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Transforming growth factor-β1 induces cell cycle arrest by activating atypical cyclin-dependent kinase 5 through up-regulation of Smad3-dependent p35 expression in human MCF10A mammary epithelial cells

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ABSTRACT

Cyclin-dependent kinases (Cdks) play important roles in control of cell division. Cdk5 is an atypical member of Cdk family with non-cyclin-like regulatory subunit, p35, but its role in cell cycle progression is still unclear. In the present study, we investigated the role of Cdk5/p35 on transforming growth factor-β1 (TGF-β1)-induced cell cycle arrest. In human MCF10A mammary epithelial cells, TGF-β1 induced cell cycle arrest at G1 phase and increased p27KIP1 expression. Interestingly, pretreatment with roscovitine, an inhibitor of Cdk5, or transfection with small interfering (si) RNAs specific to Cdk5 and p35 significantly attenuated the TGF-β1-induced p27KIP1 expression and cell cycle arrest. TGF-β1 increased Cdk5 activity via up-regulation of p35 gene at transcriptional level, and these effects were abolished by transfection with Smad3 siRNA or infection of adenovirus carrying Smad3 mutant at the C-tail (3SA). Chromatin immunoprecipitation assay further revealed that wild type Smad3, but not mutant Smad3 (3SA), binds to the region of the p35 promoter region (−1000–−755) in a TGF-β1-dependent manner. These results for the first time demonstrate a role of Cdk5/p35 in the regulation of cell cycle progression modulated by TGF-β1.

1. Introduction

Transforming growth factor-β1 (TGF-β1) is a growth inhibitory cytokine that plays an important role in the maintenance of tissue homeostasis [1]. Loss of anti-proliferative response to TGF-β1 is implicated in tumorigenesis [2]. Many types of cancer cells are refractory to growth inhibition induced by TGF-β1. Understanding the molecular mechanisms underlying TGF-β1-induced growth inhibition may provide critical insights into how cancer cells escape from cytostatic TGF-β1 response and possibly unveil novel targets for therapeutic intervention.

TGF-β1 induces cell cycle arrest at the G1 phase by suppressing cyclin-dependent kinase (Cdk) activity through the induction of Cdk inhibitors, including p15INK4B, p21WAF1 or p27KIP1 [3–5]. Smad3 plays an important role in transcriptional regulation of cell cycle regulators in response to TGF-β1. TGF-β1-activated Smad3 induces transcription of the Cdk inhibitor p21WAF1 through interaction with the transcription factors Sp1 and FoxO1 [6]. In TGF-β1-stimulated epithelial cells, Smad3, in concert with KLF11, acts as a transcriptional repressor of the c-myc oncogene, a negative regulator of Cdk inhibitors [7]. Smad3 also contributes to TGF-β1-mediated stabilization of p27KIP1 protein through decreasing Skp2 expression [8]. Primary cells from Smad3-deficient mice are insensitive to the growth inhibitory effects of TGF-β1 [9]. Ectopic expression of Smad3 in epithelial cells transformed with oncogenic Ras restores sensitivity to TGF-β1-induced growth inhibition [10]. From these observations, Smad3 has a key function in transducing anti-proliferative TGF-β1 signal.

Cyclin-dependent kinases (Cdks) and their co-activator cyclins form a family of heterodimeric kinases that play central roles in the regulation of cell cycle progression [11]. Growth factor-activated Cdk2/cyclin E and Cdk4/cyclin D exert their growth-promoting activities by stimulating cell cycle progression from the G1 to S phase [12]. Dysregulated activation of Cdks are directly linked to

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proliferation of cancer cells [13]. Inactivation of Smad3 activity by Cdk2 or Cdk4 phosphorylation confers resistance to the TGF-β growth inhibitory effects [14].

Cdk5 is an atypical Cdk that is expressed predominantly in postmitotic neurons [15]. Cdk5 is activated by binding to its non-cyclin cofactor, p35, and plays a central role in nervous system development. Recent growing evidences also indicate that beyond its role on nervous system, Cdk5 modulates diverse cellular functions. It regulates glucose-stimulated insulin secretion in pancreatic β cells [16] and controls proliferation, survival and metastatic potential of cancer cells [17,18]. However, its role in cell cycle progression still remains to be unclear. In this study, we investigated the role of Cdk5/p35 in TGF-β1-induced cell cycle arrest of MCF10A epithelial cells.

2. Materials and methods

2.1. Reagents and antibodies

The recombinant TGF-β1 was purchased from R&D systems (Minneapolis, MN). Roscovitine was obtained from Cayman Chemical (Ann Arbor, MI). SIS3 was from Sigma–Aldrich (St. Louis, MO). U0126, SP600125 and SB203580 were obtained from Calbiochem (La Jolla, CA). Smad3, Myc, p27KIP1, Cdk4, Cdk5, p35 and SKP2 antibodies, and control, p35, Cdk4 and Cdk5 siRNAs were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and transfection

MCF10A mammary epithelial cell line was obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in DMEM/F-12 containing 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml insulin, 20 ng/ml EGF, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone. Transient transfection into MCF10A cells was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer.

2.3. Real-time PCR

Total cellular RNA was extracted from cells using the phenolguanidinium isothiocyanate method. Oligo(dT) primed synthesis of cDNA from 2 μg of total RNA was made using Super-ScriptTM III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed using a SYBR Green PCR Matrix Mix (Applied Biosystems, Foster City, CA) and run on an Applied Biosystems 7300 Real-Time PCR System as described by the manufacturer. Primers used for real-time PCR as follows; human p35 (sense, 5′-GCC CTT CCT GGT GAG GAG CTG-3'; antisense, 5′-GTG TGA AGT AGT GTG GGT CGG C-3′) and control GAPDH (sense, 5′- CAATGACCCCTTCATTGACC-3'; antisense, 5′-GACAAGCTTCCCGTTCTCAG-3′). Results were expressed as a ratio: p35 mRNA/GAPDH mRNA.

2.4. Cdk5 kinase activity assay

The treated cells were lysed in 500 μl of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Proteins (500 μg) from the lysates were immunoprecipitated with 2 μg of an anti-CDK5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with protein A/G-Sepharose beads for 2 h at 4 °C. Kinase assay was performed with an ATP-GloTM kinase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, immunoprecipitates were washed three times with lysis buffer and then twice with reaction buffer containing 40 mM Tris–HCl, pH7.5, 20 mM MgCl2, 0.1 μg/μl BSA and 0.5 mM DTT. The kinase reaction was carried out for 10 min at room temperature in the presence of 200 μM ATP and 5 μg substrate histone H1. Then, 25 μl ADP-GloTM reagents were added to terminate the reaction and deplete the remaining ATP. After 50 μl kinase detection reagents were added into the mixture to incubate for 30 min, the results were recorded by measuring the luminescence with a plate-reading luminometer. Data are shown as relative light units (RLU) that directly correlate to the amount of ADP produced.

2.5. Chromatin immunoprecipitation (ChIP) assay

Cells were cross-linked with 1% formaldehyde at room temperature for 15 min, rinsed with ice-cold PBS, and collected. Cells were then resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, 1x protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)) and sonicated ten times for 10 s, followed by centrifugation for 10 min at 13,000 rpm. The sheared chromatin was incubated on a rolling shaker overnight at 4 °C with antibody (2 μg per reaction) and protein A-Sepharose beads (45 μl of a 50% slurry in 10 mM Tris–HCl [pH 8.1] and 1 mM EDTA). The antibody-bound protein/DNA complexes were precipitated and washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl [pH 8.1], 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl [pH 8.1], 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl [pH 8.1]). The captured genomic DNA was eluted, cross-linking was reversed at 94 °C for 15 min, and proteins were removed by treatment with Proteinase K at 37 °C for 1 h. Ten percent of total genomic DNA from the nuclear extract was used as input. The primers used for detection of human p35 promoter sequence as follows; ChIP1 (sense, 5′- AGGTCATATTTGTGACGGGC-3'; antisense, 5′- GTCCCTGTGGAAAGCAGCCA-3′). ChIP2 (sense, 5′-ATGTCAGGTGGAAGGAGGGA-3'; antisense, 5′-GCGCAACCCAACTCTGCCGA-3′), ChIP3 (sense, 5′-ACAAAGGCAGCACGTGTCTG-3'; antisense, 5′-CATCTAGGAGCCGGACTCCG-3′), ChIP4 (sense, 5′-CTCGAGCTGAGGACACCAAC-3'; antisense, 5′-CAGCTAGGGAGCTTCTGTCC-3′). The samples were amplified by conventional PCR, using the above p35 promoter-specific forward and reverse primers and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA).

2.6. Additional methods

Immunoblotting, flow cytometry analysis, and semiquantitative RT-PCR were as previously described [19].

2.7. Statistical analysis

Statistical analyses were performed using SigmaPlot 2001 (Systat Software, Inc, Richmond, CA). Statistical significance was assessed by comparing the means values (±SD) using a Student's *t*-test for paired data.

3. Results and discussion

3.1. Cdk5/p35 is required for TGF-β1-induced cell cycle arrest in MCF10A cells

To study the role of Cdk5 on TGF-β1-induced cell cycle arrest, we first examined the effect of roscovitine, an inhibitor of Cdk5 in human mammary epithelial MCF10A cells. Cell cycle analysis with flow cytometry revealed that pretreatment of MCF10A cells with roscovitine resulted in significant attenuation of TGF-β1-induce cell