Biochemical and Biophysical Research Communications xxx (2016) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Hypoxia-induced production of peptidylarginine deiminases and citrullinated proteins in malignant glioma cells

Taigen Sase [a, b], Mitsumi Arito [a, *], Hidetaka Onodera [b], Kazuki Omoteyama [a], Manae S. Kurokawa [c], Yayoi Kagami [d], Akihito Ishigami [d], Yuichiro Tanaka [b], Tomohiro Kato [a]

[a] Clinical Proteomics and Molecular Medicine, St. Marianna University Graduate School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan
[b] Department of Neurosurgery, St. Marianna University Graduate School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan
[c] Disease Biomarker Analysis and Molecular Regulation, St. Marianna University Graduate School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan
[d] Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan

ARTICLE INFO

*Article history:*
Received 22 October 2016
Accepted 25 October 2016
Available online xxx

*Keywords:*
Citrullinated proteins
Hypoxia
Malignant gliomas
Peptidylarginine deiminase

ABSTRACT

*Background:* Recently, it has been reported that hypoxia highly enhances expression of peptidylarginine deiminase (PAD) 4 and production of citrullinated proteins in some tumor cells. However, little is known about malignant gliomas on this issue. Therefore, we here investigated whether expression of PADs was induced by hypoxia and whether PADs citrullinated intracellular proteins if induced using U-251 MG cells of a human malignant glioma cell line.

*Methods:* Expression of PADs in U-251 MG cells, cultured under hypoxia or normoxia for 24 h, was investigated by quantitative polymerase chain reaction (qPCR). Citrullination of proteins in the cells and the cell lysates incubated for 48 h with or without $Ca^{2+}$ was detected by western blotting. Citrullinated proteins were identified by mass spectrometry.

*Results:* The mRNA levels of PAD1, 2, 3, and 4 were up-regulated by hypoxia in a hypoxia-inducible factor-1-dependent manner in U-251 MG cells. In spite of the increased expression, intracellular proteins were not citrullinated. However, the induced PADs citrullinated U-251 MG cell-derived proteins when the cells were lysed. Multiple proteins citrullinated by hypoxia-induced PADs were identified. In addition, the extracellular domain of vascular endothelial growth factor receptor 2 was citrullinated by human PAD2 *in vitro*.

*Conclusion:* Our data may contribute to understanding of pathophysiology of malignant gliomas from the aspects of protein citrullination.



## 1. Introduction

Malignant gliomas are characterized by high invasiveness, neovascularization, and necrosis [1]. These features have been reported to be triggered by hypoxia, which is a rather common condition caused by insufficient blood supply [2–5]. Generally speaking, cellular responses to hypoxia are mainly regulated by a transcriptional factor of hypoxia-inducible factor (HIF)-1 [6]. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, formation of which is triggered by hypoxia. The HIF-1 heterodimer binds to hypoxia responsive elements (HRE) of various target genes like glycolysis-related enzymes and angiogenic factor [6].

Peptidylarginine deiminases (PADs; EC 3.5.3.15) are calcium ($Ca^{2+}$)-dependent post-translational citrullination enzymes that convert an arginine residue into a citrulline residue [7]. In human, 5 isoforms of PAD have been reported (PAD1, 2, 3, 4, and 6) [7]. In *in vitro* studies, it was reported that hypoxia induced production of PAD2 and citrullinated glial fibrillary acidic protein (GFAP) in human astrocytes [8] and that hypoxia highly enhanced production of

* Corresponding author.
*E-mail addresses:* sasetaigen@marianna-u.ac.jp (T. Sase), m-ari@marianna-u.ac.jp (M. Arito), dera@marianna-u.ac.jp (H. Onodera), omoteyama@marianna-u.ac.jp (K. Omoteyama), manae@marianna-u.ac.jp (M.S. Kurokawa), yakagami@tmig.or.jp (Y. Kagami), ishigami@tmig.or.jp (A. Ishigami), tanaka@marianna-u.ac.jp (Y. Tanaka), t3kato@marianna-u.ac.jp (T. Kato).

http://dx.doi.org/10.1016/j.bbrc.2016.10.154
0006-291X/

PAD4 in various kinds of tumor cells [9–11]. In an *in vivo* study, hypoxia was found to induce production of PADs, and the produced PADs citrullinated proteins of brains in rats [12,13]. Citrullination is often critical for functions and antigenicity of proteins. Aberrant citrullination was suggested to relate to pathogenesis and pathology of some diseases [14–17]. For example, in patients with alzheimer's disease, citrullinated proteins were reported to be abnormally detected in hippocampi [14]. In sera and synovial fluid of patients with rheumatoid arthritis (RA), autoantibodies to aberrantly citrullinated proteins were reported [15–20].

In malignant gliomas, it has been poorly investigated whether hypoxia induces expression of PADs and whether PADs citrullinate intracellular proteins, if induced. In addition, it is unclear whether HIF-1 is involved in induction of PADs by hypoxia in malignant gliomas. Therefore, we here investigated whether hypoxia induced production of PADs in U-251 MG cells of a human malignant glioma cell line. We also investigated whether the induced PADs citrullinated intracellular proteins. We identified target proteins of PADs in the cell line by proteomic analysis.

## 2. Materials and methods

### 2.1. Cell culture

U-251 MG cells of a human malignant glioma cell line (DS Pharma Biomedical Co., Ltd, Osaka, Japan) were cultured in EMEM (NISSUI PHARMACEUTICAL Co., Ltd, Tokyo, Japan) containing 7% Sodium Bicarbonate (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and L-Glutamine (NISSUI PHARMACEUTICAL Co., Ltd), and supplemented with 10% fetal bovine serum (MP Biomedical LLC, Santa Ana, CA, USA). The U-251 MG cells were cultured at 37 °C in humidified air with 5% $CO_2$. For the exposure of cells to hypoxia, U-251 MG cells were cultured in a hypoxic chamber (Hypoxia incubator chamber, STEMCELL TECHNOLOGIES, Vancouver, BC, Canada) containing 1% $O_2$, 5% $CO_2$, and 94% $N_2$ for 24 h as described previously [21].

### 2.2. Activation of HIF-1

U-251 MG cells were cultured with 0, 20, and 40 μM dimethyloxaloylglycine (DMOG, Enzo life Sciences, Inc., NY, USA) of a HIF-1 activator for 24 h.

### 2.3. RNA extraction and reverse transcription-PCR (RT-PCR)

RNA extraction and RT-PCR were performed as described previously [22,23]. Briefly, extraction and purification of RNA from U-251 MG cells and reverse transcription of the RNA samples were performed using RNeasy (Qiagen, Venlo, The Netherlands) and High Capacity cDNA Reverese Transcription Kits (Life Technologies, Rockville, MD, USA), respectively. Then, 2 μg of total RNA-derived cDNA, mixed with 1 μM each of the forward and reverse primers and Premix Taq™ (Ex Taq™ Version 2.0, Takara, Shiga, Japan), was subjected to PCR. Nucleotide sequences of the primers for the amplification of a vascular endothelial growth factor (VEGF) fragment and a β-actin (ACTB) fragment are as follows: for VEGF; 5′ -GCCTCCGAAACCATGAACTTTCTGCTG and 5′ -TGGTGATGTTGGACTCCTCA, and for ACTB; 5′ -AGGCACCAGGGCGTGAT and 5′ -TGCTCCCAGTTGGTGACGAT. The thermal cycle conditions were as follows: for VEGF; 95 °C for 5 min, 25 cycles (95 °C for 15 s, 60 °C for 30 s, 72 °C for 20 s), and 72 °C for 5 min, and for ACTB; 98 °C for 5 min, 25 cycles (98 °C for 15 s, 58 °C for 30 s, 72 °C for 20 s), and 72 °C for 5 min.

### 2.4. Quantitative PCR (qPCR)

qPCR was performed as described previously [23]. Briefly, qPCR was performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems™, Foster city, CA, USA), according the manufacturer's instructions. 1 μg of total RNA-derived cDNA, mixed with TaqMan® Gene Expression Assays (PAD1: Hs00203458_m1, PAD2: Hs00247108_m1, PAD3: Hs00212088_m1, PAD4: Hs00202612_m1, PAD6: Mm00462201_m1, VEGF: Hs0090055_m1, and ACTB: Mm00607939_s1, Applied Biosystems™) and TaqMan® Gene Expression Master Mix (Applied Biosystems™), was subjected to qPCR. The thermal cycle conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

### 2.5. In vitro citrullination by endogenious PADs

The U-251 MG cells, suspended in a sonication buffer (50 mM Tris-HCl (pH 7.5), 1 mM 1, 4-dithiothreitol (DTT)), were sonicated by Bioruptor UCD-250 (SONIC BIO Corporation, Kanagawa, Japan). The cell lysates were incubated at 37 °C for 48 h, with or without 1 mM $CaCl_2$.

### 2.6. In vitro citrullination by human PAD2

Recombinant human VEGF receptor (VEGFR) 2 protein (aa1-764, abcam, Cambridge, UK) was subjected to *in vitro* citrullination in a solution (50 mM Tris-HCl (pH7.4), 1 mM $CaCl_2$, and 20 U/mg human PAD2 (abcam)) for 2 h.

### 2.7. Western blotting (WB)

The procedure for WB was described previously [24]. Briefly, an equal amount of protein (1μg/lane) was separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto a membranepolyvinylidence difluoride (PVDF membrane; Merck Millipore, Billerica, MA, USA). For detection of citrullinated proteins, citrulline residues of proteins on the PVDF membrane were chemically modified by overnight incubation at 37 °C in 0.0125% $FeCl_3$, 2.3 M $H_2SO_4$, 1.7 M $H_3PO_4$, 0.25% 2, 3-butanedion monoxime, 0.125% antipyrine, and 0.25 M acetic acid (a modification medium) [13,25]. Then, the modified citrulline residues were detected by rabbit anti-modified citrulline polyclonal antibodies diluted at 1:2000 [13,24,25] and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (Merck Millipore) diluted at 1:2000. The bound antibodies were visualized using 3,3′-diaminobenxide, tetrahydrochloride (DAB; DOJINDO, Kumamoto, Japan).

### 2.8. 2-Dimensional electrophoresis (2-DE) analysis

Proteins were extracted into a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) from each of U-251 MG samples. 2-DE was perfomed as described previously [26]. Briefly, 200 μg of the extracted protein samples were loaded onto 13 cm drystrips (Imobiline, pH range of 3–11, GE Healthcare, Buckinghamshire, UK) and the drystrips were kept at 20 °C for 12 h. Then isoelectric focusing (IEF) was performed using Ettan IPGphor (GE Healthcare). The proteins separated by IEF were further separated by 12.5% SDS-PAGE. The separated proteins, stained with SYPRO Ruby Protein Gel Stain (GE Healthcare), were transferred onto a PVDF membrane. The membrane was subjected to WB with rabbit anti-modified citrulline polyclonal antibodies to detect citrullinated proteins. Intensity of protein spots were measured by an image analyzer (Typhoon 9400, GE Healthcare).

Please cite this article in press as: T. Sase, et al., Hypoxia-induced production of peptidylarginine deiminases and citrullinated proteins in malignant glioma cells, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.10.154