Cancer Letters 371 (2016) 38–47

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Autophagy-deficiency in hepatic progenitor cells leads to the defects of stemness and enhances susceptibility to neoplastic transformation

Feng Xue [1], Lei Hu [1], Ruiliang Ge [1], Lixue Yang, Kai Liu, Yunyun Li, Yanfu Sun *, Kui Wang **

The Second Department of Liver Surgery, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, Shanghai 200438, China

ARTICLE INFO

Article history:
Received 2 September 2015
Received in revised form 13 November 2015
Accepted 14 November 2015

Keywords:
Autophagy
Hepatic progenitor cells
Stemness
DNA damage repair
Neoplastic transformation

ABSTRACT

Autophagy is a highly conserved and lysosome-dependent degradation process which assists in cell survival and tissue homeostasis. Although previous reports have shown that deletion of the essential autophagy gene disturbs stem cell maintenance in some cell types such as hematopoietic and neural cells, it remains unclear how autophagy-deficiency influences hepatic progenitor cells (HPCs). Here we report that Atg5-deficiency in HPCs delays HPC-mediated rat liver regeneration in vivo. In vitro researches further demonstrate that loss of autophagy decreases the abilities of colony and spheroid formations, and disrupts the induction of hepatic differentiation in HPCs. Meanwhile, autophagy-deficiency increases the accumulations of damaged mitochondria and mitochondrial reactive oxygen species (mtROS) and suppresses homologous recombination (HR) pathway of DNA damage repair in HPCs. Moreover, in both diethylnitrosamine (DEN) and CCl_4 models, autophagy-deficiency accelerates neoplastic transformation of HPCs. In conclusion, these findings demonstrate that autophagy contributes to stemness maintenance and reduces susceptibility to neoplastic transformation in HPCs.

Introduction

Stem/progenitor cells (SPCs) are special cells which have the ability to keep their cell population by self-replication and to differentiate into different types of downstream mature cells. In adult organisms, SPCs have the responsibility of keeping tissue homeostasis in normal conditions and repairing damaged tissues in injured conditions [1]. Moreover, in the carcinogenic circumstance, mutated SPCs including HPCs are considered as the source of tumor-initiating cells or cancer stem cells [2,3]. Therefore, control of SPCs plays an important role in self-regulation of organism. Recently several studies have shown that macroautophagy (hereafter referred to as autophagy) contributes to the maintenance of cell stemness [4].

Autophagy is an evolutionarily conserved degrading mechanism which eliminates unnecessary and damaged biomacromolecules and organelles to decrease internal consumption and prevent toxic factor accumulation, and recycle their components to support the productions of necessary metabolic precursor [5]. An increasing number of evidence show that autophagy plays a major role on cellular and tissue homeostasis and is widely involved in physiological and pathophysiological processes, such as development, metabolic disorders, neurodegenerative disease and cancer [6].

The essential role of autophagy in cell stemness was first found in adult hematopoietic stem cell (HSC) maintenance [7,8]. Then similar results were reported in the studies of neural stem cells (NSCs) and early embryo [9,10]. Recent study also presented that autophagy level of HPCs was higher than that of hepatocytes in mice. Meanwhile, autophagy inhibition impaired colony formation ability and hepatic differentiation in HPCs [11]. However, the impact of autophagy on HPCs still remains unexplored. Furthermore, both autophagy and SPCs have a close relationship with tumorigenesis [12–16], but it is not yet clear whether inhibition of autophagy affects SPC-associated tumor formation. Hence, we expected to further explore the influence of autophagy-deficiency on the stemness of HPCs and to investigate whether autophagy-deficiency could affect neoplastic transformation of HPCs.

Abbreviations: HPCs, hepatic progenitor cells; mtROS, mitochondrial reactive oxygen species; DEN, diethylnitrosamine; SPCs, stem/progenitor cells; HSCs, hematopoietic stem cells; NSCs, neural stem cells; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; shRNA, short hairpin RNA; Scr, Scramble; qPCR, quantitative real-time PCR; G6P, glucose-6-phosphatase; i.p, intraperitoneal; i.s, intrasplenically; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Tbil, total bilirubin; ALB, albumin; H&E, hematoxylin and eosin; CCK8, Cell Counting Kit-8; 8-OHdG, 8-hydroxy-2-deoxyguanosine; γ-H_2AX, phosphorylated histone H_2AX; MTDR, MitoTracker® Deep Red FM; MSR, MitoSOX™ Red Mitochondrial Superoxide Indicator; DNA-PKcs, DNA-dependent protein kinase; DSBs, double-strand breaks; IR, ionizing irradiation; NHEJ, nonhomologous end joining.

* Corresponding author. Tel.: +86 21 81875513; fax: +86 21 65562400.
E-mail address: Sunyanfu_smmu@163.com (Y. Sun).
** Corresponding author. Tel.: +86 21 81875512; fax: +86 21 65562400.
E-mail address: Wangkui_smmu@163.com (K. Wang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.canlet.2015.11.022
0304-3835/

Table 1
shRNA sequences.

shRNA	Sequence
Atg5-shRNA1	5′-CTTGGAACATCACAGTACA-3′
Atg5-shRNA2	5′-CTGTTTACAGTCAGTCTAT-3′
Atg7-shRNA1	5′-CCAAGGTCAAAGGACGAAGAT-3′
Atg7-shRNA2	5′-AGCATCATCTTTGAAGTGA-3′
Scr-shRNA	5′-TTCTCCGAACGTGTCACGT-3′

Materials and methods

Cell culture

WB-F344 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO) in a humidified incubator with 95% air and 5% CO_2 at 37 °C.

Gene silence mediated by lentivirus-delivered short hairpin RNA (shRNA)

We designed shRNA candidates with Atg5 target sequences (Atg5-shRNA1 and Atg5-shRNA2) and Atg7 target sequences (Atg7-shRNA1 and Atg7-shRNA2). Scramble shRNA (Scr-shRNA) served as the negative control (Table 1). pGCL-GFP lentiviral particles encoded GFP and shRNAs (Shanghai GeneChem, Shanghai, China). Gene expressions after lentiviral infection were revealed by quantitative real-time PCR (qPCR) and western blot at the 4th day. The transfected cells after 6~8 passages were used for subsequent experiments.

RNA extraction and qPCR

Total RNAs were extracted from cells using Trizol reagent (Invitrogen) and then were treated with RNase-free DNase (Promega, Madison, WI). The RNAs were used to synthesize cDNA by RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Specific mRNA levels were detected by qPCR using standard SYBR Green RT-PCR kit (Takara, Kyoto, Japan). The sequences of specific primers were shown in Table 2. The relative mRNA levels of specific genes were normalized to the housekeeping gene GAPDH.

Western blot

The cell lysates were subjected to SDS–PAGE. Then blots were incubated with specific primary antibodies (Table 3), followed by incubation with anti-rabbit or anti-mouse IgG peroxidase-conjugated secondary antibody (Santa Cruz) and chemiluminescent substrates. Hybridization with anti-GAPDH was used to confirm equal protein loading.

Animal models

Male F344 rats (8–10 weeks old, weighing 180–200 g) were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and were housed in a pathogen-free animal facility. Animal protocols were approved by the Second Military Medical University Animal Care Committee.

Retrorsine (Sigma-Aldrich, St. Louis, MO) and CCl_4 model: Rats received 30 mg/kg body weight retrorsine by intraperitoneal (i.p.) injection twice with a 2 week interval. After 1 month from the first retrorsine administration, single-cell suspensions (10^6 transfected WB-F344 cells/100 μl saline solution) were intrasplenically (i.s.) injected into the retrorsine-pretreated rats. Transplanted rats recovered for 1 week and then were hypodermically injected with CCl_4 (0.5 ml/kg) once every 3 days. After 6 weeks, the blood samples of rats were collected from medial angle of eye and sera were separated for biochemical analysis. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (Tbil) and albumin (ALB) were determined using FUJI DRI-CHEM7000 (Fuji Medical System, Tokyo, Japan). Meanwhile, rats were sacrificed to obtain liver samples. The percentage of GFP-positive cells in the liver section was determined on 5 randomly chosen fields at 100-fold magnification. And the average percentage of GFP-positive cells in 3 liver sections was used as the percentage of GFP-positive cells of rat livers.

Detection of neoplastic transformation: Autophagy-completed or autophagy-deficient HPCs (10^6 cells/100 μl saline solution) were i.s. injected into normal male rats. After 1 week, the rats were treated with two models: DEN (Sigma-Aldrich) induced liver tumor model and CCl_4-induced chronic liver damage model. In the DEN model, we added DEN (95 mg/l) to the drinking water of male F344 rats [17,18]. After 9 weeks and 11 weeks, the rats were sacrificed. In the CCl_4 model, the rats were hypodermically injected with CCl_4 (0.5 ml/kg) once a week. After 17 weeks, the rats were sacrificed. The rat liver sections were preserved in 10% neutral-buffered formalin for hematoxylin and eosin (H&E) and immunohistochemistry stains.

Table 2
qPCR primer sequences.

Gene	Sequence
Atg5	Sense: 5′-AGGCTCAGTGGAGGCAACAG-3′ Anti-sense: 5′-CCCTATCTCCCATGGAATCTTCT-3′
Atg7	Sense: 5′-CTTCCTGGCCAAGGTGTTTA-3′ Anti-sense: 5′-GTTGCTCAGACGGTCTCCTC-3′
HNF4α	Sense: 5′-GCAGTGCGTGGTAGACAAAGATA-3′ Anti-sense: 5′-AGTGCCGAGGGACGATGTAG-3′
G6P	Sense: 5′-GCTTGAATGTCGTCTTGTGG-3′ Anti-sense: 5′-TCAGCGAGTCAAAGAGATGC-3′
AFP	Sense: 5′-CGACATTTACATTGGACACTTG-3′ Anti-sense: 5′-CTTTCTTCCTCCTGGAGATG-3′
c-kit	Sense: 5′-CATCATGGAAGATGACGAGC-3′ Anti-sense: 5′-CAAATGTGTACACGCAGCTG-3′
GAPDH	Sense: 5′-GGATGGAATTGTGAAGGAGA-3′ Anti-sense: 5′-GTGGACCTCATGGCCTACAT-3′

Table 3
Primary antibody of western blot.

Target	Corporation	Stock No.
Atg5	CST	#12994
Atg7	CST	#8558
LC3B	CST	#3868
SQSTM1/p62	CST	#5114
Chk1	CST	#2360
Phospho-Chk1 (Ser345)	CST	#2348
p95/NBS1	Abcam	ab32074
Phospho-p95/NBS1 (S343)	Abcam	ab195927
BRCA1	Abcam	ab191042
Phospho-BRCA1 (S1524)	Bioworld	BS4027
GAPDH	Santa Cruz	sc-365062

CST, Cell Signaling Technology, Beverly, MA; Abcam, Cambridge, UK; Bioworld Technology, St. Louis, MN.

Cell Counting Kit-8 (CCK8)

The cell viability was evaluated by the CCK8 assays (DOJINDO, Kumamoto, Japan). The cells of the indicated groups were seeded at 5000 cells per well in 96 well plates for 8 hours. After that, cells were sequentially cultured for different times. Then 10 μl of CCK8 reagent was added to each well and incubated at 37 °C for 1 h. Finally, the optical density of each sample was measured at 450 nm using a microplate reader (Synergy HT, Bio-Tek, Winooski, VT).

Colony and spheroid formation assays

Transfected WB-F344 cells were plated at 500 cells per well into 6 well plates and were cultured for 2 weeks. After fixation in paraformaldehyde, the colonies were stained with crystal violet for 10 min. Then the wells were imaged using a digital camera and the number of colonies per well was counted.

Transfected WB-F344 cells were seeded at 2×10^6 cells per well into ultra-low attachment 6 well plates and cultured in DMEM/F12 (GIBCO) supplemented with 10% FBS for 10 days. The number of spheroids per well was counted.

Induction of hepatic differentiation in vitro

Transfected WB-F344 cells were seeded in 6 well plates (1×10^5 per well) and 24 well plates (2×10^4 per well) with hepatic differentiation medium (DMEM/F12 supplemented with 10% FBS, 20 ng/ml EGF and 20 ng/ml oncostatin M (both from R&D Systems, Minneapolis, MN), 10 ng/ml nicotinamide, 100 nmol/ml L-ascorbic acid and 0.1 nmol/ml dexamethasone (all from Sigma-Aldrich). This medium was changed every 3 days. After 15 days, the induced cells were detected.

Immunofluorescence and immunohistochemistry staining

The indicated cells were incubated with specific primary antibodies and corresponding secondary antibodies (Table 4). Phosphorylated histone H_2AX (γ-H_2AX) foci per cell were counted under Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany). A minimum of 100 cells per sample were counted in triplicate for each experiment.

Liver sections were stained with specific primary antibodies, followed by incubation with corresponding secondary antibodies (Table 4).