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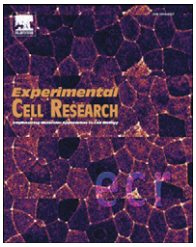

Research Article

Up- or downregulation of tescalcin in HL-60 cells is associated with their differentiation to either granulocytic or macrophage-like lineage

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ABSTRACT

Tescalcin is a 25-kDa EF-hand Ca^{2+}-binding protein that is differentially expressed in several mammalian tissues. Previous studies demonstrated that expression of this protein is essential for differentiation of hematopoietic precursor cell lines and primary stem cells into megakaryocytes. Here we show that tescalcin is expressed in primary human granulocytes and is upregulated in human promyelocytic leukemia HL-60 cells that have been induced to differentiate along the granulocytic lineage. However, during induced macrophage-like differentiation of HL-60 cells the expression of tescalcin is downregulated. The decrease in expression is associated with a rapid drop in tescalcin mRNA level, whereas upregulation occurs via a post-transcriptional mechanism. Tescalcin is necessary for HL-60 differentiation into granulocytes as its knockdown by shRNA impairs the ability of HL-60 cells to acquire the characteristic phenotypes such as phagocytic activity and generation of reactive oxygen species measured by respiratory burst assay. Both up- and downregulation of tescalcin require activation of the MEK/ERK cascade. It appears that commitment of HL-60 cells toward granulocytic versus macrophage-like lineage correlates with expression of tescalcin and kinetics of ERK activation. In retinoic acid-induced granulocytic differentiation, the activation of ERK and upregulation of tescalcin occurs slowly (16–48 h). In contrast, in PMA-induced macrophage-like differentiation the activation of ERK is rapid (15–30 min) and tescalcin is downregulated. These studies indicate that tescalcin is one of the key gene products that is involved in switching differentiation program in some cell types.

Introduction

HL-60 cell line is an established *in vitro* model to study cellular differentiation and signal transduction. These cells were originally isolated from a patient with acute myeloblastic leukemia with maturation, FAB-M2 [1,2]. HL-60 cells can be induced to terminally differentiated granulocytes or monocytes/macrophages in response to a variety of inducers [3–8].

Previous studies demonstrated that differentiation of HL-60 cells requires sustained activation of ERK1 and/or ERK2-extracellular signal-regulated kinases that belong to the mitogen-activated protein kinase (MAPK) family. ERK1 and ERK2 are activated by

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highly homologous dual specificity kinases MEK-1 and MEK-2 [9]. Sustained activation of MEKs and ERKs was observed during both cytokine- and chemically-induced myeloid differentiation [10–16]. While it is established that MEK/ERK signaling is essential for myeloid differentiation of hematopoietic cell lines and primary progenitor cells, the exact mechanisms whereby this pathway affects myelopoiesis are incompletely understood. A number of studies suggest that the MEK/ERK/MAPK pathway is central for linking various extracellular ligands to their multiple cellular target proteins that activate myeloid transcription factors and other specific mechanisms that promote differentiation [13,15,16]. For example, activated ERK1 and ERK2 phosphorylate a number of different substrates, including kinase $p90^{RSK}$, Ets family transcriptional factor Elk-1, AP-1, c-Myc, and STATs [17–21].

Tescalcin was discovered as an autosomal gene that is differentially expressed in embryonic gonads [22]. This conserved gene encodes a 24-kDa protein with a single functional EF-hand domain that can bind Ca^{2+} with micromolar affinity [23,24]. *In vitro* studies showed that tescalcin can interact with cytoplasmic tail of Na^+/H^+ exchanger [25–28] and can inhibit the phosphatase activity of Calcineurin A [23]. However, whether these observations might relate to the *in vivo* functions of tescalcin is not known. Tescalcin is expressed predominantly in the mouse heart, brain, stomach and testis, as well as in mouse and human primary hematopoietic progenitor cells and cell lines [12,23]. During differentiation and maturation of megakaryocytes, the expression of tescalcin is dramatically increased upon sustained activation of ERK1/2. Furthermore, tescalcin was shown to be a critical factor in megakaryocytic differentiation that is necessary for coupling MEK/ERK cascade with expression of Ets family transcription factors [12].

In this paper, we examined the expression of tescalcin in HL-60 cells and found that it was transcriptionally and post-transcriptionally regulated during induced differentiation of these cells and that it was required for optimal granulocytic maturation.

Materials and methods

Materials

Rabbit polyclonal antibody against GAPDH (sc-25778) and mouse monoclonal antibody against β-actin (MAB1501R) were obtained from Santa Cruz Biotechnology and Millipore, respectively. All-*trans* retinoic acid, PMA, nitro blue tetrazolium (NBT), Wright-Giemsa stain and α-naphtyl acetate esterase staining kit were purchased from Sigma-Aldrich. MEK-specific inhibitors U0126 and PD98059, antibodies to p44/42 MAPK, and Phospho-p44/42 MAPK (Thr202/Tyr204) were from Cell Signaling Technologies. The yellow-green fluorescent (505/515) 1.0 μm carboxylate-modified FluoSpheres® beads were purchased from Invitrogen. Protease inhibitor cocktail (Complete, EDTA-free; Roche) was supplemented in all cellular lysates. Protein electrophoresis reagents and markers were from Bio-Rad. Cell culture plastic and media were purchased from BD Falcon. Other chemicals were from Sigma-Aldrich. Secondary anti-mouse and anti-rabbit antibodies were obtained from Jackson Immunologicals. Lympholyte-poly™ granulocyte isolation media was obtained from Cedarlane Laboratories. Red Blood Cell lysis buffer was purchased from Roche Diagnostics. Human serum albumin was obtained from Sera Care Life Sciences.

Production of a mouse monoclonal antibody against tescalcin

Recombinant full-length tescalcin was expressed and purified from *E. coli* according to established protocol [23]. Two BALB/c mice were injected intraperitoneally with 100 μg of tescalcin in a total volume of 200 μl of 1:1 emulsion with saline adjuvant. Injection was repeated twice with 14 days interval. Three days before the day of fusion, mice were boosted via the tail vein; spleens were removed and desegregated into a single cell suspension. Resulting splenocytes were fused to a parental SP2/0 myeloma cells and plated in ClonaCell-HY hybridoma selective medium (StemCell Technologies). Ten to 14 days later, approximately 1000 colonies were picked and placed into individual wells of a 96-well tissue culture plates containing ClonaCell-HY growth medium. In the first round of screening, the reactivity against tescalcin in hybridoma supernatants was analyzed by ELISA. Positive hybridoma clones were further expanded and their ability to detect tescalcin was analyzed by Western blot. Hybridoma clone 4D94 was selected for the antibody production.

Granulocyte isolation

Human polymorphonuclear granulocytes were isolated from venous blood, taken by venepuncture from healthy consenting volunteers, on Lympholyte-poly™ separation media as described [29]. Briefly, blood was drawn and dispensed immediately into 15 ml sterile polypropylene centrifuge tubes containing heparine and mixed gently. Five milliliters of blood was then carefully layered over equal volume of separation media and centrifuged at 500×*g* and 22 °C for 35 min. The layer containing polymorphonuclear (PMN) cells was removed into the fresh tube and diluted to 10 ml with HBSS without Ca^{2+}/Mg^{2+} (Invitrogen). Cell suspension was centrifuged at 350×*g* and 22 °C for 10 min and the supernatant was discarded. Residual red blood cells (RBCs) were lysed by gentle resuspension of a pellet in 2 ml of the RBC Lysis buffer (Roche) and cells were centrifuged at 250×*g* for 5 min. Pelleted cells were washed once in HBSS without Ca^{2+}/Mg^{2+} and resuspended in HBSS with 2% human serum albumin for further analysis. Granulocyte preparations were assessed for purity using a commercial Wright-Giemsa staining kit (Sigma-Aldrich) and were found to contain >95% granulocytes.

Sample preparation and Western blot analysis

Exponentially growing suspension HL-60 cells were collected by centrifugation (300×*g*, 4 °C, 10 min.) and the cell pellet was washed twice with ice cold PBS. To obtain total cell lysate, the pellet was gently resuspended in a lysis buffer (20 mM Tris–HCl pH7.5; 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM $Na_2P_2O_7$; 1 mM beta-glycerophosphate; 1 mM Na_3VO_4; protease inhibitors) and incubated on ice for 30 min. Petri dishes with adherent macrophage-like cells were rinsed twice with PBS on ice to remove residual media and cells were gently scraped into lysis buffer. Further homogenization was performed by ultrasonic cell disruptor (Misonix) and the lysate was centrifuged at 15,000×*g* for 15 min, 4 °C. Protein concentration was measured with Bio-Rad Bradford Protein Assay unless otherwise stated. To prepare sample for gel loading, the total cell lysate was mixed with 5× SDS sample buffer (10% SDS; 62.5 mM Tris–HCl pH6.8; 25% glycerol; 125 mM