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Original Articles

Toll-like receptor-4 is a target for suppression of proliferation and chemoresistance in HepG2 hepatoblastoma cells

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ABSTRACT

Toll-like receptor-4 (TLR4) is known to influence growth and migration of hepatocellular tumors; however, its role in hepatoblastoma remains poorly understood. This study investigated the regulatory role of TLR4 in proliferation and chemoresistance of HepG2 hepatoblastoma cells. Treatment with lipopolysaccharide (LPS), a TLR4 agonist, was found to significantly upregulate TLR4 expression in HepG2 cells, but not in malignant Huh-7 and Sk-Hep1 hepatocellular carcinoma cells. Additionally, IL-6 enhanced LPS-induced TLR4 upregulation. LPS-stimulated TLR4 activation increased proliferation, nitric oxide synthase (NOS) expression, and NO production in HepG2 cells. Chemotherapeutic agents, cisplatin and doxorubicin, effectively inhibited TLR4 expression in HepG2 cells. Characterization of LPS-induced signaling activation and blockade with kinase inhibitors revealed the involvement of Akt and MAPK pathways in LPS-enhanced NO release from, and proliferation of HepG2 cells. Mechanistically, gene modifications as a result of TLR4 transfection and siRNA-mediated knockdown further demonstrated a crucial role for TLR4 in the regulation of NOS expression, cell proliferation, and chemoresistance in HepG2 cells. These findings suggest that targeting TLR4 expression and its cognate signaling may modulate proliferation and chemosensitivity in hepatoblastoma cells and serve as a potential therapeutic target.

Introduction

Toll-like receptors (TLRs) are a group of transmembrane proteins that specifically recognize conserved molecular patterns of microbial origins and play an important role in orchestrating the host defense response. In addition to initiating both innate and adaptive immune responses of hosts, TLR-mediated signaling also regulates cell proliferation and survival. Increasing evidence suggests that, despite of being predominantly expressed on immune cells, functional TLRs are also expressed in various types of human cancers, including cancers of the lung [1], colon [2], prostate [3], breast [4], and cervix [5], and melanoma [6]. Chronic inflammation is believed to elevate the risk of cancer development and progression [7,8]. TLRs modulate the release of cytokines and chemokines from cancer cells, which recruit immune cells and induce an anti-tumor immune response to suppress tumor progression [9–12]. It is also known that uncontrolled TLR signaling provides a beneficial microenvironment for tumor cells to proliferate, invade, and escape from immune surveillance; however, the precise roles of TLRs in tumor biology remain poorly understood. TLR4, a well-studied member of the TLR family of proteins in tumors, has been found to be overexpressed in cancers of the colon, prostate, and lung, and in melanomas [2,3,6,10]. Multiple lines of evidence also suggest that TLR4 expression and cognate signaling activation promote cancer progression and chemoresistance [10,13].

In the context of hepatocarinogenesis, clinical studies have revealed that TLR4 overexpression in hepatocellular carcinoma (HCC) is highly associated with alcohol intake [14] and hepatitis C virus infection [15–17]. Immunohistochemical staining results indicate that the coexistence of TLR4 and TLR9 antigenicity in HCC tissues predicts poor prognosis in patients with HCC [18]. Other lines of

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evidence reveal that translocation of intestinal bacteria results in TLR4 activation and facilitates hepatocarcinogenesis [19], while intestinal decontamination limits bacterial translocation [20], reduces serum nitric oxide (NO) levels and improves systemic hemodynamics in patients with cirrhosis [21]. Recent studies further support that exposure of HCC cells to a potent TLR4 agonist, lipopolysaccharide (LPS), not only enhances cell proliferation and survival, but also induces progression of epithelial mesenchymal transition [22–24]. Moreover, TLR4 activation is also known to facilitate invasion and migration of hepatic cancer stem cells [25], and elevates tumor-initiating activity as well as chemoresistance during development of HCC [14]. Mechanistic studies of TLR4-associated chemoresistance have shown that TLR4 signaling reduces the chemosensitivity of ovarian cancer cells to paclitaxel [26]. NO has also been shown to exhibit pleiotropic effects in tumor cells, such as induction of chemotherapeutic resistance by evading apoptosis [27,28]. High NO production reportedly contributes to sorafenib resistance in HCC [29], whereas the concomitant upregulation of nitric oxide synthase (NOS) has more recently been linked to tumor-initiating properties of colon cancer stem cells [30]. These findings support the idea that dysregulated TLR4 expression and enhanced NO levels are involved in tumor development and chemoresistance.

Hepatoblastoma (HB) is a malignant pediatric tumor occurring in the livers of infants and children [31]. To date, little is known about the role of TLR4 in HB tumorigenesis. Our previous study demonstrated that TLR4 overexpression in HB tissues was effectively suppressed by chemotherapy with cisplatin and doxorubicin and that LPS-induced TLR4 activation exerted anti-migratory effects on cultured HepG2 HB cells [32]. However, not enough is known about the overall effect of TLR4 activation on HB tumorigenesis and chemosensitivity. This study therefore aimed to elucidate the effect of LPS stimulation on TLR4 expression and cell proliferation, clarifying the involvement of elevated NOS expression and NO production therein, and determining the role of TLR4 upregulation in the proliferation and chemoresistance of HepG2 HB cells.

Materials and methods

Reagents

LPS purified from *Escherichia coli* K12 (L2654), cisplatin (P4394), doxorubicin (D1515), N^G-Nitro-L-arginine methyl ester (L-NAME; N5751) and sodium nitroprusside (SNP; 228710) were from Sigma-Aldrich (St. Louis, MO). All kinase inhibitors including SB203580 for p38 MAPK, SP600125 for JNK, PD98059 for MEK, and wortmannin for PI3K inhibition were from Sigma-Aldrich. Primary antibodies against TLR4, NOS2, NOS3, PCNA and Actin were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies for detecting phospho-Akt (Ser473), phospho-p38 MAPK (Thr180/182), phospho-ERK1/2 (Thr202/204), phospho-JNK1/2 (Thr183/185), phospho-histone H3 (Ser10) and those for above total proteins were all from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-conjugated anti-rabbit and mouse IgG antibodies were from Jackson Lab (West Grove, PA).

Cell culture

Human HepG2 HB cells (BCRC clone no. 60177) were purchased from the Bioresource Collection and Research Center (BCRC 60025, Food Industry Research and Development Institute, Hsin-Chu, Taiwan). Two HCC cell lines, Huh-7 and SK-Hep1 cells, were kind gifts from Prof. Ming-Hong Tai (Institute of Biomedical Sciences, National Sun Yat-Sen University, Taiwan). All cell lines were maintained in DMEM (Invitrogen, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin and streptomycin (Invitrogen). Cells were grown as adherent cells in a humidified atmosphere at 37 °C in a 5% CO_2 incubator.

Cell proliferation assay

HepG2 HB or HCC cells were seeded on 96-well plates in normal culture medium (10^3 cells in 100 μL of medium per well). After overnight incubation, cells were treated with LPS or anti-tumor drugs at indicated doses in replicates of four. An MTT reagent was used to measure cell proliferation and viability (chemosensitivity) as previously described [33].

Western blotting detection

Equal amounts (30 μg) of protein extracts were loaded and separated by SDS–PAGE using 10% acrylamide gels. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in TBS-T (TBS containing 0.05% Tween 20)] for 1 h. The membranes were incubated with primary antibody for overnight at 4 °C, followed by incubation with secondary detecting antibody (diluted at 1:15,000) for 1 h at room temperature. After washes for 5 times within 1 h, immunoblots were exposed to an enhanced chemiluminescence (ECL) reagent (Millipore, Temecula, CA) and digitally visualized under a BioSpectrum imaging documentation system (UVP, Upland, CA). For the purpose of semi-quantitative analysis, the images of ECL signals were measured using ImageJ software (NIH, USA). Relative protein levels were expressed as induction folds, after calculating the density ratios between interested proteins and internal control and being normalized to negative control.

Quantitative RT-PCR

Total RNA was extracted from HepG2 cells using Trizol solution (Invitrogen). Two micrograms of total RNA was used for reverse transcription reaction with Transcriptor reverse transcriptase kit (Roche Diagnostics, Mannheim, Germany) using oligo-dT and random primers. Synthesized cDNA was subjected for measurement of the dose responsive gene expression on an ABI 7500 Real-Time PCR System using the SYBR Green Master Mix kit from Applied Biosystems (Foster City, CA). Melting curves for each PCR reaction were generated to ensure the specificity of PCR reaction. Data were analyzed according to the comparative Ct method and were normalized by *β-actin* expression in each sample. Gene-specific primer pairs used for amplification were as follows: for *β-Actin*, 5′-TCC TGT GGC ATC CAC GAA ACT-3′ (forward) and 5′-GAA GCA TTT GCG GTG GAC GAT-3′ (reverse); for *TLR4*, 5′-CTT TAT TCC CGG TGT GGC CA-3′ (forward) and 5′-GCA GGG TCT TCT CCA CCT TC-3′ (reverse).

Nitric oxide detection

Total amount of NO production including nitrite/nitrate contents in conditioned media was measured by using Griess reagent as previously described [34]. In brief, supernatants were collected and centrifuged to remove cell debris. Equal amount of samples and sodium nitrate standards were loaded into microtiter plates and replenished with reaction buffer (50 mM MOPS, 1 mM EDTA, pH 7.0), followed by addition of nitrate reductase and reduced β-NADH (Sigma). Reaction with orbital shaking was done by incubation at room temperature for 20 min. Afterwards, an equal volume of color reagent A (sulfanilamide) and reagent B [*N*-(1-naphthyl)ethylenediamine dihydrochloride] were added and incubated at room temperature for 5 min. Optical density at 540 nm was detected with a microplate reader (Sunrise, Tecan Group, Mannedorf, Switzerland) and value of blank control (medium without cells) was subtracted. The total nitrite/nitrate concentrations were calculated with calibrated standard curve.

TLR4 gene modifications and chemosensitivity

A pcDNA3.1 expression vector carrying full-length human *TLR4* gene (Addgene, Cambridge, MA, plasmid #13086) was used to overexpress TLR4 gene in clone-9 hepatocytes, a cell line with constitutive low TLR4 expression. In brief, the cells were transfected with 2 μg plasmid DNA or empty vector DNA by using Lipofectamine™ 2000 *in vitro* transfection reagent (Invitrogen) according to the manufacturer's instructions. Clone-9 cells receiving 48 h of transfection with TLR4-carrying or empty plasmids were exposed to LPS or anti-tumor drugs for another 24 h. Chemosensitivity was determined by using an MTT-based cell viability assay and detecting caspase-3 cleavage. For TLR4 gene silencing experiments, HepG2 cells at 60%–70% confluence were transfected with either 100 nM of RNA oligonucleotides interfering *TLR4* gene expression (Ambion Silencer Select pre-designed and validated siRNA S14195, Life Technologies, Grand Island, NY) or equivalent scrambled siRNA control through Lipofectamine. After 48 h of gene silencing, the cells were exposed to LPS or anti-tumor drugs for another 24 h, followed by cell viability assay and caspase-3 detection.

Statistical analysis

The quantitative results were expressed as mean ± SD. Student's *t*-test was used to calculate statistically significant differences between the two study groups.

Results

Upregulation of TLR4 expression in HepG2 cells by LPS and IL-6 treatment

In order to investigate the association of TLR4 expression with hepatoma malignancy, western blotting was used to compare TLR4