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Original article

Involvement of prolyl isomerase PIN1 in the cell cycle progression and proliferation of hepatic oval cells

Prabodh Risal[a,b], Nirajan Shrestha[a], Lokendra Chand[a], Karl G. Sylvester[c], Yeon Jun Jeong[a,d,*]

[a] Laboratory of Liver Regeneration, Research Institute of Clinical Medicine of Chonbuk National University-Biomedical Research Institute of Chonbuk National University Hospital, Geuman-dong, Duckjin-gu, Jeonju, 561-712, South Korea
[b] Department of Biochemistry, School of Medical Sciences, Kathmandu University, Dhulikhel, Kavre, Nepal
[c] Department of Surgery, Division of Pediatric Surgery, Stanford University School of Medicine, PSRL, 257 Campus Drive, Stanford, CA 94305-5148, USA
[d] Department of Surgery, Chonbuk National University Medical School, 634-18 Geuman-dong, Duckjin-gu, Jeonju, 516-712, South Korea

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ABSTRACT

Liver regenerates remarkably after toxic injury or surgical resection. In the case of failure of resident hepatocytes to restore loss, repopulation is carried out by induction, proliferation, and differentiation of the progenitor cell. Although, some signaling pathways have been verified to contribute oval cell-mediated liver regeneration, role of Peptidyl-prolyl *cis*-trans isomerase NIMA-interacting 1(Pin1) in the oval cells proliferation is unknown. In the present study, we evaluate the role of Pin1 in oval cells proliferation. In our study, the expression of Pin1 in the mice liver increased after three weeks feeding of 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) diet along with the proliferation of oval cells. The expression of Pin1 was higher in oval cells compared to the hepatocytes.Pin1 inhibition by Juglone reduced oval cell proliferation, which was restored to normal when oval cells were treated with IGF-1. Consistent with increased cell growth, expression of Pin1, β-catenin and PCNA were increased in IGF-1 treated cells in a time dependent manner. In FACS analysis, siRNA-mediated knockdown of the Pin1 protein in the oval cells significantly increased the numbers of cells in G0/G1 phase. Furthermore, hepatocyte when treated with TGF-β showed marked reduction in cell proliferation and expression of Pin1 whereas this effect was not seen in the oval cells treated with TGF-β. In conclusion, Pin1 plays important role in the cell cycle progression and increase oval cells proliferation which may be crucial in chronic liver injury.

1. Introduction

Pin1 is an enzyme of paravulin family of peptidyl-prolyl isomerase (PPIase). Pin1 specifically binds phosphorylated Ser/Thr-Pro protein motifs and catalyzes the cis/trans isomerization of the peptide bonds. Phosphorylation of its substrates by enzyme kinase creates binding sites for the Pin1 [12], inducing conformational changes that, regulates the function of target protein [13,20].

Pin1 is present in almost all cells but its expression is strongly correlated with the proliferative activity of normal tissues [10]. Pin1 plays important role in chromosome condensation during mitosis. Pin1 interaction with chromatin increases during G2/M phase. It has been shown that Pin1 and cdc2/cyclin B kinase are by themselves sufficient to induce condensation [33]. Deletion of Pin1/Essl from yeast or HeLa cells induces mitotic arrest, whereas HeLa cells that over-express Pin1 are arrested in the G2 phase of the cell cycle [32]. Hence, Pin1 presumably regulates mitosis by interacting with mitotic phosphorylated proteins including Cdc25, NIMA, and Wee-1, and by attenuating its mitosis-promoting activities [12]. Knockout model of Pin1 revels that Pin1 is important for survival and proliferation of retina and mammary epithelial cells [10]. Further, Pin1 depletion causes impairment in primordial germ cell expansion, and leads to a profound decrease in fertility. Primordial germ cell had a marked increase in duration of the cell cycle owing to a prolonged G1-S transition [1]. Analyses of Pin1-null embryo fibroblasts (MEFs) provide additional support for a role of Pin1 in proliferation that is unrelated to its function in mitosis. It was reported that Pin1-null MEFs shows slower growth than wild-type and Pin1-null MEFs rendered quiescent owing to serum

Abbreviations: Pin1, peptidyl-prolyl *cis*-trans isomerase; DDC, 5-diethoxycarbonyl-1, 4-dihydrocollidine; PCNA, proliferating cell nuclear antigen; IGF-1, insulin-like growth factor 1; TGF-β, transforming growth factor-β.

* Corresponding author at: Department of Surgery, Chonbuk National University Medical School, 634-18 Geuman-dong, Duckjin-gu, Jeonju, 516-712, South Korea.
E-mail address: surgeon@jbnu.ac.kr (Y.J. Jeong).

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deprivation are markedly delay in cell cycle re-entry in response to insulin-like growth factor- 1 (IGF-1) [6]. The expression of Pin1 is enhanced by oncogenic *Neu/Ras* signaling through the activation of transcriptional factor E2F [22]. Moreover, it is a target of several oncogenic pathways and its expression is increased in transformed cell lines and some human cancer tissues. Molecular marker analysis has revealed close relationship between Pin1 and cyclin D1, which is known to play a key role in cancer [31]. Furthermore, Pin1 also regulates turnover and subcellular localization of β-catenin by binding to its pThr 246-Pro motif by preventing β-catenin to interact with the tumour suppressor APC (adenomatous polyposis coli gene product) [17,18,23].

Liver possess tremendous capacity to regenerate after toxic injury or surgical resection [4,14,24,28]. Under conditions of severe liver injury when hepatocyte proliferation is inadequate, the liver is repopulated by induction, proliferation, and differentiation of the progenitor cell compartment [5,15,25,27]. Although, different factors have been verified that contribute to oval cell-mediated liver regeneration, the signaling pathways that control oval cells proliferation still remains unclear. The aim of the present study is to find the role of Pin1 in the mechanism of liver repair through oval cell proliferation that may lead to the successful manipulation of liver biology for therapeutic purpose.

2. Material and methods

2.1. Chemicals and reagents

The chemicals and reagents used in this study were: Carbon tetrachloride (Sigma Aldrich, St. Louis, MO, USA), light mineral oil (Sigma Aldrich, St. Louis, MO, USA), Human TGF-β1 (R&D system, MN, USA), 0.1%, 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) diet grain based (BioServe, Frenchtown, NJ, USA), insulin like growth factor 1(IGF-1) (GroPep, Adelaide, Australia), juglone (Sigma Aldrich, St. Louis, MO, USA), OCT compound (Miles, Elkhart, IN, USA), Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12, Gibco, London, UK), RPMI (Hyclone, South Logan, UT, USA), Phosphate Buffered saline (Gibco, London, UK), trypsin EDTA (Hyclone, South Logan, UT, USA), penicillin-streptomycine solution (Hyclone, South Logan, UT, USA), ITS^{TM} premix containing insulin, transferrin, and selenium (BD Biosciences, Bedford, MA), dexamethasone (Sigma Aldrich, St. Louis, MO, USA) and 4′,6′-diamidino-2-phenylindole (DAPI, Molecular Probes, CA, USA).

2.2. Preparation of the recombinant adenovirus

To prepare Pin1-expressing adenovirus, the mouse Pin1 cDNA was cloned into the KpnI and XhoI sites of pENTR 2B (Invitrogen, CA, USA), and the Pin1 cDNA insert was transferred to the pAd/CMV/V5-DEST vector (Invitrogen, CA, USA) by the Gateway system using LR Clonase (Invitrogen, CA, USA). The plasmids linearized with PacI (Promega, WI, USA) were transfected into 293A cells using Lipofectamine 2000. Then 293A cells were cultured for 1–2 weeks in RPMI 1640 containing 10% FBS, with replacement of the medium every 2 days. Viruses were purified form the supernatants of 293A cells cultures by cesium chloride density gradient centrifugation. Virus titers were determined using a plaque assay using serial dilution. As a control, the pAd/CMV/V5-GW/LacZ vector (Invitrogen, CA, USA) was used to produce LacZ-bearing adenovirus.

2.3. Animals

Eight weeks old male FVB mice were purchased from Koatech (Pyeongtake, South Korea). The mice were house in 12-h light/dark cycles, with free access to food and water. The animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. The study was carried out in accordance with the ethical rules of the Chonbuk National University's institutional Animal Care and Use committee.

2.4. DDC diet model for chronic liver injury and oval cell induction

The mice were fed with a normal diet for two days after acclimatization and then the rodent diet was enriched with the porphyrinogenic liver stimulant 0.1% DDC which was given for indicated time as described by Burstein and Jakubowski et al. [2,9]. Oval cell induction and proliferation was confirmed by microscopic study of liver section prepared after two and three weeks of DDC diet feeding, by staining with Hematoxylin and eosin and also by immunohistochemistry using oval cell specific marker, CD44.

2.5. Cell culture and cells treatment

AML12 cells (ATCC, Manassas, VA, USA), was maintained in DMEM/F12 containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/mL streptomycin, 1000 μl/L of ITS^{TM} premix and 40 ng/mL of dexamethasone. The oval cell line was a generous gift from Dr. Karl G. Sylvester (Stanford University, USA) which was maintained in RPMI medium with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 μg/mL streptomycin as described previously [8]. AML12 and oval cells were seeded separately in 10 cm tissue culture dish and at around 70–80% confluency, media was changed to serum free medium for 12 h to arrest growth and synchronize cell activity. Cells were treated with 2 ng/mL of TGF-β or 50 ng/mL of IGF-1 in serum free medium for different time point to check the expression of Pin1 and cell cycle related proteins.

2.6. Cell proliferation and viability assay

For cell viability assay, oval cells (5×10^3) and AML12 (1×10^4) were cultured separately in the 96 well plates. After 24 h medium was changed to serum free and further incubated for 12 h. The cells were treated with either TGF-β, IGF-1or Juglone for indicated time and, the medium was aspirated and continued to culture. At different time point cell viability was assessed by using EZ-Cytox cell viability assay kit (iTSBiO, Seoul, Korea) according to the manufacturer's instructions.

2.7. BrdU incorporation after Pin1 knockdown

To knock-down Pin1, mouse Pin1 siRNA (ON-TARGETplus, SMARTpool, Thermo Fisher Scientific., Lafayette, CO) was used. In all experiments, control RNAi (ON-TARGETplus, GAPD Control pool, Thermo Fisher Scientific., Lafayette, CO) was used as a control. 5×10^3 oval cells were plated 96 well culture plate in antibiotics free medium containing 10% serum. After 12 h, transfection was carried out using lipofectamine reagents (Invitrogen, Carlsbad, CA) according to the vendor's protocol. Six hours after transfection, the medium was changed. BrdU at the final concentration of 10 mmol/L was added for the last 24 h. The tissue culture plates were washed, and a colorimetric BrdU cell proliferation assay was performed according to the manufacturer's instructions (Boehringer-Mannheim). BrdU incorporation in virus-transfected cells was expressed as percentage of BrdU incorporation in control wells.

2.8. Cell cycle analysis

The cells were seeded in 10 cm dishes and transfected with either, Ad-Pin1, Ad-LacZ, control RNAi or Pin1 siRNA. Six hours