ELSEVIER

CYTOKINE

www.elsevier.com/locate/issn/10434666

Cytokine 37 (2007) 155–162

TGFβ regulates the expression and activities of G2 checkpoint kinases in human myeloid leukemia cells

Xiaotang Hu [a,e,*], Dongming Cui [a], Lynn C. Moscinski [a,c], Xiaohong Zhang [a], Vivian Maccachero [e], Kenneth S. Zuckerman [a,b,d]

[a] *Interdisciplinary Oncology Program, University of South Florida, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA*
[b] *Department of Biochemistry and Molecular Biology, University of South Florida, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA*
[c] *Department of Pathology, University of South Florida, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA*
[d] *Department of Internal Medicine, University of South Florida, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA*
[e] *School of Natural and Health Sciences, Barry University, 11300 Northeast Second Avenue, Miami Shore, FL 33161, USA*

Received 29 October 2006; received in revised form 18 March 2007; accepted 20 March 2007

Abstract

Transforming Growth Factor-beta (TGFβ) is known to be a negative regulator of G1 cyclin/cdk activity. It is not clear whether TGFβ has any effect on G2 checkpoint kinases. We have found that TGFβ downregulated the expression of several G2 checkpoint kinases including cdc2, cyclin B1, and cdc25c without causing cell accumulation in G2/M phases in two human leukemia cell lines. The inhibition was time-dependent with a maximal inhibition being observed by 24 h for cyclin B1 and cdc2 and by 48 h for cdc25c. The inhibition was not a result of G1 arrest but a direct effect of TGFβ which downregulates their expression at mRNA level. In proliferating cells, there was a significant formation of cdc2–pRb complexes, which was decreased to 30% of control levels by 48 h after initiating TGFβ treatment. Cdc2 showed a marked kinase activity on GST-Rb protein in proliferating cells detected by *in vitro* kinase assay, which was downregulated in response to TGFβ. In addition, TGFβ caused a rapid and transient dephosphorylation of cdc2 (Tyr15) and cdc25c (Ser216) for about 2–3 h before a dramatic decrease of both molecules by 48 h. Taken together, our data suggest that TGFβ has a direct inhibitory effect on G2 checkpoint kinases, which is regulated at mRNA level. The transient activation of cdc2 and cdc25c and subsequent inhibition of cdc2, cyclin B1, and cdc25c could amplify TGFβ-induced G1 arrest and growth inhibition.

Keywords: Leukemia cells; Growth factors; Cell proliferation; Cell cycle; Cancer; Cytokine; Signalling

1. Introduction

All growing cells undergo a cell cycle. In mammalian cells, Gap phases exist between the replication of DNA in S phase and the segregation of chromosomes during M phase. The Gap phases termed G1 and G2 serve as important check points to integrate information regarding the control of a cell to progress into S or M, which is regulated by the action of cyclin-dependent kinases (cdks) and their regulatory subunits, the cyclins. D-type cyclins-associated cdk4 and/or cdk6 kinase activities have been detected in mid G1, prior to the activation of any other known cdk [1–3]. The cyclin E-bound cdk2 exhibits kinase activities at the G1/S transition [1,4–7]. Thus, these kinases are termed G1 checkpoint kinases. pRb is the product of the retinoblastoma tumor suppressor gene. pRb acts as a signal transducer connecting the cell cycle clock with the transcriptional machinery. Dephosphorylation of pRb negatively regulates cell cycle progression by binding to transcription factor, E2F. In contrast, phosphorylation of pRb results in the loss of its growth suppression property

* Corresponding author. Address: School of Natural and Health Sciences, Barry University, 11300 Northeast Second Avenue, Miami Shore, FL 33161, USA. Fax: +1 305 899 3225.
E-mail address: xthu@mail.barry.edu (X. Hu).

1043-4666/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2007.03.009

[8,9]. G1 checkpoint kinases regulate pRb phosphorylation [10]. When cells receive a growth signal, cdk4 and cdk6 phosphorylate pRb and release transcription factor E2F from pRb complexes. As a result, E2F-dependent DNA transcription is activated and the proteins required for the cell cycle progress are produced. The effects of E2F on cell cycle progress have been well established. For example, when overproduced in cultured cells, E2F can activate DNA transcription, drive G1-arrested cells into S phase, and act as an oncogene. Conversely, inhibition or absence of E2F activity blocks cells in G1 phase [10–16].

The critical role of cdc2 in cell cycle control has been reported. In the yeast strain *Schizosaccharomyces pombe*, both the G2-M and G1-S transitions are triggered by the activity of a single protein kinase, cdc2. In higher eukaryotes, cdc2 only regulates the G2-M transition, primarily in association with cyclin B. Thus, cdc2 is termed G2 checkpoint kinase. Activation of cdc2 is controlled at several steps including cyclin binding and phosphorylation of Thr161 [17]. However, the critical regulatory step in activating cdc2 during progression into mitosis appears to be dephosphorylation of Tyr15 and Thr14, catalyzed by members of the cdc25 family of phosphatases [18,19].

TGFβ is a potent regulator of cellular proliferation, differentiation, and morphogenesis. A major effect of TGFβ is its ability to inhibit cell proliferation. Previous studies have demonstrated that the inhibitory role of TGFβ is a result of retaining cells in G1 phase and blocking the G1/S transition, which is linked to destruction of cyclin E–cdk2 assembly and the inhibition of cdk4 synthesis [20–23]. In addition, TGFβ has an inhibitory effect on the phosphorylation of pRb, leading to the inhibition of E2F-initiated DNA transcription [24,25]. The observations described above have led to the conclusion that TGFβ-induced inhibition of G1 checkpoint kinase activities and dephosphorylation of pRb are the major mechanisms linked to G1 arrest and cell proliferation inhibition [24,25]. There are few reports regarding the regulatory role of TGFβ1 on G2 cyclins/cdks with an exception, in which TGFβ-induced downregulation of cdc2 has been observed in mouse epithelial cells [26]. However, it is not clear whether the downregulation of cdc2 had any impact on cell cycle progress and cell proliferation.

In order to gain further insight into the inhibitory effects of TGFβ on cell proliferation, we examined the effects of TGFβ on several G2 checkpoint kinases with a focus on cdc2. We show here that TGFβ1 inhibited not only G1 but also G2 checkpoint kinases, which is regulated at mRNA level. We also detected that cdc2 is associated with pRb and affected pRb phosphorylation.

2. Materials and methods

2.1. Cytokines and antibodies

Recombinant human transforming growth factor-beta1 (TGFβ1) was purchased from R&D Systems (Minneapolis, MN). Antibodies used and their sources were as follows: p34 cdc2, cdc25c, cyclin B1, cdk4, cdk7, cyclin D2, cyclin D3 , and actin (Santa Cruz Biotechnology, Santa Cruz, CA); pRb, HRP-conjugated anti-rabbit IgG (New England Biolabs, Beverly, MA), and HRP-conjugated anti-mouse IgG (Amersham, Arlington Heights, IL).

2.2. Reagents

The kits for assays of cell proliferation (MTT) were purchased from Boehringer Mannheim (Indianapolis, IN). Protein A-Sepharose and protein G-Agarose were obtained from Pharmacia (Piscataway, NJ) and Invitrogen/Life Technology (Grand Island, NY), respectively. [γ-^{32}P]ATP was supplied by ICN (Costa Mesa, CA). Both radioactive and non-radioactive RNase Protection Assay kits were purchased from BD Biosciences (San Diego, CA). Aphidicolin and Nocodazole were obtained from BioMol (Polymouth Meeting, PA). The culture media and fetal bovine serum (FBS) were purchased from Invitrogen/Life Technology (Grand Island, NY).

2.3. Maintenance of cell lines

Human MV4-11 and TF-1 cell lines were purchased from ATCC (Manassas, VA). These cell lines were routinely maintained in Iscove's Modified Dulbecco's Medium (IMDM) (for MV4-11) or RPMI 1640 (for TF-1) supplemented with 10% fetal bovine serum (FBS) in the absence (for MV4-11) or presence of GM-CSF (for TF-1).

2.4. Assays of cell proliferation

Cell proliferation was examined by counting cells directly with a hemocytometer and indirectly by MTT colorimetric assays. Cells were cultured in RPMI 1640 (for TF-1) or IMDM (for MV4-11) supplemented with 10% fetal calf serum in the presence or absence of GM-CSF or TGFβ1. The tetrazolium salt MTT is metabolized by NAD-dependent dehydrogenase activity to form a colored reaction product. The amount of dye formed directly correlates with the number of viable cells. Briefly, cells were grown in microplates in a final volume of 100 μl of RPMI 1640 or IMDM supplemented with 10% or 20% fetal calf serum in the presence or absence of GM-CSF or TGFβ1. After 48–72 h incubation, 10 μl of the MTT labeling reagent was added to the cells and incubated for 4 h in a humidified atmosphere air containing 5% CO_2. After solubilization, the formazan dye was quantitated using a microplate (ELISA) reader at 550 nM, as recommended by the manufacturer.

2.5. RNA isolation

Total RNA was isolated from MV4-11 and TF-1 cell lines, using Trizol reagent (Invitrogene/GibcoBRL) following the manufacturer's instruction.