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Analytical methods for measuring collagen XIX in human cell cultures, tissue extracts, and biological fluids

J.B. Oudart [a,b,1], S. Brassart-Pasco [a,1], E. Luczka [a], A. Dupont-Deshorgue [a], G. Bellon [a,b], S.P. Boudko [c], H.P. Bächinger [c], J.C. Monboisse [a,b], F.X. Maquart [a,b], L. Ramont [a,b,*]

[a] Université de Reims Champagne-Ardenne, FRE CNRS No. 3481, Reims, France
[b] CHU de Reims, Laboratoire Central de Biochimie, Reims, France
[c] Research Department, Shriners Hospital for Children, Portland, OR 97239, USA

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ABSTRACT

Type XIX collagen is a minor collagen associated with basement membranes in vascular, neuronal, mesenchymal, and epithelial tissues. We demonstrated that the NC1, C-terminal, domain of collagen XIX inhibits the migration capacities of tumor cells and exerts a strong inhibition of tumor growth. Other basement membrane collagens or derived fragments were measured in biological fluids such as blood and urine of patients and appeared to be useful for diagnosis, prognosis, or treatment monitoring. The aim of this study was to develop and validate methods to measure collagen XIX and its fragments in human cell cultures, tissue extracts, and human biological fluids. For that purpose, we developed real-time PCR, Western blot, and competitive enzyme-linked immunosorbent assays. We demonstrated that the methods developed in this paper are specific for collagen XIX. We showed that it is expressed in human cell cultures, tissue extracts, and various biological fluids. These methods may be used in various human tissue extracts and biological fluids such as serum, amniotic fluid, cord blood, and many other fluids. Collagen XIX or its fragments could constitute new biomarkers for human diseases as well as for diagnosis and/or prognosis.

The basement membrane is a complex structure [1], long regarded as a simple cell support. However, it exerts many important biological functions, well described today, and its roles in many physiological and pathological situations are now well established [2]. Measurement of several basement membrane components are used as diagnostic and prognostic markers in many diseases, and some of them are used as tumor markers. For example, measurements of type IV collagen, the main component of the basement membranes, or of type XVIII collagen, a minor component of basement membranes, were used as tumor markers [3,4]. Enzyme-linked immunosorbent assays (ELISA)[2] have been developed to measure fragments of these collagens in various human biological fluids [5].

Type XIX collagen is a minor collagen associated with basement membranes. It is a homotrimer of 400 kDa, composed by the association of three α1(XIX) chains. Each chain comprises 1142 residues, including six noncollagenous domains (NC1-NC6) separated by five collagenous domains (Col1-Col5) [6,7]. It is localized in the basement membrane zone of vascular, neuronal, mesenchymal, and epithelial tissues, associated with type IV or type XVIII collagens [8]. Collagen XIX is ubiquitously expressed during embryogenesis, where it participates in extracellular matrix assembly. During breast cancer progression, collagen XIX disappears from basement membrane at invasive stages [9]. Previously, we demonstrated that the NC1, C-terminal, domain of collagen XIX exerts a strong inhibition of tumor growth in an experimental murine melanoma model [10]. *In vitro*, NC1(XIX) also inhibits the migration capacities of tumor cells [11].

The aim of this study was to develop and validate methods for measuring collagen XIX expression. These methods can be used to investigate the expression of collagen XIX in cell cultures, in human fluids, or in tissue extracts. For that purpose, we developed a real-time PCR assay as well as Western blot and competitive enzyme-linked immunosorbent assays.

Materials and methods

Reagents

Culture media and molecular biology products were from Life Technologies (Invitrogen, Strasbourg, France). All other reagents were purchased from Sigma (St. Quentin-Fallavier, France). Rabbit

* Corresponding author. Address: Laboratoire Central de Biochimie, CHU de Reims, Rue Serge Kochman, 51092 Reims Cedex, France. Fax: +33 326788539.
E-mail address: lramont@chu-reims.fr (L. Ramont).

[1] These two authors contributed equally to the study.

[2] *Abbreviations used:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assays; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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anti-human NC1 α1(XIX) was produced by Covalab (Villeurbanne, France). The NC1(XIX) peptide, NPEDCLYPVSHAHQRTGGN, was purchased from Proteogenix (Oberhausbergen, France). It was obtained by solid-phase synthesis using a FMOC (*N*-(9-fluorenyl)methoxy-carbonyl) derivative procedure and further purified by reverse-phase high-performance liquid chromatography with a C18 column eluted by a gradient of acetonitrile in trifluoroacetic acid, and then lyophilized [12]. A trimeric mini collagen containing 6 GPO triplets coupled to a NC1(XIX) domain was produced as described [13].

Cell cultures

MRC-5, a human lung embryonic fibroblast cell line, was provided by RD-Biotech collection. BZR and MG-63 cells, two human bronchial epithelial and osteosarcoma cell lines, were provided by the American Type Culture Collection (ATCC, Manassas, VA). They were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) in Nunclon 25 cm^2 flasks (Dutscher, Brumath, France) at 37 °C in a humid atmosphere composed of 5% CO_2 and 95% air.

Sample preparation

Kidney tissue samples were obtained from Laboratory of Anatomy of the Faculty of Medicine. Extraction of collagen XIX from tissue samples was performed according to Myers et al. [14]. The anatomical piece was rinsed several times in a solution containing 50 mM Tris-HCl, 4.5 M NaCl, 20 mM EDTA, pH 7.5, 10 mM *N*-ethylmaleimide, 1 μg/mL aprotinin (w/v). It was then cut into multiple fragments of less than 0.5 cm, and placed in an extraction buffer at 4 °C for 24 h (30 ml, 10/1 v/w) containing 50 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, pH 7.5, and protease inhibitors: 5 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 0.1% azide. The tissue was homogenized on ice using an Ultra-Turrax T25 (8 × 1 min). The tissue suspension was stirred slowly for 24 h at 4 °C. After 24 h, the extract was centrifuged at 32,000 rpm for 30 min. The supernatant was collected and the protein supernatant was then precipitated with ammonium sulfate to 40% saturation, and then centrifuged again at 32,000 rpm for 30 min at 4 °C. The protein pellet thus obtained was resuspended in 20 mL of extraction buffer containing 0.1% Triton X-100 and slowly stirred overnight at 4 °C and then dialyzed overnight at 4 °C against 500 mL of solution containing 25 mM Tris-HCl, 0.4 M NaCl, 2.0 mM EDTA, pH 7.4, Triton X-100 (0.1%), and protease inhibitors: 5 mM *N*-ethylmaleimide, 0.5 mM PMSF, 5 mM benzamidine, 0.1% azide. A second dialysis was then performed against 500 ml of the same buffer except pH (7.2) and NaCl (0.1 M) concentration. The dialyzate was centrifuged at 20,000 rpm for 15 min at 4 °C. The supernatant was incubated in a batch procedure with Sepharose beads (Sepharose Fast Flow Resin S, Amersham Biosciences), preequilibrated in the final dialysis buffer containing 0.1 M NaCl, overnight at 4 °C with gentle stirring. The suspension was centrifuged at 480*g* for 5 min at 4 °C.

The resin was washed with a buffer containing 25 mM Tris-HCl, 2.0 mM EDTA, pH 7.2, 5 mM *N*-ethylmaleimide, 0.5 mM PMSF, 5 mM benzamidine, 0.1% azide. Elutions were carried out stepwise at 30-min intervals using increasing concentrations of NaCl (0.1 to 1 M) with centrifugation at 480*g*, 5 min at 4 °C.

Conventional PCR

RNA isolation was performed using the Qiagen RNeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. cDNA was prepared from 1 μg of total RNA by reverse transcription (RT) at 42 °C for 45 min. The primers were [5′-ctggtcaaaagggagagcaa-3′] and [5′-ctccttttatgccctttct-3′] for COL19A1, [5′-ctggagccaagtgctaacatgcc-3′] and [5′-ccgggtttgagaaaccagtc-3′] for EEF1A1. The primers were designed using the e-primer 3 software and the specificity of the sequences was checked using the NCBI Blast software. The PCR was performed in an Eppendorf thermocycler and the cycling program (45 cycles) included a 20-s denaturation step at 95 °C and a 30-s annealing step at 60 °C, followed by a 30-s elongation step at 72 °C. The amplified DNA fragments were visualized after agarose gel electrophoresis in the presence of ethidium bromide and image acquisition was performed using the Bio-Capt and Bio-1D software (Vilber Lourmat, Marne la Vallée, France).

Quantitative real-time PCR

RNA isolation was performed as previously described. SYBR Premix Ex Taq (Ozyme, Saint Quentin en Yvelines) was used for the PCR. The primers used were the same as those above. Mx 3005P (Stratagene) was used for amplification and data collection. A first denaturation step was performed for 10 min at 95 °C, and then the cycling program (45 cycles) included a 5-min denaturation step at 95 °C, a 30-s annealing step at 60 °C, followed by a 15-s elongation step at 72 °C. Fluorescence acquisition was carried out at 72 °C in single mode at the end of the elongation step. After real-time PCR, a melting-curve analysis was performed by continuously measuring fluorescence during heating from 55 to 95 °C at a transition rate of 0.2 °C/s. Product specificity was evaluated by melting-curve analysis and by electrophoresis in 2% agarose gel. Fluorescence was analyzed by the Data Analysis software (Stratagene). Crossing points (Cp or Ct) were established using the second derivative method. The qPCR efficiency was calculated from the slope of the standard curve. Target gene expression levels were normalized to reference gene. The results were calculated using the delta-delta method.

Western blot

For Western blot analysis, samples, reduced or not by 10 mM dithiothreitol, were subjected to SDS-PAGE (0.1% SDS, 10% polyacrylamide gel) (100 μg total protein per lane), and then transferred onto Immobilon-P membranes (Millipore, St. Quentin en Yvelines, France). Membranes were blocked by incubation with 5% nonfat dry milk, 0.1% Tween 20 in 50 mM Tris-HCl buffer, 150 mM NaCl, pH 7.5, for 2 h at room temperature. They were incubated overnight with a 1/1000 diluted rabbit anti-human NC1(XIX) antibody and then for 1 h with the 1/10000 diluted corresponding peroxidase-conjugated second antibody at room temperature. Immune complexes were visualized using the ECL prime chemiluminescence detection kit (GE Healthcare, Orsay, France).

Competitive ELISA procedure

For determining ELISA conditions, 96-well microtiter plates were used for enzyme-linked immunosorbent assay. Serial dilutions were performed from a single stock solution to obtain the different dilutions of antibody and peptide concentrations. NC1(XIX) peptide (0 to 20 μg/well) was adsorbed to the 96-well microtiter plate in carbonate buffer (0.2 M sodium carbonate, 0.2 M sodium bicarbonate, pH 9.6) overnight at 4 °C. After washing with TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, Tween 20 0.05% (v/v)), each well was blocked with TBST-BSA 1% (TBST-bovine serum albumin) for 2 h at 37 °C. After washing the plate with TBST, 100 μl of the primary antibody solution (IgG rabbit anti-NC1 (XIX)) at different dilutions (1/100 to 1/800) in TBST-1% BSA was added to each well, and then incubated for 1 h at room