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JLP-JNK signaling protects cancer cells from reactive oxygen species-induced cell death

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Oxidative stress, which can be caused by an overproduction of reactive oxygen species (ROS), often leads to cell death. In recent years, c-Jun NH_2-terminal kinase (JNK)-associated leucine zipper protein (JLP, also known as SPAG9 or JIP4), a scaffold protein for JNK mitogen-activated protein kinase (MAPK) signaling pathways, was found to serve as a novel biomarker for cancer. However, although JNK MAPK pathways are reported to be activated in response to various stimuli, including oxidative stress, whether JLP is involved in ROS signaling remains unknown. In this study, we examined the role of JLP in hydrogen peroxide (H_2O_2)-induced cancer cell death, and found that *JLP* knockdown (KD) cells exhibit a substantially enhanced cell death response, along with increased intracellular ROS levels. This is the first demonstration of a protective role for JLP in response to cell-death stimulation. We also found that the H_2O_2-induced JNK activation was attenuated in *JLP* KD cancer cells. The decreases in cell viability and JNK activation in the *JLP* KD cells were almost completely reversed by expressing wild-type JLP, but not a mutant JLP lacking the JNK-binding domain. These data collectively suggest that the JLP-JNK signaling pathway counteracts ROS-induced cancer cell death.

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1. Introduction

Aerobic organisms have antioxidant defense systems to maintain reactive oxygen species (ROS) homeostasis. ROS were once thought to be toxic by-products of oxygen consumption and metabolism that exclusively damaged cellular macromolecules. However, extensive studies have revealed that ROS also act as mediators of intracellular signaling to regulate a variety of cellular processes, including proliferation and differentiation [1,2]. Furthermore, altered redox balance and dysregulated redox signaling are found to occur in human diseases, including cancer [3,4]. In fact, tumor cells are typically characterized as having elevated levels of ROS, which are thought to contribute to malignant tumor progression [5]. Tumor cells also frequently express high antioxidant activity to remove excessive ROS while remaining tumorigenic, suggesting that the regulation of redox homeostasis is complex. In addition, ROS overproduction (i.e., oxidative stress), which can be caused by an imbalance in the oxidant–antioxidant system, is known to cause cell death, including that of cancer cells.

Mammalian mitogen-activated protein kinase (MAPK) intracellular signal transduction pathways play important roles in many cellular functions, including proliferation and apoptosis [6]. Recent

Abbreviations: DHE, dihydroethidium; FBS, fetal bovine serum; GST, glutathione S-transferase; HA, hemagglutinin; HCC, hepatocellular carcinoma; H_2O_2, hydrogen peroxide; JBD, JNK-binding domain; JLP, JNK-associated leucine zipper protein; PARP, poly(ADP-ribose) polymerase; JNK, c-Jun NH_2-terminal kinase; KD, knockdown; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; ROS, reactive oxygen species; shRNA, short hairpin RNA.

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studies showed that c-Jun NH_2-terminal kinase (JNK)-associated leucine zipper protein (JLP, also known as SPAG9 or JIP4), a scaffold protein for JNK MAPK cascades [7,8], is a multifunctional protein that is also involved in kinesin-mediated axonal transport and cytokinesis [9,10]. Furthermore, JLP is used as a biomarker for various types of cancers [11,12], and has been suggested to play a role in the invasion and proliferation of cancer cells [13,14]. However, although JNK pathways are reported to be activated in response to various stimuli, including oxidative stress [15], whether JLP is involved in ROS signaling has remained unknown. In this study, we investigated whether and how JLP is involved in ROS-induced cancer cell death.

2. Material and methods

2.1. Cell culture and reagents

Hepatocellular carcinoma (HCC) Huh7 cells were cultured in Roswell Park Memorial Institute 1640 medium (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), HCC HepG2 cells were cultured in Eagle's minimum essential medium (Wako) with non-essential amino acids (Wako) and 10% FBS, and cervical cancer HeLa and colon carcinoma HCT116 cells were cultured in Dulbecco's modified Eagle's medium (Wako) with 10% FBS. All cells were incubated in a 5% CO_2 atmosphere at 37 °C. Hydrogen peroxide (H_2O_2) and puromycin were obtained from Wako and Sigma-Aldrich (St. Louis, MO, USA), respectively. In some experiments, dihydroethidium (DHE) (Sigma-Aldrich) was dissolved in dimethyl sulfoxide and added to the culture medium (see Fig. 2).

2.2. Plasmids and viral vector preparation

The expression plasmid for Flag-tagged JNK2 was described previously [16]. The pLVTH lentivirus plasmid vectors for short hairpin RNAs (shRNAs) were constructed as previously described [17]. The following annealed oligonucleotides were used to express shRNAs against human JLP: shJLP-1, forward, 5′-GATCCCCCTT-GAGCTGAAAGCGAAAAACCTCGAGGTTTTTCGCTTTCAGCT-CAAGTTTTTGGAAA-3'; reverse, 5′-AGCTTTTCCAAAAACTTGAGCTGAAAGCGAAAAACCTCGAG GTTTTTCGCTTTCAGCTCAAGGGG-3'; shJLP-2, forward, 5′-GATCCCCGCATCACAGTGGTTGGTTGTTCAAGAGA-CAACCAACCACTGTGATGCTTTTTGGAAA-3'; reverse, 5′- AGCTTTTC-CAAAAAGCATCACAGTGGTTGGTTGTCTCTTGAA-CAACCAACCACTGTGATGCGGG -3.' The control vector pLVTH-shLuc and the mammalian expression vector for hemagglutinin (HA)-tagged protein, pCL20c-CMV-HA, were described previously [18]. Total RNA was prepared from primary normal human astrocytes (Lonza, Basel, Switzerland), converted to cDNA by reverse transcriptase with random hexamers (Takara, Shiga, Japan), and the coding sequence of JLP (RefSeq accession number NM_001130528) was amplified by polymerase chain reaction (PCR). Nucleotide substitutions at positions 2529 (A to C), 2532 (G to C), 2535 (T to C), and 2538 (T to C) (causing no amino acid change) were introduced into the shJLP-2-target sequence by overlapping PCR as previously described [16], and the JLP cDNA was inserted into pCL20c-CMV-HA to generate pCL20c-CMV-HA-JLP-WT^{shR}. Nucleotide +1 is the A of the ATG-translation initiation codon of the human *JLP* gene. To express the HA-JLP-WT^{shR} protein at suboptimal levels in cells, a 518-bp deletion was made in the CMV promoter/enhancer region according to the findings by Morita et al. [19], referred to as CMVΔ6, and the resulting plasmid was named pCL20c-CMVΔ6-HA-JLP-WT^{shR}. To generate the expression plasmid for a mutant JLP lacking the JNK-binding domain (JBD), pCL20c-CMVΔ6-HA-JLP-$ΔJBD^{shR}$, a deletion of the JBD [amino acid residues 197–213 of human JLP (RefSeq accession number NP_001124000)] was introduced using overlapping PCR as previously described [16]. All PCR products were verified by sequencing. Lentiviral vectors were produced as previously described [17].

2.3. Cell viability, death, and ROS assays

Cell viability was evaluated by crystal violet staining. Cells were plated in 12-well plates at 3×10^5 cells per well, washed with phosphate buffered saline (PBS), incubated with a crystal violet solution (0.1%, w/v) for 5 min, washed with water, and dried. Stained cells were dissolved in methanol, and the absorbance at 595 nm was measured. To assay cell death, cells were plated in a 35-mm dish at a density of 2.5×10^5 cells, cultured overnight, and incubated with 5 μg/ml Hoechst 33342 (Sigma-Aldrich) for 20 min. The culture medium was then replaced with medium containing 2 μg/ml propidium iodide (PI) (Sigma-Aldrich) and H_2O_2 (0.5 mM for Huh7 or 2 mM for HeLa), and time-lapse analysis was performed. Images were captured using a BZ-9000 microscope (Keyence, Osaka, Japan) at 1-h intervals for 6 h (Huh7) or 8 h (HeLa). The ROS levels were measured as described by Wojtala et al. [20]. Briefly, sub-confluent cells were treated with H_2O_2 (0.5 mM for Huh7, and 2 mM for HepG2 and HeLa) in culture medium for 30 min. The medium was then replaced by modified Krebs-Ringer buffer (135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM KH_2PO4, 1 mM $MgSO_4$, 20 mM HEPES, 5.5 mM Glucose, pH 7.4) containing 20 μM DHE for 15 min. Images were captured using a Keyence BZ-9000 microscope, and the mean fluorescence intensity was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

2.4. Immunoprecipitation and Western blotting

To analyze protein-protein interactions, HEK293T cells were co-transfected with expression plasmids as described previously [21]. The cells were then lysed in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.1% sodium deoxycholate) containing 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and Protease Inhibitor Cocktail (Sigma-Aldrich), immunoprecipitated with anti-HA antibody (Ab) beads (Wako), and analyzed by Western blotting using a horseradish peroxidase-conjugated anti-Flag Ab (Sigma-Aldrich). Total cell lysates were prepared and analyzed by Western blotting as previously described [17], using anti-Phospho-JNK (#9251), anti-pan-JNK (#9252), anti-cleaved caspase-3 (#9661), anti-poly(ADP-ribose) polymerase (PARP) (#9542) (each diluted 1:1000, from Cell Signaling, Boston, MA, USA), and anti-α-tubulin (1:3000; T5168) (Sigma-Aldrich) Abs. To obtain an Ab against JLP (GenBank accession number AB047782), glutathione S-transferase (GST)-JLP (residues 18–160) and His-JLP (residues 18–160) proteins expressed in *Escherichia coli* were purified with glutathione Sepharose (GE Healthcare, Little Chalfont, UK) and Ni-NTA Agarose (Qiagen, Hilden, Germany) according to the manufacturers' instructions, respectively. The GST-JLP protein was injected into rabbits, and the resulting antisera were affinity purified using the His-JLP protein and used at 0.25 μg/ml.

2.5. Statistical analysis

Significance was determined using a two-tailed unpaired Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

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