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Discovery of novel poly(ADP-ribose) glycohydrolase inhibitors by a quantitative assay system using dot-blot with anti-poly(ADP-ribose)

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ABSTRACT

Poly(ADP-ribosyl)ation, which is mainly regulated by poly(ADP-ribose) polymerase (PARP) and poly (ADP-ribose) glycohydrolase (PARG), is a unique protein modification involved in cellular responses such as DNA repair and replication. PARG hydrolyzes glycosidic linkages of poly(ADP-ribose) synthesized by PARP and liberates ADP-ribose residues. Recent studies have suggested that inhibitors of PARG are able to be potent anti-cancer drug. In order to discover the potent and specific Inhibitors of PARG, a quantitative and high-throughput screening assay system is required. However, previous PARG assay systems are not appropriate for high-throughput screening because PARG activity is measured by radioactivities of ADP-ribose residues released from radioisotope (RI)-labeled poly(ADP-ribose). In this study, we developed a non-RI and quantitative assay system for PARG activity based on dot-blot assay using anti-poly (ADP-ribose) and nitrocellulose membrane. By our method, the maximum velocity (V_{max}) and the michaelis constant (k_m) of PARG reaction were 4.46 μM and 128.33 μmol/min/mg, respectively. Furthermore, the IC50 of adenosine diphosphate (hydroxymethyl) pyrrolidinediol (ADP-HPD), known as a non-competitive PARG inhibitor, was 0.66 μM. These kinetics values were similar to those obtained by traditional PARG assays. By using our assay system, we discovered two novel PARG inhibitors that have xanthene scaffold. Thus, our quantitative and convenient method is useful for a high-throughput screening of PARG specific inhibitors.

Introduction

Poly(ADP-ribosyl)ation, which is catalyzed by PARP and PARG, is one of post-translational modifications of proteins. PARP family consists of seventeen members in human, while PARG family consists only two members, PARG and ADP-ribosyl-(arginine)-hydrolase 3 (ARH3) [1]. PARP cleaves NAD^+ into nicotinamide and ADP-ribose and polymerizes the later onto various nuclear proteins including PARP itself and histones. PARG hydrolyzes glycosidic linkages of poly(ADP-ribose) and liberates ADP-ribose residues. The physiological roles of poly(ADP-ribosyl)ation in DNA damage response have been studied and discussed well. PARP1 recognizes single-stranded DNA breaks by DNA damage-inducers such as ultraviolet and alkylating agents, and its activity dramatically increases. In the synthesizing step of poly(ADP-ribose) by PARP1 or PARP2, it is thought that the acute response is a "flag" for efficient recruitment of DNA repair factors to DNA damage loci, because depletion of PARP1 leads in a delay of DNA repair [2]. In addition, we and others have suggested that the degradation of poly(ADP-ribose) by PARG supplies ATP at DNA damage loci [3,4]. That is, the ADP-ribose produced by PARG-mediated degradation of poly(ADP-ribose) can be converted to ATP that is used in a DNA ligase III-mediated DNA ligation step in base excision repair. Recently, it was also reported that acceptor-free poly(ADP-ribose) induces cell death *via* the release of apoptosis-inducing factor (AIF) from mitochondria [5,6]. This shows that accumulated poly(ADP-ribose) itself has an important physiological function.

Thus, degradation of poly(ADP-ribose) by PARG has crucial roles in cellular stress responses. Therefore, PARG inhibitors may have potential as anti-cancer drugs or their sensitizers. So far, some PARG inhibitors have been searched. Our group has identified tannin and lignin derivatives as potent PARG inhibitors [7–10]. However, their derivatives are not adequate to structural development. On the other hand, poly(ADP-ribose) analogues, ADP-HPD and poly(etheno-ADP-ribose), have been identified as potent PARG inhibitors, but these compounds lack cell-permeability [11,12]. At present, PARG inhibitors that are drug-like small molecules have not been found yet.

Recently, we are trying to create PARG specific inhibitors by structure based virtual screening and structure based-drug design. However, we have felt that radiometric PARG assay is quantitative but not appropriate for screening of large chemical libraries. In the

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radiometric PARG assay, ^{3}H or ^{32}P-labeled poly(ADP-ribose) is used, and the PARG activity is analyzed by a thin layer chromatography or a liquid-scintillation counter. Although these methods have good sensitivities, it is not appropriate for handling of a lot of compounds during long term. In the past, several methodologies about non-radiometric PARG assay have been published [13,14]. The fluorometric method is multi-well format and is effective in high-throughput screening. However, the fluorescent properties of chemical compounds may affect assay results, because the method utilizes changes of fluorescent property based on chemical conversion of ADP-ribose. The dot-blot assay using anti-poly(ADP-ribose) is also appropriate for inhibitor screening. Immunoblotting with anti-poly(ADP-ribose) clone 10H, which recognizes longer poly(ADP-ribose) than ADP-ribose dimer, have been used in detection of poly(ADP-ribose) in living cells or organs. In the traditional dot-blot assay with anti-poly(ADP-ribose) antibody, positive charged nylon membrane (PCN-membrane) has been used to fix poly(ADP-ribose), which has a large negative charge, while general dot-blot methods for protein detection are used nitrocellulose membrane (NC-membrane). However, it has not been shown whether accurate kinetics of PARG activity can been obtained by the method using PCN-membrane. Furthermore, it may be not appropriate to use PCN-membrane for poly(ADP-ribose) fixation in high-throughput screening, because charged compounds could affect retention of poly(ADP-ribose) onto the membrane, leading inaccurate evaluation of assay results.

Although PARG assay using dot-blot with anti-poly(ADP-ribose) is a simple and useful method, there is less evidence in calculation of accurate kinetics. Therefore, in this study we developed traditional PARG dot-blot assay into a more quantitative method and tried to search for novel PARG inhibitors.

Materials and methods

Reagents and buffers. Bovine PARG and control poly(ADP-ribose) were purchased from Biomol (USA). ADP-HPD were from Calbiochem (USA). Compounds (including ADP-HPD) used for membrane choice were dissolved at 10 mM in distilled H_2O (dH_2O) or dimethyl sulfoxide (DMSO). NC- and PCN-membranes were from GE Healthcare (USA). The compositions of buffers used in this method are shown as follows. Hypotonic buffer; 10 mM Tris–HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM 2-mercaptoethanol, and 1% protease inhibitor cocktails. Poly(ADP-ribosyl)ation buffer; 82.5 mM Tris–HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, and 3.3 mM 2-mercaptoethanol. TTBS; 25 mM Tris–HCl (pH 7.4), 140 mM NaCl, 8.1 mM KCl, and 0.1% Tween20. High salt TTBS; 25 mM Tris–HCl (pH 7.4), 1 M NaCl, 8.1 mM KCl, and 0.1% Tween20. PARG assay buffer; 50 mM KPO_4 (pH 7.2), and 10 mM 2-mercaptoethanol.

Poly(ADP-ribose) calibration curve. Several concentration (51–3300 nM) of control poly(ADP-ribose) was prepared in PARG assay buffer (20 μl). The solution (10 μl) was blotted onto a NC-membrane, and the membrane was left at room temperature (RT) for 20 min to dry up. After washing once with 70 mM Na_2HPO_4 (pH 9.3) to remove ADP-ribose monomer and once with TTBS, the membrane was incubated in 2.5% non-fat dry milk and 0.25% bovine serum albumin in TTBS for 1 h at RT. The blocked membrane was incubated with monoclonal anti-poly(ADP-ribose) antibody for 2 h at RT. After washing thrice with TTBS, the membrane was probed with horseradish peroxidase-conjugated anti-mouse IgG for 1 h at RT. After washing thrice with TTBS, the membrane was incubated with Supersignal West Femto Chemiluminescent Substrate (PIERCE, USA). The membrane-bound poly(ADP-ribose) was visualized by LAS-3000 (Fuji Film, Japan). The quantitative data was analyzed using Multigauge (Fuji Film, Japan).

Poly(ADP-ribosyl)ation of chromosomal proteins. Poly(ADP-ribosyl)ated nuclear proteins were prepared as previously described with minor modifications [15,16]. HeLa S3 cells (1×10^7 cells) lyzed by 1 ml of Hypotonic buffer containing 0.1% Nonidet P40 were incubated on ice for 10 min and homogenized. Nuclei were collected by centrifugation at 1000*g* at 4 °C for 5 min, and then washed with Hypotonic buffer. The isolated nuclei were resuspended in 500 μl of poly(ADP-ribosyl)ation buffer and sonicated. Poly(ADP-ribosyl)ation of nuclear proteins was started by the addition of 500 μl of poly(ADP-ribosyl)ation buffer containing 0.6 mM NAD^+, and incubated for 30 min at RT. Poly(ADP-ribosyl)ated chromatin were collected by centrifugation at 10,000*g* for 5 min at 4 °C and washed twice with 1 ml of 10 mM KPO_4 (pH 7.2).

Preparation of acceptor-free poly(ADP-ribose). Acceptor-free poly(ADP-ribose) whose average chain length is approximately 15 mer was prepared as described previously [15–17] with following modification. Poly(ADP-ribosyl)ated chromatin prepared as above was resuspended in 500 μl of 10 mM KPO_4 (pH 7.2), boiled for 30 min, and left on ice for 5 min. After centrifugation at 10,000*g* at RT for 10 min, the supernatant was added 150 μl of 10 kuniz units/ml deoxyribonuclease I (DNase I) (SIGMA, USA) and incubated at 37 °C for 30 min to digest genomic DNA. And then, to completely release poly(ADP-ribose) from chromatin proteins, 150 μl of 10 mg/mL proteinase K (Pro K) (WAKO, Japan) was added and incubated at 37 °C for 1 h. Acceptor-free poly(ADP-ribose) polymer were extracted by phenol/chloroform. After ethanol precipitation, the polymer was resuspended by 10 mM KPO_4 (pH 7.2). No contamination of proteins and nucleic acids in the poly(ADP-ribose) polymer was confirmed by SDS–PAGE followed by

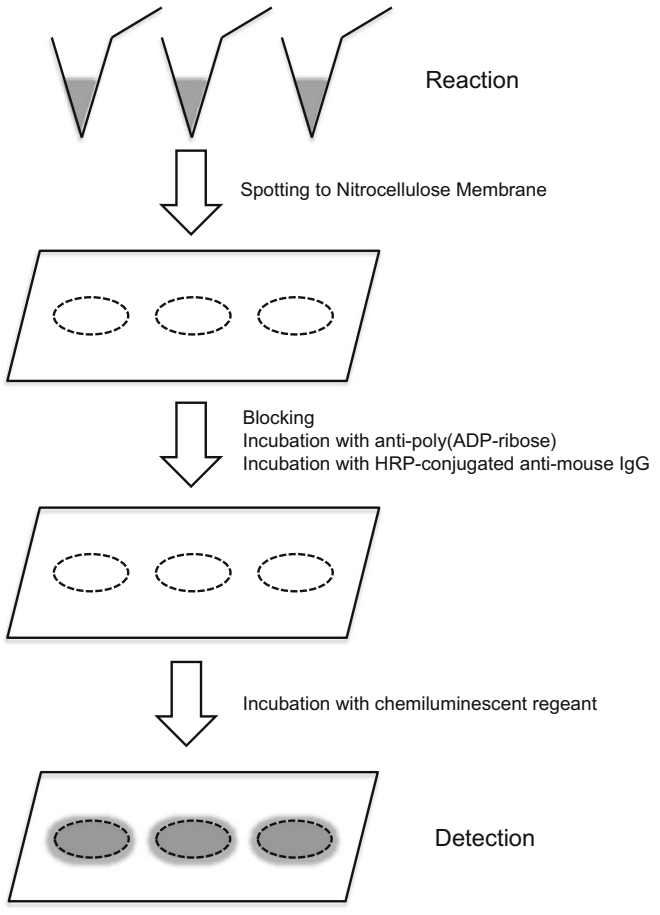

Fig. 1. Schematic procedures of PARG enzymatic activity assay.