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CD147-induced cell proliferation is associated with Smad4 signal inhibition

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ABSTRACT

CD147 is a multifunctional trans-membrane glycoprotein, which is highly expressed in many cancers. However, the mechanism by which CD147 modulates cell proliferation is not fully understood. The aim of this study is to investigate the role of CD147 in cell proliferation associated with the TGF-β/Smad4 signaling pathway. Here, we used cell viability and clone formation assays in LNCaP prostate cancer cells to demonstrate that CD147 promotes cell proliferation. The luciferase assay and western blotting show that silencing CD147 using shRNA enhances transcription and expression of p21[WAF1]. Using immunofluorescence and nuclear-cytoplasmic separation, we show that this is primarily attributed to transport of Smad4 from the cytoplasm to nucleus. Other assays (GST pull-down, co-immunoprecipitation and immunofluorescence) demonstrate that Smad4 is a new interaction partner of CD147, with the Smad4 MH2 domain and CD147 intracellular domain (CD147-ICD) being involved in the interaction. Furthermore, we report that a phosphoserine (pSer) in CD147 (pSer252) is responsible for this interaction and inhibition of the Smad4/p21[WAF1] signal that promotes cell proliferation. Our results provide a novel molecular mechanism for CD147-induced cell proliferation associated with Smad4 signal inhibition.

1. Introduction

CD147 (or BSG) is a 65 kDa transmembrane glycoprotein that belongs to the immunoglobulin (Ig) superfamily [1]. CD147 plays a pivotal role in normal tissue development [2,3], Alzheimer's disease (AD) [3], and various cancers [4–7]. The broadly expressed CD147 is a two Ig-like domain isoform of CD147 and is composed of 269 residues composed of an extracellular domain (206 residues), a transmembrane domain (24 residues) [8], and an intracellular domain (ICD, 39 residues) [9,10]. Although the CD147 gene encodes for a 29 kDa protein, it has a highly glycosylated mature form that migrates on SDS-PAGE like a 65 kDa species [11]. In addition, a three Ig-like domain isoform of CD147 has also been reported; however this isoform is rarely expressed [12]. Increasing evidence indicates that CD147 is involved in numerous processes related to tumor progression. For instance, CD147 directly regulates tumor cell adhesion [13], angiogenesis [14], chemoresistance [5], cell migration [15], invasion and metastasis [14–16]. However, its function in the regulation of cell proliferation is still poorly understood. Recently, CD147 has also been identified as a mediator of anti-apoptotic function and chemoresistance in several cancer cell lines [17,18], once again with the underlying mechanism of action being poorly understood.

Transforming growth factor-β (TGF-β) plays a pivotal role in both homeostasis and cell differentiation. Studies have found that the TGF-β signaling pathway not only inhibits tumor proliferation, but also promotes tumor progression, a function that mainly depends on Smad4 activation and deactivation [19,20]. Recently, crosstalk between TGF-β and CD147 has been reported. Here, activation of the TGF-β1-CD147 complex was shown to induce a positive feedback loop in the regulation of hepatic stellate cells that facilitate liver fibrosis [21]. However, the correlation between CD147 and Smad4 remains unclear.

The inhibition of cell proliferation induced by Smad4 is mediated by p21[WAF1] that are inhibitors of cyclin-dependent kinase (CDK) [22]. p21[WAF1] is one of the Smad4/DPC4-regulated downstream target genes, and over-expression of the Smad4 gene can bypass TGF-β receptor activation and re-establish one of the key regulatory controls

Abbreviations: CD147, cluster of differentiation 147; PCa, prostate cancer; TGF-β, transforming growth factor-β; CD147-ICD, CD147 intracellular domain; FBS, fetal bovine serum; PFA, paraformaldehyde; PMSF, phenylmethanesulfonyl fluoride

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of cell proliferation [23]. Following TGF-β and receptor binding, phosphorylated R-Smads form complexes with the common-mediator (Co-) Smad4 which is translocated to the nucleus to activate cell cycle inhibition genes, such as $p21^{WAF1}$ [24].

Until now, direct evidence for the crosslink between CD147 and the Smad4/$p21^{WAF1}$ pathway has been lacking. Therefore, we initiated the current study to understand the correlation between CD147 and the Smad4/$p21^{WAF1}$ pathway in mediating the inhibition of cell proliferation inhibition and examining the roles of CD147 in $p21^{WAF1}$ regulation, one of the downstream target genes of the Smad4 pathway. We also explored the CD147 phosphorylation site involved in Smad4/$p21^{WAF1}$ pathway regulation.

2. Materials and methods

2.1. Plasmids construction

The Flag-tagged Smad4 was a gift from Dr. Hsiu-Ming Shih (Taipei, China) [25]. The MH1、Linker and MH2 domain of Smad4 were amplified by PCR and then cloned into pGEX4T−1 vector. The CD147/GFP expression plasmid was kindly provided by Bryan P. Toole [26]. CD147 intracellular domain was constructed to pGEX4T−1 vector (GST-CD147-ICD), and then used site-directed mutagenesis to construct GST-CD147-ICD/S246A and GST-CD147-ICD/S252A. The same strategy was used to construct CD147/S246A and CD147/S252A. A double-stranded oligonucleotide corresponding to each of the human CD147 sequences (5′-gggcctcccagagtgaaggct-3′, 5′-gggccggtcagagctacacat-3′, 5′-gggctccgaccaggccatcat-3′ was synthesized and cloned into the pBS/U6 vector, to make each of these short hairpin RNA (shRNA) constructs of CD147). A double-stranded oligonucleotide corresponding to the human Smad4 cDNA sequence (5′-gggcagccaatgtgaatgact-3′) was synthesized and cloned into the pBS/U6 vector, to make Smad4-shRNA construct.

2.2. Cell culture and transient transfection

The human prostate cancer cell line, LNCaP, was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China) and cultured in T-medium (GIBCO, Grand Island, NY, USA; custom formula # 02−0056DJ, 80% DMEM, 20% F12K, 3 g/L $NaHCO_3$, 5 mg/ml insulin, 13.6 pg/ml triiodothyronine, 5 mg/ml transferrin, 0.25 mg/ml biotin, 25 mg/ml adenine) plus 10% FBS. Human benign prostate hyperplasia epithelial cells (BPH-1) and human prostate cancer PC-3 cells, were originally purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Science (Shanghai, China) and maintained in DMEM medium with 10% FBS.

Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were seeded into 48-well plates for 16 h and co-transfected with each of four reporter plasmids 100 ng, including two $p21^{WAF1/Cip1}$ promoter reporters, p21P-luc and p21PΔp53-luc, in the presence of Renilla luciferase control pREP7 vector 25 ng, firefly luciferase activities were measured by using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) and the ratio of firefly luciferase activity to Renilla luciferase activity was calculated as relative luciferase activity.

2.3. Cell viability and clone formation assays

Cell viability was assessed by using the MTS assay. Cells (4×10^3/well) were seeded in 96-well plates. 24 h later after transfected, MTS was added to each well, and measured at 490 nm wavelength after incubated for 4 h at 37 °C. Transfected cells (50 cells/dish) were seeded on 35 mm dishes and cultured. After half a month, the colonies were fixed in 3.7% PFA, and dyed with 0.1% crystal violet, counted the number of colonies under the microscope after air dried the plates.

2.4. Co-immunoprecipitation and western blotting

The cells transfected for 2 days were lysed on ice with NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 8.0, 1% NP-40 (v/v), 1 mM PMSF). Lysates were collected by centrifugation at 15,000 g for 10 min at 4 ℃, then proteins were subjected to specific immunoprecipitation with 1 μg/ml of the appropriate antibody, CD147 or Smad4 (Santa Cruz Biotechnology, USA) for 4 h at 4 ℃ , added 50 μl of protein A/G-Sepharose beads to the mixture and then incubated for 1 h at 4 ℃ with gentle shaking. The beads were washed four times with ice-cold NP-40 lysis buffer. The bound proteins were eluted using SDS-PAGE sample buffer boiling for 10 min. The immunoprecipitated proteins were analyzed by western blotting.

In the immunoblotting assays, cells were washed twice with ice-cold PBS before lysed on ice for half an hour in RIPA buffer containing 1% Sodium deoxycholate (w/v), 150 mM NaCl, 0.1% SDS (w/v), 1% Triton X-100 (v/v), 10 mM Tris-HCl (pH 7.4), 1 mM PMSF. Total lysates were fractionated by SDS-PAGE and transferred to PVDF membranes, the membranes were incubated with 5% (w/v) skimmed milk in TBST, followed incubated with the indicated primary antibodies, including CD147, Smad4, p21WAF1, p53 and β-actin (Santa Cruz Biotechnology, USA) antibodies, at room temperature for 2 h, and the HRP conjugated secondary antibodies were used at room temperature for 1 h. The signal was detected with ECL Plus kit (Beyotime Biotechnology, Beijing, China) in terms of the manufacturer's instructions.

2.5. Immunofluorescence

Cells were co-transfected with each CD147 or CD147-shRNA and Smad4 plasmids and then fixed with 3% formaldehyde for 30 min. After being blocked in PBS-BSA buffer for 15 min, the cells were incubated with monoclonal Smad4 antibodies for 2 h, followed by incubation with fluorophore-conjugated secondary antibodies (Proteintech Group, Chicago, IL, USA). DAPI staining for 1 min was carried out after secondary antibody incubation.

2.6. Nuclear and cytosolic separation

The separation of the nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.7. GST pull-down assay

To generate GST-fusion proteins, E.coli strain BL-21 cells were transformed with corresponding plasmids by adding 0.1 mM IPTG at 30 ℃ for 5-h, and centrifuge at 15,000 g for 15 min at 4 ℃. Glutathione-Sepharose Beads can be used for affinity purification of proteins. Cells were plated in 6 wells plate, and lysed after transfected 48 h in RIPA buffer on ice for 30 min. Followed, same amounts of GST or GST-intracellular domain of CD147 fusion proteins were captured on Glutathione-Sepharose Beads, and then incubated with cell lysates for 2 h at room temperature or overnight at 4 ℃. Finally, Beads were washed four times with GST washing buffer (120 mM NaCl, 50 mM Tris-HCl at pH8.0, 0.25% NP-40, 1 mM DTT, 1Mm PMSF) and analyzed by western blot.

2.8. Statistical analysis

All results are presented as the mean ± S.D. The Student's *t*-test was used to compare means of two independent groups. A One-way ANOVA was applied to analyze the difference of means of more than two groups. Statistical significance was assumed for a two-tailed $P < 0.05$ using SPSS17.0 (Chicago, IL, USA).