FEBS Letters 580 (2006) 5718–5722

Mechanism of hypoxia-specific cytotoxicity of procaspase-3 fused with a VHL-mediated protein destruction motif of HIF-1α containing Pro564

Hiroshi Harada[a,b], Shinae Kizaka-Kondoh[a,c,*], Masahiro Hiraoka[a]

[a] *Department of Radiation Oncology and Image-applied Therapy, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Nano-Medicine Merger Education Unit, Kyoto University, Japan*
[c] *COE Formation for Genomic Analysis of Disease Model Animals with Multiple Genetic Alterations, Kyoto University Graduate School of Medicine, Japan*

Received 29 July 2006; revised 9 September 2006; accepted 11 September 2006

Available online 22 September 2006

Edited by Veli-Pekka Lehto

Abstract **Under normoxic conditions the alpha-subunit of hypoxia-inducible factor (HIF-1α) protein is targeted for degradation by the von Hippel-Lindau (VHL) tumor suppressor protein acting as an E3 ubiquitin ligase. Recently, we developed a hypoxia-targeting protein, TOP3, which consisted of procaspase-3 with the VHL-mediated protein destruction motif of HIF-1α. This design enables procaspase-3 to be regulated similarly with HIF-1α, being degraded under normoxia while stabilized under hypoxia. Furthermore, stabilized TOP3 was cleaved by the hypoxic stress-induced endogenous caspases and thus the procaspase-3 was converted to active caspase-3 specifically under hypoxic conditions. These data demonstrated that the VHL-mediated protein destruction motif of HIF-1α endowed procaspase-3 with hypoxia-specific cytotoxicity.**
© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Hypoxia-inducible factor-1; von Hippel-Lindau; Caspase-3; Hypoxia; Apoptosis

1. Introduction

Hypoxia-inducible factor (HIF) is a transcriptional complex that mediates a broad range of cellular and systemic responses to hypoxia [1]. HIF-1 is a heterodimer composed of α and β subunits, and α subunit of HIF-1 (HIF-1α) is regulated in an oxygen-dependent manner at the post-translational level [2–4]. HIF-1α contains oxygen-dependent degradation (ODD) domains, which contains proline residues, proline-402 and proline-564. In normoxia, HIF-1α is hydroxylated at these proline residues by prolyl-4-hydroxylases [5,6]. The modification accelerates the interaction of the HIF-1α protein with the von Hippel-Lindau (VHL) tumor suppressor protein, resulting in the rapid ubiquitination and subsequent degradation of the protein by the 26S proteasome [7–10].

Recently, we screened ODD domain mutants of human HIF-1α protein and determined the $ODD_{548–603}$, which endowed fusion proteins with sufficient oxygen-dependent degradation regulation [11]. In order to specifically eradicate HIF-1α-expressing hypoxic cells in solid tumors, we constructed procaspase-3 fused with $ODD_{548–603}$ and HIV-Tat protein-transduction domain (PTD). The final product was named TOP3 (Tat-ODD-Procaspase-3) [11–14]. The HIV-Tat PTD domain is derived from human immunodeficiency virus type-1 Tat protein and efficiently delivers TOP3 to any tissue in vivo. The procaspase-3 comes from human caspase-3 protein [15] and confers cytocidal activity to TOP3. We already reported that TOP3 had anti-tumor activity in xenografts model with various cancer cells [11–14] and induced apoptosis to hypoxic tumor cells in xenografts [14].

In the present study, we clarify the mechanism of TOP3 activation and confirm its hypoxia-specific cytotoxity.

2. Materials and methods

2.1. Cell culture and hypoxic treatment in vitro

CFPAC-1 and MIA PaCa-2 human pancreatic cancer cell lines, HeLa human cervical epithelial adenocarcinoma cell line, A549 human lung adenocarcinoma cell line, WiDr human colorectal adenocarcinoma cell line and 786-O human renal cell carcinoma cell line were purchased from the American Type Culture Collection. HeLa/EF-Luc and HeLa/5HRE-Luc cell clones were isolated as described previously [14]. WT8, a 786-O cell clone stably transfected with a plasmid coding hemagglutinin (HA)-tagged VHL, was a kind gift from Dr. William G. Kaelin Jr. [16]. CFPAC-1 was maintained as described previously [11], and the other cells were maintained at 37 °C in 5% FBS-Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

Hypoxic condition of $<0.02\%$ of oxygen tension was attained by the use of a Bactron Anaerobic Chamber, BACLITE-1 (Sheldon Manufacturing Inc., Cornelius, OR). Cells were incubated in the chamber for at least 6 h before various treatments.

2.2. Formulation of TOP3 fusion protein

The Tat-ODD-Procaspase-3 fusion protein (TOP3) was prepared and dissolved in 10 mM Tris–HCl buffer (pH 8.0), as described previously [11]. The final concentration of TOP3 preparation was 15 μg/ml for in vitro experiments if not indicated, and 10 mM Tris–HCl (pH 8.0) was used as the buffer in both in vivo and in vitro experiments if not indicated.

2.3. Experimental procedures for analyses of TOP3 activation in vitro

Cells were pre-incubated under aerobic or hypoxic condition for 6 h, added with TOP3, and incubated further for 20 h before analyses.

As for the Western blot analysis, cells were seeded at 1×10^5 cells/well in a 6-well plate, treated as above and the lysates were prepared by suspending cells from each well in 100 μl of 1 × loading buffer. Twenty μl of the lysate was electrophoresed per lane on a 15% (for

*Corresponding author. Fax: +81 75 771 9749.
E-mail address: skondoh@kuhp.kyoto-u.ac.jp (S. Kizaka-Kondoh).

0014-5793/$32.00 © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2006.09.025

TOP3/caspase-3), a 10% (for VHL) or a 7.5% (for HIF-1α) SDS-polyacrylamide gel. TOP3/caspase-3, VHL and HIF-1α were detected by polyclonal anti-caspase-3 antibody (Cell Signaling Technology Inc., Osaka, Japan), monoclonal anti-HA antibody (Roche Diagnostics Japan, Tokyo, Japan) and monoclonal anti-HIF-1α antibody (BD Bioscience Pharmingen, San Diego, CA), respectively. The polyclonal and the monoclonal primary antibodies were then reacted with anti-rabbit and anti-mouse IgG horseradish peroxidase linked antibodies (Amersham Biosciences Corp., Piscataway, NJ), respectively. Detection was carried out with a chemiluminescence-based method using the ECL-PLUS system (Amersham Biosciences Corp.).

As for the DNA fragmentation analysis, genomic DNA was isolated from 6×10^5 cells with Quick Apoptotic Ladder Detection Kit (BioVision Research Products, Mountain View, CA). DNA was electrophoresed in a 1.5% agarose gel.

As for the FACS analysis, the cells were harvested, gently suspended in PBS and mixed with equal volume of 2 × hypotonic fluorochrome solution (100 μg/ml propidium iodide in 0.2% sodium citrate–0.2% Triton X-100) immediately before the analysis with a flow cytometry using CELLQuest (BD Biosciences, Franklin Lakes, NJ).

As for the analysis of caspase-3 after TOP-3 treatment, the cells were seeded at 2×10^4 cells/well in a 24-well plate, pre-incubated for 16 h and treated with TOP3 for 0, 2, 4, 6 or 8 h. As for the analysis of endogenous capsase-3 and -9 activities, similarly seeded cells were cultured with the medium, which was pre-exposed to hypoxic conditions. Then the cells were incubated under hypoxic or aerobic conditions and harvested after indicated time of incubation. Caspase-3 and -9 activities in 50 μl lysates were measured by using Colorimetric Protease Assay Kit according to the manufacturer's instructions (MBL, Nagoya, Japan). The experiments were done in triplicate and the mean of $OD_{405\,nm}$/50 μg was calculated.

3. Results

3.1. Hypoxia-dependent activation of TOP3

We previously showed citocidal effect of TOP3 on hypoxic cells in the xenografts of a human pancreatic cancer cell line [11,14]. TOP3 was designed to be degraded in normoxic cells through the function of ODD domain of human HIF-1α protein which is sensitive to the VHL-mediated destruction in normoxia. The design allows the conversion of the procaspase-3 domain to active caspase-3 under hypoxia, executing apoptotic killing of the cells.

These expectations were tested as follows. Firstly, the stability of TOP3 was compared with that of HIF-1α protein under aerobic and hypoxic conditions (Fig. 1A). CFPAC-1 cells were cultured under aerobic (lanes 1 and 2) and hypoxic (lanes 3 and 4) conditions and the protein levels of TOP3 and HIF-1α were tested with (lanes 2 and 4) or without (lanes 1 and 3) addition

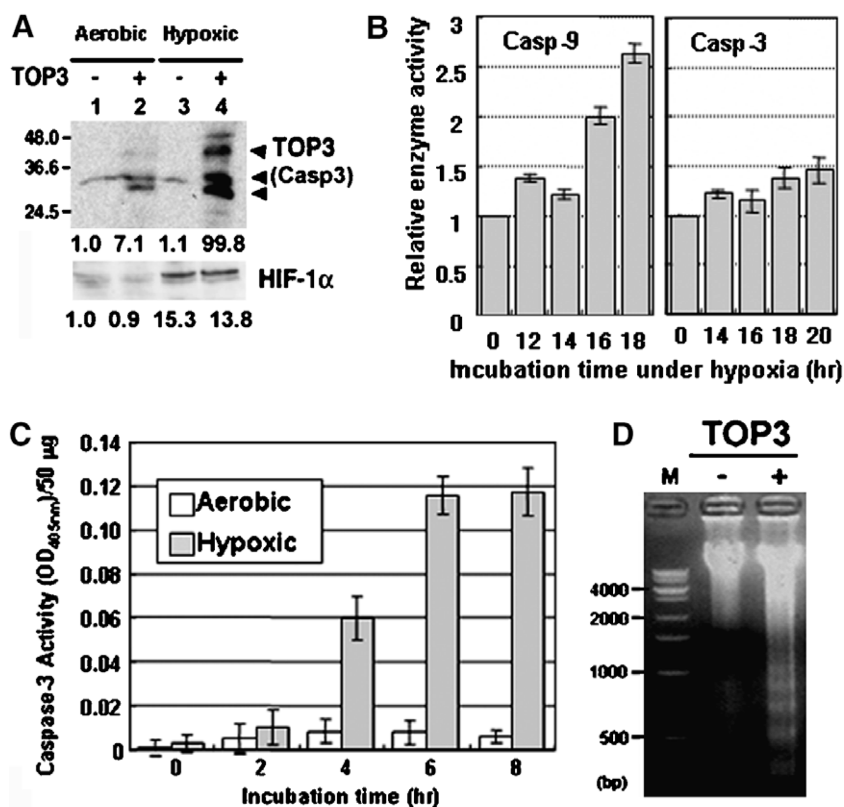

Fig. 1. Hypoxia-dependent stabilization and activation of TOP3 in vitro. (A) CFPAC-1 cells were treated with the buffer only (−; lanes 1 and 3) or TOP3 (+; lanes 2 and 4) under aerobic (lanes 1 and 2) or hypoxic (lanes 3 and 4) conditions for 20 h. TOP3 (upper panel) and HIF-1α (lower panel) proteins in the cells were analyzed by Western blotting with a polyclonal anti-caspase-3 and a monoclonal HIF-1α antibody, respectively. TOP3, its major derivative proteins and endogenous caspase-3 are indicated by arrowheads. The relative density of the bands of TOP3 (upper panel) and HIF-1α (lower panel) proteins of the lanes 2–4 to the lane 1 are indicated below each panel, respectively. (B) CFPAC-1 cells were cultured under hypoxic conditions for indicated period of time and then cell lysates were prepared. Caspase-9 (left) and -3 (right) activities in the cell lysates were measured and relative caspase activities to the 0 h are indicated. (C) CFPAC-1 cells were cultured under aerobic (open bars) and hypoxic (gray bars) conditions for 16 h and then treated with TOP3 or the buffer for the indicated period of time. Total cell lysates were prepared and then $OD_{405\,nm}$ of the lysates was measured. TOP3-derived caspase-3 activity was calculated using the following formula: ($OD_{405\,nm}$/50 μg of the TOP3-treated cells) − ($OD_{405\,nm}$/50 μg of the buffer-treated cells). Results are the means of three independent experiments ± S.D. (D) The cells were treated with the buffer (−) and TOP3 (+) for 20 h under hypoxic conditions. Then the genomic DNA was isolated and analyzed by electrophoresis with a 1.5% agarose gel.

Download English Version:

https://daneshyari.com/en/article/2050894

Download Persian Version:

https://daneshyari.com/article/2050894

Daneshyari.com