Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
The International Journal of Biochemistry & Cell Biology 37 (2005) 1921–1929
IJBCB
www.elsevier.com/locate/biocel

Investigation of cell cycle arrest effects of actinomycin D at G1 phase using proteomic methods in B104-1-1 cells

Hyae-Kyeong Kim [a], Mi-Young Kong [a], Moon-Jin Jeong [a], Dong-Cho Han [a], Jung-Do Choi [b], Hak-Yong Kim [b], Kab-Seok Yoon [a], Jung-Min Kim [a], Kwang-Hee Son [a], Byoung-Mog Kwon [a,c,*]

[a] *Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Republic of Korea*
[b] *Department of Biochemistry, Chungbuk National University, Cheongju 361-763, Republic of Korea*
[c] *Biomolecular Science, University of Science and Technology in Korea, Taejeon, Republic of Korea*

Received 28 February 2005; received in revised form 20 April 2005; accepted 28 April 2005

Abstract

Actinomycin D was previously reported as an inhibitor of Shc/Grb2 interaction in B104-1-1 cells. Actinomycin D arrested the cell cycle at the G1 phase at 1 nM, which is about 10 times lower than the inhibition of Shc/Grb2 interactions in B104-1-1 cells. To evaluate other mechanisms of actinomycin D affected suppression of tumors and cell growth, except inhibition of Shc/Grb2 interactions, we examined the proteomic expression profile by proteomic technology. We found up-regulation of MEKK3 and down-regulation of Hsp70 expression from proteomic analysis, which is a very interesting observation because MEKK3 is strongly related with G1 arrest of cell cycle and Hsp70 is also involved in cell cycle regulation. These results indicate that the anti-tumor effects of actinomycin D is due to synergic effects of various proteins regulated by the compound including inhibition of the Shc/Grb2 interaction and other signaling pathways in the cytoplasm. Here we provide a mechanism-based explanation for growth inhibition by actinomycin D using proteomic technology. Thus, this approach may be a potentially useful method to reveal new mechanisms of active compounds or drugs with unknown cellular function.

Keywords: Actinomycin; Proteomics; Cell cycle; MEKK3; Hsp70

1. Introduction

ErbB-2 is often overexpressed in adenocarcinomas of the breast, stomach, and ovary (Harris, Eichholtz, Hiles, Page, & O'Hare, 1999) and involved in action of

Abbreviations: AMD, actinomycin D; Hsp, heat shock protein; hTM, human tropomyosin; MEK, MAP kinase kinase

* Corresponding author. Tel.: +82 42 860 4557; fax: +82 42 861 2675.
E-mail address: kwonbm@kribb.re.kr (B.-M. Kwon).

1357-2725/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2005.04.015

the Ras signaling pathway. Tyrosine phosphorylation of Shc and formation of the Shc/Grb2 complex occurs in B104-1-1 (*neu**-transformed NIH3T3) cells that express mutation-activated ErbB-2, and in human breast cancer cells that overexpress the ErbB-2 protein (Xie, Li, & Hung, 1995; Xie, Pendergast, & Hung, 1995). The Shc/Grb2/SOS pathway plays an important role in oncogenic signaling of mutation-activated ErbB-2 (Xie et al., 1995a,b). Recent studies have indicated that the cellular signals from EGFR to Ras/MAP kinase can be inhibited by antagonists of the Grb2 SH2 domain (Williams et al., 1997). Thus, compounds that can disrupt the interaction between Grb2 SH2 and some proteins can inhibit Ras activation, leading to blockage of malignant cell growth that is activated by the Ras signaling pathway (Gay et al., 1999).

From screening for Shc/Grb2 interaction inhibitors, AMD (actinomycin D), AMC2 (actinomycin C2), and AMVII (actinomycin VII) were identified from microbial origins (Nam et al., 1998).

AMD being our main concern, we examined its effect on the growth of B104-1-1 tumor xenografts implanted into nude mice. Tumor growth was inhibited in vivo after treatment with the inhibitor. Efficacy was correlated with a reduction in the levels of Shc/Grb2 binding in excised tumors (unpublished results). These results indicate that AMD may be a potentially useful agent for treatment of specific tumors caused by EGFR or the ErbB-2 aberrance.

Whereas the concentration of DNA intercalation requires a high dose (3–10 μM), cytotoxicity in different tumor cell lines appears at low doses (<10 nM). The mechanism of low dose inhibition is not well defined. AMD, as a novel SH2 domain ligand, inhibited Shc/Grb2 interaction in B104-1-1 cells with an IC_{50} value of 10 nM (Kim et al., 1999). However, the cytotoxicity of AMD against B104-1-1 cells appeared at lower concentrations (GI_{50} value of 1 nM) than that of inhibition of Shc/Grb2 protein-protein interactions. These results indicate that AMD affected multiple cellular events as well as the inhibition of Shc/Grb2 interactions. To unveil the cellular regulatory mechanism of AMD, except previously known functions, we compared proteome profiles in AMD-treated and untreated B104-1-1 cells by 2-DE and MALDI-TOF MS.

2. Materials and methods

2.1. Cell lines and cell cycle analysis

B104-1-1 and NIH3T3 cells were purchased from ATCC. B104-1-1 and NIH3T3 were grown in DMEM, supplemented with 10% FBS and 50 μM 2-mercaptoethanol at 37 °C in a humidified 5% CO_2 atmosphere. B104-1-1 cells were seeded in T-75 cm^2 flasks and treated the next day for 24 h with either AMD or 0.1% DMSO. Single-cell suspensions were collected using Trypsin-EDTA and cell pellets were fixed in ice-cold 70% ethanol for 24 h at −20 °C. The next day the samples were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4). After centrifugation, propidium iodide (30 μg/ml) and RNAse (125 μg/ml) were added to the pellets for 30 min at 20 °C. Analysis of the cell cycle was performed using a FACS Calibur instrument (Becton Dickinson, Franklin Lakes, NJ, USA).

2.2. Two-dimensional electrophoresis

B104-1-1 cells were seeded in T-185 cm^2 flasks and treated the next day for 48 h with either AMD or 0.1% DMSO. After treatment, cells were washed twice with phosphate-buffered saline and collected directly in a sample buffer (8 M urea, 4% CHAPS, 0.5% dithiothreitol (DTT), IPG buffer pH 4–7 or pH 5–8). The lysates were aliquotted and stored at −70 °C. Protein concentrations were determined by the Bradford assay.

Isoelectrofocusing was performed in strips with immobilized pH gradient (pH 4–7 or 5–8 linear gradient, 17 cm, Bio-Rad). Samples (1 mg of proteins) were applied with the rehydration techniques. First-dimension electrophoresis was performed in IEF Cell (Bio-Rad) according to the manufacturer's recommendation and according to Gorg et al. (2000). After the isoelectrofocusing, strips were equilibrated in 50 mM Tris–HCl, pH 8.8, 6 M urea, 2.0% SDS, 30% glycerol, with 130 mM DTT, (10 min) and then in the same solution without DTT but with 135 mM iodoacetamide (10 min). SDS-PAGE of equilibrated strips was performed for 14–18 h. After electrophoresis, gels were stained in colloidal staining solution using coomassie brilliant blue G-250.