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Early response as shown by enhancement of transglutaminase 1 expression after cisplatin-induced acute kidney injury

Kentaro Furukawa, Miki Yamane, Hideki Tatsukawa, Kiyotaka Hitomi*

Graduate School of Pharmaceutical Sciences, Nagoya University, Nagoya, 464-8601, Japan

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ABSTRACT

Acute kidney injury (AKI) is caused by drugs and other stimuli, which limits the use of several therapeutic approaches. The AKI mouse model generated by intraperitoneal administration with cisplatin, one of the most widely used anti-cancer drugs, is generally applied to study on this disease. Transglutaminases are posttranslational modifying enzymes that catalyze irreversible cross-linking reactions between proteins in several biological events such as skin formation and blood coagulation. In this study, we found an increase in the expression level of transglutaminase (TG1) in the kidney of mice which had been injected with cisplatin and underwent progressive nephrotoxicity. Before the appearance of the tentative symptoms of renal failure, which is apparent by morphological damage in the kidney and increases in blood creatinine levels, both the expression level and activity of TG1 rapidly increased mainly at the proximal tubule. On the other hand, the protein expression level of another major isozyme (TG2) remained mostly unaltered. This investigation will provide a possible basal-level biomarker and also information on progression of renal failure from the aspect of the protein-modifying enzyme, transglutaminase.

1. Introduction

Transglutaminases (TGase: EC 2.3.2.13) are a family of enzymes that catalyze a covalent cross-linking reaction between proteins in several tissues [1,2]. In calcium dependent manner, peptide-bound glutamine residues in substrate proteins form γ-glutamyl-ε-lysine covalent isopeptide bonds with lysine residues in identical or different substrates. Instead of lysine residues, primary amine or water molecules also react with glutamine by this enzymatic reaction resulting in transamidation or substitution into glutamic acid, respectively. These protein modifications participate in several biological events such as blood coagulation, epidermis formation, transcriptional regulation and extracellular matrix stabilization. Among the eight isozymes in the TGase family, TG1, TG2 and Factor XIII are the major enzymes expressed in mammals. TG1 is expressed mainly in keratinocytes and is essential for their terminal differentiation by cross-linking structural proteins underneath the outermost layer of the epidermis [3]. TG2, expressed ubiquitously, plays multiple roles in metastasis, stabilization of extracellular matrix, and transcription regulation, through modifications of several functional proteins [4]. Factor XIII polymerizes fibrin molecules to form a clot upon coagulation [5]. Thus, these catalytic reactions contribute to various post-translational modifications of numerous functional proteins, as well as having other possible, unknown significant functions. Upon cellular responses to stimulating factors and/or changes in intracellular environment, the level of the enzymatic activity fluctuates at the transcriptional regulation level.

In recent studies on kidney, TGase appeared to regulate several proteins, although the precise mechanisms are under investigation. It has been recently described that TG1 protects renal epithelial cells from apoptosis and also promotes proliferation by modulating signaling molecules such as Stat-3 and AKT [6,7]. TG2 is involved in renal diseases, such as fibrosis and IgA nephropathy, resulting in modification of extracellular matrix and immunoglobulin (IgA) receptors, respectively [8,9]. Thus, the molecular events affecting protein modification by catalytic

Abbreviations: AKI, acute kidney injury; BUN, blood urea nitrogen; FITC, fluorescein-isothiocyanate; GAPDH, glyceroaldehyde-phosphodehydrogenase; HE, hematoxylin and eosin; PAS, Periodic acid-Schiff staining; PBS, phosphate-buffered saline; sCr, serum creatinine; TBS, Tris-based buffered saline.

* Corresponding author. Graduate School of Pharmaceutical Sciences, Nagoya University, Chikusa, Nagoya, 464-8601, Japan.
E-mail address: hitomi@ps.nagoya-u.ac.jp (K. Hitomi).

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cross-linking reactions have been under investigation in renal diseases.

Cisplatin is a widely used anti-cancer drug that is highly effective against several types of cancers when used alone or combination with other drugs [10]. This compound undergoes hydrolysis in specific intracellular circumstances and then cross-reacts with nuclear DNA leading to impairment of the cell cycle, resulting in apoptosis of cancer cells. However, upon clinical application, patients receiving cisplatin have severe kidney damage as a side effect [11]. In cisplatin chemotherapy, the acute kidney injury (AKI) has been the main limiting factor. Hence, it is necessary to clarify the mechanism of this damage and also to detect the symptoms for cellular alterations upon treatment with cisplatin.

So far, we have identified TG1-and TG2-specific and highly reactive glutamine-donor substrate peptides which enabled establishment of the detection systems for *in vitro* and *in situ* TGase activities in various cells or tissues [12–14]. TG2 is thought to contribute to the repair from renal damage because it plays a role in the recovery of the damaged cells. Additionally, upon apoptosis or inflammation in various tissues, the enzymatic activity of TG2 increased to prevent cellular components from being released into the extracellular area [15]. Although there is less information about the role of TG1 upon damage to epithelial cells, cross-linking of structural proteins may contribute to the recovery. In this study, by the detection systems using isozyme-specific peptides, we investigated the expression and activities for TG1 and TG2 in the kidney of the cisplatin-treated mice. This series of experiments will contribute to diagnosis and give more information on the involvement in the enzyme activity of AKI. Furthermore, detection of possible variations in these TGase activities will clarify their physiological significance on these protein modifications.

2. Materials and methods

2.1. Reagents

Most reagents were obtained from WAKO (Wako, Osaka, Japan). The labeled peptides, (pepK5: YEQHKLPSSWPF, pepT26: HQSYVDPWMLDH) were synthesized from Biosynthesis (Lewisville, TX).

2.2. Production of renal injured model mouse and its evaluation

Eight to 10 weeks ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Cisplatin was dissolved in phosphate-buffered saline (PBS). For production of AKI model mice, mice were given cisplatin by intraperitoneal injection of 20 mg/kg body weight/day and kept for 1–3 days [16].

Upon sacrifice, the serum was prepared from heart drawing blood. According to the manufacture's method, colorimetric assays were carried out to measure the levels of blood nitrogen (BUN) (Arbor assays™ MI, USA) and serum creatinine (sCr) (Cayman Chemical Co. Ann Arbor, MI, USA).

2.3. Immunochemical analysis

Polyclonal antibodies against mouse TG1 and TG2 were prepared from rabbit by immunization with the recombinant enzymes as full-length proteins (Europhin, Tokyo, Japan). These antigens were produced as hexahistidine-tagged proteins in *E. coli* and purified to homogeneity. From the serum, the specific antibodies were affinity purified as IgG fractions using the Sepharose gel which immobilized each recombinant protein to avoid cross-reactivities.

For Western blotting, the kidney from the mice were homogenized in lysis buffer containing 10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.1 mM EDTA and the protease inhibitors cocktail (Merck Millipore, Darmstadt, Germany), and the soluble fraction was treated with SDS-buffer. The samples were subjected to 10% SDS-PAGE and transferred to the polyvinylidene difluoride (PDVF) membrane (Merck Millipore). The membrane was reacted with primary antibody and the specific signal was detected by the secondary antibody-conjugated with peroxidase and chemiluminesence reagent (Thermo Scientific, Rockford, IL). The polyclonal antibody against GAPDH (glyceroaldehyde-3-phosphodehydrogenase) was purchased from Millipore. For the analyses of TG1 and TG2 in the urine, samples were prepared from the sacrificed mice and were diluted by PBS and then treated with SDS-buffer.

2.4. In vitro and in situ detections of the enzymatic activities

To the kidney soluble extract (50 μg), biotin-labeled peptides (pepK5 and pepT26) were added at the final concentration of 100 μM in the presence of 5 mM $CaCl_2$. Then, the reaction products were subjected to 10% SDS-PAGE and blotted to PVDF membrane. The biotin-incorporated proteins were detected using streptavidin-conjugated peroxidase and chemiluminesence reagent. As negative controls, the mutant peptides where glutamine-residues were exchanged to asparagine residues (pepK5QN and pepT26QN) were used.

The *in situ* activity was visualized using FITC-labeled peptides for the unfixed kidney section as reported previously [14]. From the frozen tissue, 10 μm section was prepared and the reaction mixture containing 100 mM Tris-Cl (pH 8.0), 1 mM dithiothreitol, and 5 mM $CaCl_2$ in the presence of 5 μM FITC-labeled peptide was added. After washing by PBS, the mounting solution SCMM R2 (Leica Microsystems Co. Ltd.) was added and the section was observed under the fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The signal intensity in the images were adjusted with maintaining linearity by imaging software (Adobe Photoshop CS).

2.5. Immunohistochemical analysis

The kidney was treated with 4% paraformaldehyde. The section was blocked (diluted goat serum) and then reacted with antibodies against mouse TG1 or TG2. As a negative control, the same amounts of IgG (Sigma) was used. To amplify the immunoreaction, biotin-labeled secondary antibody followed by adding avidin and biotin-peroxidase were used. Color development was performed using 3, 3′-diaminobenzidine staining.

2.6. Staining on tissue section

HE (hematoxylin and eosin, Leica Microsystems Co. Ltd.) and PAS (Periodic acid-Schiff, Muto pure Chemicals Co., Tokyo, Japan) staining were performed by standard methods.

2.7. Statistical analysis

The results for immunoblotting and *in vitro* enzymatic activities were statistically analyzed by Student t-test using the densitometric values of signals (Fig. 2B and D). Regarding the other results including picture data, statistical analyses were not carried out (Figs. 1 and 3–5).