Analytical Biochemistry 482 (2015) 16–21

Contents lists available at ScienceDirect

Analytical Biochemistry

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

A competitive enzyme-linked immunosorbent assay for quantification of tetrastatin in body fluids and tumor extracts

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ARTICLE INFO

Article history:
Received 17 November 2014
Received in revised form 25 March 2015
Accepted 23 April 2015
Available online 29 April 2015

Keywords:
Tetrastatin
Collagen IV
Competitive ELISA
Pathologies

ABSTRACT

Basement membrane collagens or derived fragments are measured in biological fluids such as blood and urine of patients and appear to be useful for diagnosis, prognostication, or treatment monitoring as proposed for endostatin, a fragment of collagen XVIII, or tumstatin, a fragment of collagen IV. Tetrastatin, the NC1 alpha 4 collagen IV domain, was previously reported to inhibit tumor growth and angiogenesis. The aim of this study was to develop and validate a method to measure tetrastatin concentrations in human fluids. We developed a competitive enzyme-linked immunosorbent assay (ELISA). It allowed measuring tetrastatin levels in human serum, bronchial aspiration and bronchoalveolar lavage fluids, and lung tissue extracts. The tetrastatin level was significantly higher in tumor tissues than in healthy lung tissues. Tetrastatin competitive ELISA could be useful to quantify tetrastatin in tissues and biological fluids for the diagnosis or prognostication of diseases in which basement membrane metabolism may be altered, especially tumor progression.

Basement membranes are thin and amorphous specialized extracellular matrices that play roles in various biological events, including embryonic development. They are mainly composed of laminins, nidogens, heparan sulfate proteoglycans (perlecan and/or agrin), and type IV collagen. More recently, associations between basement membrane and minor collagens such as types XVIII, XV, and XIX have been reported and named basement membrane zone [1]. Basement membranes were long considered simple structural supports to the tissue as the constitutive proteins form an organized scaffold. However, they were reported to exert important biological functions, and their roles in many physiological and pathological situations are now well established [2]. Basement membrane components were suggested as diagnostic or prognostication markers in many diseases [3], and some of them were proposed as cancer biomarkers [4]. For example, type XVIII collagen, a basement membrane-associated collagen, was proposed as a cancer biomarker [5,6].

Collagen IV is the major component of basement membranes. It is formed by the association of three α(IV) chains among six possible, α1(IV) to α6(IV), each encoded by a different gene. Tissue distribution of α(IV) chains is variable. Whereas α1(IV) and α2(IV) chains are ubiquitous, the other four chains are expressed in specialized basement membranes. The α3(IV) and α4(IV) chains occur only in [α3(IV); α4(IV); α5(IV)] heterotrimers in the aorta, the pulmonary alveoli, the glomerulus, the cochlea, or the lens capsule. From the N terminus to the C terminus, each α(IV) chain comprises (i) an N-terminal domain comprising the 7S domain, (ii) a long central triple-helical domain with several interruptions, and (iii) a C-terminal NC1 domain of approximately 230 residues [7]. Different α(IV) NC1 domains were reported to exert antitumor or antiangiogenic activities [8].

The NC1 alpha 4(IV) domain, also named tetrastatin, induced inhibition of *in vivo* tumor growth in a mouse melanoma xenograft model and inhibition of *in vitro* tumor cell proliferation and invasion [9]. Peptides reproducing several tetrastatin sequences and named tetrastatin-1, -2, and -3 strongly inhibited endothelial cell migration without any significant effect on their proliferation [10,11].

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http://dx.doi.org/10.1016/j.ab.2015.04.023
0003-2697/

Tumstatin, the NC1 alpha 3(IV) domain, was also reported to exert antiangiogenic and antitumor properties [12–14]. Circulating tumstatin was first detected in mouse serum [15]. It was confirmed in human serum by Luo and coworkers, who also studied tumstatin amount in lung tissue extract by enzyme-linked immunosorbent assay (ELISA)[2] [16]. No studies on circulating tetrastatin have been reported to date. The aim of the current work was to develop and validate a rapid and convenient method allowing tetrastatin detection and its quantification in human fluids and tissue extracts. A competitive ELISA was developed to achieve this goal. Unselected patients were used to detect tetrastatin in body fluids (serum, bronchial aspiration fluids, and bronchoalveolar lavage fluids). We also tried to detect tetrastatin in tissue extracts of patients with lung cancer.

Materials and methods

Reagents

Biochemical reagents were purchased from Sigma (St. Quentin–Fallavier, France). Rabbit anti-human tetrastatin antibody was produced by Covalab (Villeurbanne, France) by immunizing the TLKESQAQRQKISRC sequence in rabbit. The His-Tag monoclonal antibody was obtained from GenScript (Piscataway, NJ, USA). pQE-31 vector and Ni-NTA resin were obtained from Qiagen (Courtaboeuf, France).

Patients and specimens

Blood samples from 53 unselected patients (22 female and 31 male, age range = 10–89 years, mean = 55 years) were obtained from the Central Laboratory of Biochemistry at Reims University Hospital (CHU de Reims). Sera were then separated and stored at −80 °C.

Bronchoalveolar lavage fluids from 20 patients (8 female and 12 male, age range = 24–81 years) and bronchial aspiration fluids from 22 patients (11 female and 11 male, age range = 27–81 years) (mean = 59 years), were obtained from the Department of Respiratory Diseases at Reims University Hospital and centrifuged at 2000*g* for 10 min at 4 °C, and supernatants were stored at −80 °C.

Tissue extracts from 12 patients (cancer tissue and healthy tissue taken at a distance from the tumor and checked as normal by a collaborating pathologist) were obtained from the Department of Pathology at Reims University Hospital and stored at −80 °C.

Collection and use of human specimens were approved by the institutional review board of Reims University Hospital and conducted according to the Declaration of Helsinki principles. Written informed consent was obtained from all subjects.

Protein extraction from tissues

Tissues were cut into small pieces, sonicated on ice in a Tris buffer (50 mM Tris [pH 7.6], 0.5 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.02% NaN_3, 1 mM phenylmethanesulfonyl fluoride [PMSF], 5 mM ethylenediaminetetraacetic acid [EDTA], and 1 mM iodoacetamide), homogenized overnight at 4 °C, and centrifuged at 10,000*g* for 10 min at 4 °C. Supernatants were kept frozen until use.

[2] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; rh tetrastatin, recombinant human tetrastatin; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; TBS-T, Tween 20 in Tris–HCl buffer and NaCl; BSA, bovine serum albumin; TMB, tetramethylbenzidine; CV, coefficient of variation; HUVEC, human umbilical vein endothelial cell.

Expression and purification of recombinant human tetrastatin

The sequence encoding the complete human tetrastatin (NC1[α4(IV)1–229]) was amplified by reverse transcription–polymerase chain reaction from kidney messenger RNA (mRNA) using the following sets of primers: 5′-TTTGGCCCTGGATACCTCGGT-3′and 5′-CGCATTCTCTAGCTATACTTC-3′. The resulting complementary DNA (cDNA) fragments were cloned into a pQE-31 vector. The orientation and complete sequence of the insert were checked by sequencing. Recombinant human tetrastatin (rh tetrastatin) was expressed in *Escherichia coli* JM109, DE3 strain (Promega, Charbonnière-les-bains, France). Transformed bacteria were grown at 37 °C in 100 ml of Luria–Bertani medium containing 50 μg/ml ampicillin until the absorbance at 600 nm was approximately 0.6. Protein expression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 h of culture at 37 °C under agitation, bacterial suspension was harvested by centrifugation at 4000*g* for 15 min at 4 °C. The pellets were resuspended in 5 ml of 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 8.0). The cells were disrupted by thaw/freeze cycle and sonication. After centrifugation at 10,000*g* for 30 min, supernatant (4 ml) was incubated for 1 h with 1 ml of Ni-NTA Superflow resin. The resin was then poured into a chromatography column and washed with 4 ml of 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 8.0). Finally, rh tetrastatin was eluted with 3 × 0.5 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0). After dialysis against distilled water, its purity was assessed by SDS–PAGE (polyacrylamide gel electrophoresis) and by Western blotting using an anti-tetrastatin polyclonal antibody or an anti-6 His-Tag monoclonal antibody.

Western blot

For Western blot analysis, samples were reduced by 10 mM dithiothreitol and subjected to SDS–PAGE (0.1% SDS and 10% polyacrylamide gel) (100 μg total protein/lane) and then transferred onto Immobilon-P membranes (Millipore, St. Quentin en Yvelines, France). Membranes were blocked by incubation with 5% nonfat dry milk and 0.1% Tween 20 in 50 mM Tris–HCl buffer and 150 mM NaCl (pH 7.5) (TBS-T) for 2 h at room temperature. They were incubated overnight with a 1/5000 diluted rabbit anti-human tetrastatin polyclonal antibody or with a 1/5000 anti-6 His-Tag monoclonal antibody and then for 1 h with the 1/10,000 diluted corresponding peroxidase-conjugated secondary antibody at room temperature. Immune complexes were visualized using the ECL Prime Chemiluminescence Detection Kit (GE Healthcare, Orsay, France).

Competitive ELISA procedure

Direct ELISA development

Serial dilutions were performed from a single stock solution to obtain the different dilutions of antibody or rh tetrastatin concentrations. rh tetrastatin (0–10 μg/well) was adsorbed to the 96-well microtiter plate in carbonate buffer (0.2 M sodium carbonate and 0.2 M sodium bicarbonate, pH 9.6) overnight at 4 °C. After washing with TBS-T, the plate was blocked with TBS-T supplemented with 1% bovine serum albumin (BSA) for 1 h at 37 °C and washed with TBS-T. Then, 100 μl of the primary antibody solution (rabbit anti-human tetrastatin polyclonal antibody) at different dilutions (1/100 to 1/3200) in TBS-T containing 1% BSA was added to each well and incubated for 1 h at room temperature. After three washes with TBS-T, 100 μl of the secondary antibody solution (anti-rabbit IgG coupled to peroxidase) diluted 1/10,000 was added to each well and incubated for 1 h at room temperature. After three