NASH, and (iv) HCCs with high lipid accumulation in nonfibrotic NASH (Supplementary Fig. S10). Interestingly, COX2 expression was predominantly detected in senescent HSCs in the HCCs with high lipid accumulation arising from nonfibrotic NASH, whereas lower COX2 expression was observed in other three groups (Fig. 7A). The SASP factor expression and the excess PGE\textsubscript{2} biosynthesis was observed in the COX2-expressing HSCs in HCCs with high lipid accumulation in nonfibrotic NASH (Fig. 7A). These results suggest a similar mechanism could occur in human NASH-associated HCCs, and high COX2 expression and PGE\textsubscript{2} overproduction were observed specifically in HCCs with high lipid accumulation in nonfibrotic NASH.

DISCUSSION

HCC is the second leading cause of cancer mortality worldwide (43). Although long-term infection with HBV or HCV is a well-known risk factor for HCC, obesity-associated NAFLD and NASH have recently emerged as important causes of HCC (6). Owing to the increased incidence of obesity-associated HCC (42), the mechanisms involved in this disease have been extensively investigated. Previous reports have suggested that endoplasmic reticulum stress, p62/SQSTM accumulation, and increased mitochondrial ROS levels in hepatocytes are important causes of hepatocyte carcinogenesis (3). Although these reports have focused on the initial onset of liver carcinogenesis (44), the progression mechanisms of obesity-associated liver cancer are still incompletely understood, particularly in the context of the gut–liver axis.

In this study, we showed that the gut–liver axis plays an important role in obesity-associated liver cancer development. The hepatic translocation of DCA, an obesity-induced Gram-positive gut microbial metabolite, promoted cellular senescence in HSCs. The Gram-positive gut microbiota also provided its component LTA to the liver to synergistically induce the expression of SASP factors and the innate immune receptor TLR2 in senescent HSCs in HCCs with high lipid accumulation in nonfibrotic NASH, which are important causes of hepatocyte carcinogenesis (3). Although these reports have focused on the initial onset of liver carcinogenesis (44), the progression mechanisms of obesity-associated liver cancer are still incompletely understood, particularly in the context of the gut–liver axis.

In this study, we showed that the gut–liver axis plays an important role in obesity-associated liver cancer development. The hepatic translocation of DCA, an obesity-induced Gram-positive gut microbial metabolite, promoted cellular senescence in HSCs. The Gram-positive gut microbiota also provided its component LTA to the liver to synergistically induce the expression of SASP factors and the innate immune receptor TLR2 in senescent HSCs. Furthermore, LTA upregulated COX2 expression in the senescent HSCs to promote local production of PGs. In particular, PGE\textsubscript{2} was likely to suppress the antitumor immune response through the PTGER4 receptor on immune cells, and we found that an PTGER4 antagonist significantly suppressed obesity-induced HCC development with the reactivation of antitumor immunity (Fig. 7B).
Recent studies have provided insight into the effects of the gut–liver axis in some liver diseases, such as fatty liver, hepatitis, liver fibrosis, and HCC with fibrosis (4, 28, 45); these effects are thought to be mediated by absorption of intestinal contents, including gut microbiota–related metabolites and components, which directly affect liver homeostasis (46).

The gut microbiota not only alters the form of metabolites to be absorbed, but also supplies ligands for the activation of innate immunity, which could stimulate proinflammatory responses in the liver (46). Other groups have reported that LPS, a cell surface component of the Gram-negative gut microbiota, promotes liver fibrosis and fibrotic liver cancer.
in mice (26). In this study, we clarified that DCA and LTA derived from the obesity-induced Gram-positive gut microbiota cooperated to upregulate the expression of SASP factors and COX2 in DCA-induced senescent HSCs, suggesting a unique role of the gut microbiota in the development of obesity-associated HCC.

Our results also suggested the importance of excess biosynthesis of PGE\(_2\) through the COX2 pathway in a TLR2-dependent manner in obesity-associated liver carcinogenesis. PGE\(_2\) production was confirmed in the senescent HSCs, suggesting that senescence-associated lipid mediators also play pivotal roles with other SASP factors in the tumor-promoting microenvironment. This COX2-mediated product, PGE\(_2\), is known to be a key factor in cancer progression through directly activating Wnt signals (33–35). Moreover, PGs are known to affect immune cells (29). Indeed, in our study,
PGE2 production in the senescent HSCs, consistent with NASH-associated HCC with high levels of lipid accumulation. The PTGER4 pathway significantly reduced the numbers of Tregs and AAT-008. We further showed that inhibition of the PTGER4 receptor in vitro significantly reduced the ratio of PD-1–expressing CD8+ T cells in our setting. These findings provide valuable new insights into the regulation of the tumor microenvironment and the development of potent anticancer drugs, and may facilitate the prevention and treatment of obesity-associated liver cancer, although further studies are needed to identify the functioning PGE2 receptor in human noncirrhotic NASH-associated HCC.

**METHODS**

**Mice and Diet**

C57/Bl6 mice were purchased from CLEA Japan Inc. The TLR2−/− mice (C57/Bl6) were purchased from Oriental Yeast Co. Ltd. Male mice were used for all the experiments in this study. The mice were maintained under specific pathogen-free conditions, on a 12-hour light–dark cycle, and fed a normal diet (Labo MR) or high-fat diet (D12492, Research Diet Inc.) ad libitum. Mice weighing more than 45 g at 30 weeks old were used as obese mice for all experiments. The sample size used in this study was determined based on the expense of data collection and the need to have sufficient statistical power. Randomization and blinding were not used in this study. All animal experiments followed protocols approved by the Animal Care and Use Committee of Tokyo University of Science (Tokyo, Japan).

**Chemically Induced Carcinogenesis**

DMBA (Sigma) treatments were performed as described previously (4). In brief, 50 μL of a solution of 0.5% DMBA (Sigma) in acetone was applied to the dorsal surface of mice on postnatal days 4 to 5. After this application, mother mice with pups were fed ND or HFD. At the age of 4 weeks old, pups were weaned and continuously fed either ND or HFD until euthanized. Evaluation of tumor number and size was determined by counting the number of visible tumors and measuring the size of the tumor.

**Treatment of Deoxycholic Acid and Antibiotics In Vivo**

DCA (Wako) was dissolved in absolute ethanol and diluted in 66% propylene glycol (Wako) to reduce the concentration of alcohol to 5%. Mice treated with DMBA at neonatal stage were fed with ND supplemented with 0.3% DCA or vehicle-supplemented ND (control) at the age of 8 weeks old until euthanized. ND-fed mice treated with DMBA at neonatal stage were intraperitoneally treated with 200 μL of a solution of 5 mg/mL LTA (Sigma, L2515) or water (vehicle) every day at the age of 22 weeks old until euthanized. HFD-fed mice...
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Treated with DMBA at neonatal stage were treated with vancomycin (500 mg/L) in drinking water. HFD-fed mice treated with DMBA at neonatal stage were administered with 40 μg per g (weight) of DCA or vehicle three times per week using a plastic feeding tube and treated with 200 μL of a solution of 5 mg/mL. LTA or vehicle every day. The administration schedules of vancomycin, DCA, and LTA in HFD-fed mice are described in each figure legend.

Treatment with PTGER4 Antagonist

A PTGER4 receptor specific antagonist, AAT-008, was kindly provided by AskAt Inc. AAT-008 is 4-[1-(5-chloro-2-(5-fluorophenox)-pyridin-3-yl]carbonylaminomethyl] benzoic acid (Ki = 0.97 nmol/L for human PTGER4), and its activity against human, mouse, and rat PTGER4 receptors was examined and proven equivalent (49). AAT-008 was dissolved in 0.5% methycellulose solution, and HFD-fed mice treated with DMBA at the neonatal stage were orally administered AAT-008 (30 mg/kg) or vehicle everyday using a feeding tube, at the age of 19 weeks old until euthanized.

Histology and Immunofluorescence Analysis

Hematoxylin and eosin staining and immunohistochemical analysis were performed as previously described (4). The primary antibodies for mouse bodies were as follows: α-SMA (Sigma A5228, mouse monoclonal), o-SMA (Abcam, ab5964, rabbit polyclonal), p21 (Abcam, ab2961), 53BP1 (Santa Cruz, sc22760), IL6 (Abcam ab6672), Groo (Abcam, ab17882), I1.L (R&D Systems, AF-401-NA), F4/80 (Invitrogen, BM8), CD45 (Millipore, 05-1416), LTA (Lifespan Biosciences, LS-C204288), PGE2 (Abcam, ab2318), COX2 (Abcam, ab15191), Glypican 3 (Abcam, ab66396), and phospho-IκBα (Cell Signaling Technology, 9246). The primary antibodies for human samples were as follows: o-SMA (Dako, M0851), COX2 (Abcam, ab15191), and PGE2 (Abcam, ab2318). PGE2 staining was performed as previously reported (50).

Oil Red O Staining

Frozen sections (5-μm thick) of livers were washed in 60% isopropanol and were incubated with six parts oil red O solution (0.3 g oil red O in 100 mL isopropanol) and 4 parts H2O for 13 minutes at 37°C. After washing in 60% isopropanol and tap water, the sections were counterstained with hematoxylin.

Western Blot Analysis

Tissue lysates were prepared using a homogenizer in lysis buffer (50 mmol/L HEPEs (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 10% glycerol, 0.1% Tween 20, and 10 mmol/L β-glycerophosphate) containing protease inhibitor cocktail (Nacalai Tesque). The transfected membranes were immunoblotted directly with antibodies, and the signals were detected using an enhanced chemiluminescence system (GE Healthcare). The first antibodies used were as follows: 15-PGDH/HPGD (Novus Biologic, NB200-179) and GAPDH (Abcam, ab9485).

Bacterial 16S rRNA Amplicon Sequencing and Analysis

Bacterial genomic DNA was isolated from fecal samples using a QiAamp DNA Stool mini kit (QiAGEN), following the manufacturer’s instructions. DNA from fecal samples was amplified using the universal 16S rRNA primers F515 5′-GTGCTACGGCAGCGCTCTAAT-3′ and R806 5′-GGACTACHVGGGTWTCTAAT-3′ targeting the V4 hyper variable region of the 16S rRNA gene using KOD FX DNA polymerase (TOYOBO). 16S rRNA libraries for all samples were preanalyzed reads having at least 97% identity were subjected to OTU analysis and removed putative chimeric reads using UCLUST and UCHIME. Representative sequences from each OTU were blasted to the database in Ribosomal Database Project (RDP) and aligned by the RDP Classifier. The obtained OTU sequences were grouped at the phylum level.

Mediator Lipidomics

LC/MS-MS-based lipidomics analyses were performed using a high-performance liquid chromatography system (Waters UPLC) with a linear ion-trap quadrupole mass spectrometer (QTRAP5500; AB SCIEX) equipped with an Acquity UPLC BEH C18 column (Waters) as described previously (30). MS-MS analyses were conducted in negative-ion mode, and fatty-acid metabolites were identified and quantified by multiple reaction monitoring.

Primary Cell Culture

Murine primary hepatic stellate cells were isolated by the following procedure. Briefly, the livers were isolated from the blood-removed 10-week-old mice. The gall bladder was removed, and then the livers were carefully excised into small pieces. The small liver pieces were incubated in PBS containing 0.05% trypsin and 0.53 mmol/L EDTA (Nacalai Tesque) for 14 minutes at 37°C with gentle mixing at every 2 minutes. The cells were passed through 70-μm cell strainers (BD Biosciences), washed with DMEM supplemented with 10% FBS at least two times, and cultured in tissue culture dishes. After five days, α-SMA-positive cells were confirmed by cell staining and quantitative PCR analysis, and used as active hepatic stellate cells. The murine primary HSCs were cultured in the absence or presence of DCA (100, 200, or 300 μmol/L) for 8 days, and then were treated with LTA (10 or 25 μg/mL, Invivogen) for 6 hours until harvested. Primary hepatocytes were collected using the following methods. The mouse livers were perfused slowly via the inferior vena cava with 30 mL of warm liver perfusion medium [33 mmol/L glucose, 100 mmol/L NaCl, 2.3 mmol/L KCl, 1.2 mmol/L KH2PO4, 25 mmol/L HEPEs, 1.5% FBS, 1% GlutaMAX supplement (Gibco), and 0.5 mmol/L EDTA] at a rate of 5 mL/min, and then digested with 40 mL of liver digest medium (liver perfusion buffer containing 6.5 mmol/L CaCl2 and 50 μg/mL Liberase TM) at a rate of 5 mL/minute. After the gall bladder was removed, the livers were carefully excised and incubated in liver digest medium for 5 min at 37°C, and passed through a 100-μm cell strainer (BD Biosciences). Hepatocytes were collected by centrifuging the cell suspension at 50 × g for 2 minutes and used for flow cytometric analysis. The collected hepatocytes were also confirmed by their morphology and hepatocyte marker expression by quantitative PCR analysis, and used as hepatocytes. These primary murine cells were prepared every time for each experiment and were not passaged. Therefore, they were not authenticated for their clonality. These cells were tested each time by PCR-based assay to verify that they were free of Mycoplasma contamination.

Quantitative PCR

Total RNA was extracted from mouse tissues or cultured cells using TRIzol reagent (Life Technologies) and reverse transcription and quantitative PCR were performed as previously described (23). Primers used were as follows: mouse Gapdh, 5′-CACTACATGTTGCTACAGTT-3′ (forward) and 5′-CCAGAATGATCCTCAAGGAC-3′ (reverse); mouse Cdkn2a, 5′-GGCTGATTGGAGTCCGATC-3′ (forward) and 5′-TCTGTTGCGGAAATGACAAATCT-3′ (reverse); mouse Il6, 5′-GAAGAGGGTGGCTAGCAAGAAGC-3′ (forward) and 5′-AAGC
CCTAGGTTTGGCCAGTA-3′ (reverse); mouse G3′, 5′-GCTGG
GATGATCTCAGGAAG-3′ (forward) and 5′-AGTGCACATGCAGAC
TCT-3′ (reverse); mouse Th2, 5′-GGGACATGGTTCTGACACCCG-3′ (forward) and 5′-GGGCAACAGATCCAGGAAACG-3′ (reverse); mouse Coxl, 5′-TGTCGGGTTGACATTGCTGGAG-3′ (forward) and 5′-TGGG
CTCGATTGCCCGT-3′ (reverse); mouse Cox2, 5′-AACCGGTATTG
CTCTGAAAT-3′ (forward) and 5′-CATGGTTTATAGGCCGCTT-3′
(reverse); mouse Pgr1, 5′-GGGATCTGCTGAACACAGAAG-3′ (forward) and 5′-TGGGGTTTATAGGCCGCTT-3′ (reverse); mouse Pgr2, 5′-CTTAAAGCTATGCAGAAG-3′ (forward) and 5′-GATAATGGG
CCCTGGTGAAGA-3′ (reverse); mouse Pgr3, 5′-TGTCGGGTGAAG
TGAACACAG-3′ (forward) and 5′-TCTGCAAGAATCCCCAGGAA
AGGA-3′ (reverse); mouse Pgr4, 5′-TACTCTCATACGACACTG
GGA-3′ (forward) and 5′-TGCTGCTAGCATGTAAGGACCGAG-3′
(reverse); mouse Isg15, 5′-ACGCGATCTCAGGATTTCCATGCA
CA-3′ (forward) and 5′-CAAGGGCTTGGGAAATGACA-3′ (reverse);
Firmcutes 16S rRNA, 5′-GGGATTTTGTGGGAAAGTGACG-3′ (forward) and 5′-AGGATCTGACAGAATCCCACAGC-3′ (reverse).

Immune Cell Isolation, Stimulation, and Multiplex Bead-Based Assay

Livers obtained from mice were minced with scissors and incubated in PBS (supplemented with 3% fetal bovine serum, 0.1 mg/mL CaCl2, and 0.1 mg/mL MgCl2) containing 61.5 μg/mL Liberase TM (Roche) and 10 mg/mL DNase I (Roche) for 30 minutes at 37°C. Digested tissues were further incubated for 5 minutes in the presence of 5 ml/L EDTA (Dojin Laboratories) and passed through a 100-μm cell strainer (BD Biosciences). Red blood cells were lysed by RBC lysis buffer (BioLegend), and the cells were filtered through a 70-μm cell strainer (BD Biosciences). The cells were cultured in the presence of 1 μg/mL LPS, 1 μg/mL PGE2, or PGE2 (Cayman Chemical) with or without 10 ng/mL LTA (InvivoGen) for 24 hours. Supernatants were assayed for cytokines by multiplex assay using the LEGENDplex Mouse Th Cytokine Panel (13-plex; BioLegend), according to the manufacturer’s instructions.

Flow Cytometry

Cells were preincubated with unlabelled anti-CD16/32 mAb (2.4G2; TONBO) to avoid nonspecific binding of antibodies to FcR. Cells were then incubated with the antibodies for CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-67), CD11b (M1/70), CD11c (N418), CD45.2 (104), CD69 (H1.2F3), CD103 (2E7), I-A/E (M5/114.15.2), KLRG1 (2F1/KLRG1), NK1.1 (PK136; all from BioLegend), PD-1 (J43; BD Biosciences), and TLR2 (T2.5; eBioscience). Cells were fixed and permabilized using a FOXP3 staining buffer kit (eBioscience), and then intracellularly stained for FOXP3 (FJK-16s; eBioscience). Murine primary HSCs and freshly isolated hepatocytes were preincubated with unlabelled anti-CD16/32 mAb (2.4G2), and then incubated with the antibody for TL2 (T2.5). Stained cells were analyzed by Attune Nxt (Life Technologies) and data were processed by FlowJo Version 10 software (FlowJo). Dead cells were excluded by using Zombie NIR fixable viability dye (BioLegend).

Quantification of Ptgfr4 mRNA Levels

CD11b+ DCs (CD11b+CD103-CD11c-MHC class II+), CD103+ DCs (CD11b+CD103+CD11c-MHC class II+), double-negative DCs (CD11b+CD103−CD11c-MHC class II−), CD11b+FoxP3+ macrophages, CD4+CD25+ Tregs, CD4+CD25− T cells, CD8+ cells, and NK1.1+ cells were sorted from mouse livers by FACS Aria II cell sorter (BD Biosciences). Total RNA was extracted using a RNeasy Plus Micro kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized and amplified with Smart-Seq v4 Ultra Low input RNA kit (Clontech) according to the kit manual. Total RNA extraction from murine primary HSCs and freshly isolated hepatocytes and quantitative PCR were performed as described above. Primers used were as follows: mouse Gapdh, 5′-CACTACATAGTC TACTATGTC-3′ (forward) and 5′-CACCAGTAGTACTCCAGCAG-3′ (reverse); mouse Ptgfr4, 5′-TGTCCTACCTCCGTCGTG-3′ (forward) and 5′-GCACAGTCTCCGGAAGAAGG-3′ (reverse).

Human Subjects

Written informed consent was obtained from all patients according to the protocol reviewed and approved by the board members of the ethics committee of the Japanese Foundation for Cancer Research (JFCR) in accordance with the Declaration of Helsinki. The protocols for the analysis of human liver samples were also reviewed and approved by the board members of the ethics committee of the Tokyo University of Science in accordance with the Declaration of Helsinki.

Statistical Analysis

Data were analyzed by an unpaired t test with Welch correction (two-sided) or the Mann–Whitney test (two-sided). P values less than 0.05 were considered statistically significant. “NS” indicates not significant.

Disclosure of Potential Conflicts of Interest

S. Narumiya is a consultant/advisory board member for Astellas Pharma. No potential conflicts of interest were disclosed by the other authors.

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