Abstract: Interleukin-1α (IL-1α) is produced inside cells in its precursor form (pIL-1α). Enzymatic cleavage yields mature (mIL-1α) and the propeptide of IL-1α (pPI-1α), which are thought to be localized in the nucleus, because of the presence of nuclear localizing signals. Studies of pPI-1α function have been hampered by the lack of a pPI-1α-specific antibody (Ab). In the present study, the authors generated anti-pPI-1α Ab by using recombinant histidine-tagged pPI-1α (His-pPI-1α) as an immunogen. Rabbits were immunized with His-pPI-1α, and affinity-purified Ab was obtained. Ab reactivity and specificity were examined by Western blotting. The antibody successfully recognized transfectant-derived green fluorescence protein (GFP)-tagged pPI-1α but not GFP. A sandwich enzyme-linked immunosorbent assay (ELISA) system established by biotinylating the anti-pPI-1α Ab successfully detected GFP-pPI-1α. The Ab and ELISA system allows functional analysis of pPI-1α and improves understanding of pPI-1α.

Keywords: alarmin, ELISA, propiece IL-1α

Introduction

The interleukin (IL)-1 family includes seven agonistic ligand members [1], among which IL-1α was cloned in 1985 [2]. IL-1α is initially synthesized intracellularly as a precursor (pPI-1α). pPI-1α is cleaved by calcium-dependent protease calpain to generate N-terminal half-propeptide IL-1α (pPI-1α) and C-terminal half-mature mIL-1α [1] (Fig. 1). Secretion of mIL-1α triggers downstream inflammatory reactions through its receptors. In contrast, pPI-1α and pPI-1α were reported to be preferentially localized in the nucleus because of its nuclear localizing sequence and to upregulate IL-6 and IL-8 expression by nuclear factor kappa beta and activator protein-1 activation [3].

Alarmins are molecules rapidly released from damaged cells [4]. They function as early warning signals to activate innate and adaptive immunity. pPI-1α is an alarmin released from damaged cells in response to danger signals such as hypoxic shock, oxidative stress, and heat shock [5]. Intriguingly, pPI-1α is released from necrotic cells but not from apoptotic cells [6,7]. The functional differences between pPI-1α and mIL-1α have been investigated in studies using recombinant proteins [8]. The results indicate that both molecules induce IL-6 secretion from the human lung carcinoma cell line, A549 [8]. As described above, the roles of mIL-1α and pPI-1α are becoming clearer. Because mIL-1α is generated by cleavage of pPI-1α, the amount of pPI-1α generated must be equivalent to the amount of mIL-1α released from cells. However, the roles of pPI-1α are unknown because antibodies that can detect pPI-1α are not available. Recombinant pPI-1α and its detection systems might be useful in clarifying the biological functions of pPI-1α. In the present study, pPI-1α-specific Ab was obtained by immunizing rabbits with a histidine-tagged recombinant pPI-1α (His-pPI-1α) protein. The Ab successfully detected His-pPI-1α and transfectant-derived pPI-1α on a Western blotting. Using biotinylated Ab, the authors developed a sandwich enzyme-linked immunosorbent assay (ELISA) system that allows for analysis of pPI-1α functions in the extracellular milieu.

Materials and Methods

DNA construction

Full-length human IL-1α open reading frame was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using the total RNA of HeLa cells. The fragment was cloned to the bacterial expression vector pTrcHis (Invitrogen, Waltham, MA, USA) with T/A cloning by adding the A residue during incubation with Taq polymerase (Takara, Shiga, Japan) and designated the pTrc-pPI-1α vector (Fig. 2a). This vector was used as a template to generate pPI-1α and mIL-1α, which were then cloned to the same vector and designated pTrc-pPI-1α (Fig. 2b) and pTrc-mIL-1α (Fig. 2c), respectively. The constructs were transformed to Escherichia coli (E. coli.) strain BL21 (Agilent, Santa Clara, CA, USA) and used for protein synthesis. In transfection experiments, pPI-1α was cloned to green fluorescence protein (GFP)-containing mammalian expression vector pEGFP-C3 (Takara Bio USA, Mountain View, CA, USA) by an infusion method (In-Fusion HD Cloning Kit, Takara). The EcoRI site was added to the primers (pEGFP-pPI-1α vector; Fig. 2d). The pEGFP-C3 vector used was as a control vector (Fig. 2e). pPI-1α, pPI-1α, and mIL-1α were amplified by PCR and cloned to the EcoRI site of the pEGFP-C3 vector (Fig. 2f, g, h, respectively). The sequences of all inserts were confirmed, and all plasmids were transformed to E. coli strain DH5α.

Cell culture and transfection

HeLa cells were cultured with 10% fetal calf serum (FCS)-Dulbecco’s minimal essential medium supplemented with penicillin and streptomycin (Sigma). For transfection, the HeLa cells were seeded (5 × 10^5 cells/dish) on the day before the experiments. Each vector was mixed with OPTI-MEM and Plus 3000 reagent (Invitrogen). The Lipofectamine 3,000 reagent (Invitrogen) was diluted with OPTI-MEM. Each mixture was mixed and incubated for 15 min at room temperature (RT). The reaction mixture was added to the HeLa cultures and incubated for 18 h at 37°C. The transfectants were washed with phosphate-buffered saline (PBS), and cell lysates were collected by using cell lysis buffer (1% Triton X-100/10 mM Tris-HCl buffer [pH 8.0]). The samples were centrifuged (10,000 × g, 3 min at 4°C), and supernatants were subjected to Western blotting and sandwich ELISA.

Correspondence to Dr. Masatake Asano, Department of Pathology, Nihon University School of Dentistry. 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan Fax: +81-33219-8340 E-mail: asano.masatake@nihon-u.ac.jp

Color figures can be viewed in the online issue at J-STAGE.

DN/JST/JSTAGE/josnusd/19-0477

doi.org/10.2334/josnusd.19-0477

Color figures can be viewed in the online issue at J-STAGE.
Recombinant protein synthesis and purification

Transformed bacterial cells were incubated in Luria-Bertani media for 18 h at 37°C. The cells were stimulated with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO, USA) for another 18 h and harvested (15,000 ×g, 10 min) with a centrifuge (Beckman, Indianapoli, IN, USA). The harvested cells were suspended in an 8-M urea solution (8 M urea, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl [pH 6.3]) and sonicated with a sonication apparatus (American Laboratory Trad, San Diego, CA, USA). The samples were centrifuged (15,000 ×g, 10 min), and the supernatants were transferred to new tubes. Nickel resin (GE Healthcare, Chicago, IL, USA) was added to the samples and rotated for 18 h at 4°C. The samples were centrifuged (10,000 ×g for 5 min at 4°C), and the supernatants were discarded. The Ni resin was extensively washed with 8-M urea buffer, after which Ni resin-bound recombinant proteins were eluted with a 250-mM imidazole solution (250 mM imidazole in 8 M urea). The obtained proteins were dialyzed against PBS and used for immunization.

Ab preparation

The prepared recombinant His-ppIL-1α (0.4 or 0.2 mg/0.5 mL) was mixed with 0.5 ml of Freund’s complete adjuvant (Rockland Immunochemicals, Limerick, PA, USA). The mixture was subcutaneously injected (5 times, at 2-week intervals) into two rabbits. After the final immunization, all the blood was collected from the common carotid artery and incubated at 37°C for 1 h and further incubated at 4°C overnight. The samples were centrifuged (1,200 ×g for 15 min), after which antisera were obtained and subjected to affinity purification. His-ppIL-1α was dialyzed against 0.1 M NaHCO₃ overnight. The sample was mixed with Affi Gel-10 (Bio-Rad, Hercules, CA, USA) for 1 h at RT. The prepared affinity column was equilibrated by PBS, and the obtained antiserum was applied. The bound Ab was eluted with 3M MgCl₂ and dialyzed against PBS. This fraction was used as an affinity-purified Ab.

Western blotting

For Western blotting, cell lysates prepared from each transfectant were subjected to 15% or 12% SDS-PAGE. Western blotting was performed as described previously [9]. Primary Abs against ppIL-1α (×10,000) and anti-GFP Ab (×1,000) (Abcam, Cambridge, UK) were diluted with 1% BSA-PBST (0.1% Tween 20/PBS), while the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) Ab (×10,000) (Abcam) was diluted with 1% BSA-PBST. The bands were detected with an ECL kit (GE Healthcare, Tokyo, Japan).

ELISA

The 96-well plates were coated with anti-ppIL-1α Ab (×1,000, 50 μL/well) for 18 h at RT. The plate was washed with 0.01% Tween 20/PBS by using an automated plate washer (Bio-Rad). Nonspecific binding was blocked by incubating the plate with 100 μL of 1% bovine serum albumin (BSA)-PBS for 1 h. The blocking buffer was discarded, and the samples were applied and incubated for 1 h. After the wash, biotinylated anti-ppIL-1α Ab (×10,000, 50 μL/well) was added to each well and incubated for 1 h. The plates were washed and further incubated with streptavidin-HRP (×1,000, 50 μL/well, Merck-Millipore, Temecula, CA, USA) for 30 min. After the wash, the color reaction was performed by adding SureBlue (SeraCare, SeraCare, Milford, MA, USA) (200 μL/well) and incubating for 10 min. The reaction was stopped with 2 M H₂SO₄ (50 μL/well), and optical density was measured with a microplate reader (450 nm) (Bio-Rad).

Statistical analysis

A normality test with statistical software showed a non-normal distribution. Therefore, the nonparametric Mann-Whitney U-test was performed (n = 7). The results are presented as mean ± SD. A P value of less than 0.05 was considered to indicate statistical significance.
Results

Purification of His-ppIL-1α and His-mIL-1α
The purity of His-ppIL-1α and His-mIL-1α were confirmed by Coomassie brilliant blue staining. As shown in Fig. 3, each protein was detected as 16-kDa and 17-kDa single bands. His-ppIL-1α migrated slightly faster than His-mIL-1α and was used for rabbit immunization.

ppIL-1α-specific Ab
Rabbit antisera obtained by immunizing His-ppIL-1α were subjected to affinity purification. The reactivity of the purified Ab was examined by Western blotting. Cell lysates obtained from GFP and GFP-ppIL-1α transfectants were loaded onto 15% SDS-PAGE and subjected to Western blotting. When the membrane was blotted with anti-GFP Ab, GFP and GFP-ppIL-1α were detected as 27-kDa and 43-kDa bands (Fig. 4a), respectively. In contrast, only GFP-ppIL-1α was detected by anti-ppIL-1α Ab (Fig. 4a). Moreover, cell lysates were prepared from pcDNA (mock), pcDNA-mIL-1α, pcDNA-pIL-1α, and pcDNA-ppIL-1α transfectants and subjected to Western blotting (12% SDS-PAGE) (first Ab: the Ab obtained in this study ×10,000; 2nd Ab: goat anti-rabbit IgG (H+L) ×10,000). Representative data from three independent experiments are shown.

ELISA
The standard curve was drawn by applying serially diluted His-ppIL-1α (Fig. 5a); the detection limit of the system was 3.1 ng/mL. The amount of ppIL-1α in cell lysates of GFP and GFP-ppIL-1α transfectants was measured with this system. As shown in Fig. 5b, GFP-ppIL-1α transfectants contained 19.2 ng/mL of GFP-ppIL-1α.
Discussion

In the present study, the ppIL-1α-specific Ab was obtained by using recombinant His-ppIL-1α generated in *E. coli*. The Ab was biotinylated, and a sandwich ELISA system against ppIL-1α was established. Anti-ppIL-1α Ab detected GFP-ppIL-1α but not GFP (Fig. 4) on a Western blot, indicating specific detection of ppIL-1α. A monoclonal Ab against pIL-1α was generated by Carlsen et al. [10]. Moreover, Ross et al. reported identification of nuclear pIL-1α with a monoclonal Ab via flow cytometry [11]. However, a ppIL-1α ELISA system has never been established.

Most studies of IL-1α focused on mIL-1α because it can be secreted outside the cell and evokes immunological reactions. For these reasons, reagents that can detect mIL-1α but not ppIL-1α are more commonly available. The presence of nuclear localizing sequence has facilitated investigation of the biological functions of ppIL-1α in the nucleus. Werman et al. reported that ppIL-1α functions as a transcriptional regulator [3]. In addition, ppIL-1α affects cell growth in various cancer cell lines [12,13]. These studies were conducted mainly by using a transfection method with tagged ppIL-1α; detection was achieved by Ab against tag molecules. A specific Ab against ppIL-1α might allow direct detection of the molecule and clarify the functions of ppIL-1α in the body.

Factors released in response to danger are called alarmins [4]. These molecules inform surrounding cells about conditions, thus triggering various reactions. Although IL-1α is a known alarmin [14], it is unclear whether ppIL-1α, itself, acts as an alarmin. If it does, it is released in the extracellular space and exerts its functions on surrounding cells. However, the extracellular functions of ppIL-1α have never been examined. Generation of His-ppIL-1α in the present study might aid in examining the effects and functions of ppIL-1α. His-ppIL-1α might be used to stimulate culture cells or artificially generate wounds in animals. In such experiments, accurate detection systems are indispensable. The present ELISA system might be useful for measuring ppIL-1α in extracellular milieu or in other experimental samples. Future studies should attempt to clarify the biological functions of ppIL-1α.

Acknowledgments

This study was supported in part by research grants from the Sato Fund of the Nihon University School of Dentistry and by a grant from the Dental Research Center of the Nihon University School of Dentistry.

Conflict of interest

None.

References

1. Di Paolo NC, Shayakhmetov DM (2016) Interleukin 1α and the inflammatory process. Nat Immunol 17, 906-913.
2. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V et al. (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315, 641-647.
3. Werman A, Werman-Venkert R, White R, Lee JK, Werman B, Krelin Y et al. (2004) The precursor form of IL-1 alpha is an intracellular proinflammatory activator of transcription. Proc Natl Acad Sci USA 101, 2434-2439.
4. Oppenheim JJ, Yang D (2005) Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 17, 359-365.
5. Rider P, Carmi Y, Gottman O, Braiman A, Cohen I, Voronov E et al. (2011) IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol 187, 4835-4843.
6. Cohen I, Rider P, Carmi Y, Braiman A, Dotan S, White MR et al. (2010) Differential release of chromatin-bound IL-1alpha discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. Proc Natl Acad Sci USA 107, 2574-2579.
7. England H, Summenegger HR, Edye ME, Rothwell NJ, Brough D (2014) Release of interleukin-1α or interleukin-1β depends on mechanism of cell death. J Biol Chem 289, 15942-15950.
8. Kim B, Lee Y, Kim E, Kwak A, Ryoo S, Bae SH et al. (2013) The interleukin-1α precursor is biologically active and is likely a key alarmin in the IL-1 family of cytokines. Front Immunol 4, 391.
9. Omagari D, Mikami Y, Suguro H, Sunagawa K, Asano M, Samuki E et al. (2009) Poly I:C-induced expression of intercellular adhesion molecule-1 in intestinal epithelial cells. Clin Exp Immunol 156, 294-302.
10. Carlsen TG, Kjærgaard P, Jørgensen TL, Foldbjerg R, Nielsen ML, Poulsen TB et al. (2015) Interleukin-1α activation and localization in lipopolysaccharide-stimulated human monocytes and macrophages. J Immunol Methods 422, 59-71.
11. Ross R, Grimmel J, Goedicke S, Mösbs AM, Bulan AM, Butter P et al. (2013) Analysis of nuclear localization of interleukin-1 family cytokines by flow cytometry. J Immunol Methods 387, 219-227.
12. Stevenson FT, Tuck J, Locksley RM, Lovett DH (1997) The N-terminal propiece of interleukin 1 alpha is a transforming nuclear oncoprotein. Proc Natl Acad Sci USA 94, 508-513.
13. Zhang Y, Yu X, Lin D, Lei L, Hu B, Cao F et al. (2017) Propiece IL-1α facilitates the growth of acute T-lymphocytic leukemia cells through the activation of NF-κB and SP1. Oncotarget 8, 15677-15688.
14. Voronov E, Dinarello CA, Apte RN (2018) Interleukin-1α as an intracellular alarmin in cancer biology. Semin Immunol 38, 3-14.