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Biochemical and Biophysical Research Communications 341 (2006) 958–963
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An ELISA method for the detection and quantification of human heparanase

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Received 9 January 2006
Available online 24 January 2006

Abstract

Heparanase is a mammalian endo-β-D-glucuronidase that cleaves heparan sulfate side chains at a limited number of sites. Heparanase enzymatic activity is thought to participate in degradation and remodeling of the extracellular matrix and to facilitate cell invasion associated with tumor metastasis, angiogenesis, and inflammation. Traditionally, heparanase activity was well correlated with the metastatic potential of a large number of tumor-derived cell types. More recently, heparanase upregulation was detected in an increasing number of primary human tumors, correlating, in some cases, with poor postoperative survival and increased tumor vascularity. The present study was undertaken to develop a highly sensitive ELISA suitable for the determination and quantification of human heparanase in tissue extracts and body fluids. The assay preferentially detects the 8 + 50 kDa active heparanase heterodimer vs. the latent 65 kDa proenzyme and correlates with immunoblot analysis of heparanase containing samples. It detects heparanase at concentrations as low as 200 pg/ml and is suitable for quantification of heparanase in tissue extracts and urine.

Keywords: Heparanase; ELISA; Antibody; Urine

Heparanase is an endoglycosidase that specifically cleaves heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPG) [1–3]. HSPG consist of a protein core to which HS side chains are covalently attached. These complex macromolecules are highly abundant in the extracellular matrix (ECM) and are thought to play an important structural role, contributing to ECM integrity and insolubility [4]. In addition, HS side chains can bind to a variety of biological mediators such as growth factors, cytokines, and chemokines, thus providing a readily available reservoir of active molecules that can be liberated upon local or systemic cues [5]. Moreover, HSPG on the cell surface participates directly in signal transduction cascades by potentiating the interaction between certain growth factors and their receptors [6–8]. HS-degrading activity is thus expected to affect several fundamental aspects of cell behavior under normal and pathological settings. Traditionally, heparanase activity was implicated in cellular invasion associated with angiogenesis, inflammation, and cancer metastasis [9–12]. This notion recently gained further support by employing siRNA and ribozyme technologies, clearly depicting heparanase-mediated HS cleavage and ECM remodeling as critical requisites for metastatic spread [13]. Since the cloning of the heparanase gene and the availability of specific molecular probes, heparanase upregulation was documented in an increasing number of primary human tumors, correlating with reduced postoperative survival, enhanced local and distant metastasis, and increased microvessel density [14–21]. The enzyme has also been implicated in diabetic nephropathy [22,23] and immune responses [2,11,12,24]. Collectively,

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doi:10.1016/j.bbrc.2006.01.048

these studies provide compelling evidence for the clinical relevance of the enzyme, making it an attractive target for drug development. Heparanase gene induction in human malignancies, as well as in several other pathologies such as cirrhosis, nephrosis, and diabetes [22,23,25], further implies the enzyme as a valuable clinical diagnostic marker. Several assays have been reported for measuring heparanase enzymatic activity, utilizing its HS-degrading capacity [26–31]. However, a method for the detection and quantification of small amounts of heparanase in tissue extracts and body fluids has not been reported. Here, we report the development of a sensitive ELISA method suitable for determination and quantification of human heparanase. The assay preferentially detects the 8 + 50 kDa active heparanase heterodimer vs. the 65 kDa latent proenzyme. It correlates with immunoblot analysis of heparanase containing samples, detects heparanase at concentrations as low as 200 pg/ml, and is suitable for quantification of heparanase in tissue extracts, plasma, and urine samples. A 4- to 5-fold average increase in heparanase levels was found in urine collected from cancer and diabetes patients vs. healthy donors, further supporting the notion that heparanase may be considered as a diagnostic and prognostic marker, and a valid target for drug development.

Materials and methods

Antibodies and reagents. Monoclonal anti-heparanase antibody 1E1 was generated by immunizing Balb/C mice with the entire 65 kDa heparanase protein. Hybridomas were obtained by routine procedure and were selected by an ELISA screen using the 65 kDa heparanase for coating. Several hybridomas that reacted positively with recombinant human heparanase were selected for further characterization. Anti-heparanase1453 polyclonal antibody was raised in rabbit against the entire 65 kDa heparanase precursor isolated from the conditioned medium of heparanase-transfected 293 cells [32], and has been shown to recognize both the latent and active forms of heparanase [32–34]. HRP-conjugated goat anti-rabbit antibody was purchased from Jackson ImmunoResearch (West Grove, PA). Microtiter 96-well plates (Maxisorp) were from Nunc (Roskilde, Denmark). HRP colorimetric substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) was from Biological Industries (Beit Haemek, Israel). Single chain (GS3) active heparanase, comprised of the 8 and 50 kDa heparanase heterodimer gene construct, was kindly provided by Dr. Christian Steinkuhler (IRMB/Merck Research Laboratories, Pomezia, Italy) [30], and the protein was purified from the conditioned medium of baculovirus infected insect cells [30].

ELISA procedure. Wells of microtiter plates were coated (18 h, 4 °C) with 2 μg/ml of 1E1 monoclonal anti-heparanase antibody in 50 μl of coating buffer (0.05 M Na_2CO_3, 0.05 M $NaHCO_3$, pH 9.6) and were then blocked with 2% BSA in PBS for 1 h at 37 °C. Samples (200 μl) were loaded in duplicate and incubated for 2 h at room temperature, followed by the addition of 100 μl antibody 1453 (1 μg/ml) for additional 2 h at room temperature. HRP-conjugated goat anti-rabbit IgG (1:20,000) in blocking buffer was added (1 h, room temperature) and the reaction was visualized by the addition of 50 μl chromogenic substrate (TMB) for 30 min. The reaction was stopped with 100 μl H_2SO_4 and absorbance at 450 nm was measured with reduction at 630 nm using ELISA plate reader. Plates were washed five times with washing buffer (PBS, pH 7.4, containing 0.1% (v/v) Tween 20) after each step. As a reference for quantification, a standard curve was established by a serial dilution of recombinant 8 + 50 GS3 active heparanase enzyme (390 pg/ml–25 ng/ml).

Sample preparation and immunoblotting. Liver and lung tissues were harvested from control and heparanase transgenic mice and homogenized by Polytron homogenizer (Kinamatica, Luzerne, Switzerland) in 10 volumes of PBS supplemented with protease inhibitors. Lysate samples were centrifuged and the supernatant was applied onto ^{35}S-labeled ECM to evaluate heparanase activity (see below), or pre-absorbed with concanavalin A (Con A)–Sepharose beads to concentrate the samples and reduce non-specific reactivity, and subjected to SDS–PAGE under reducing conditions. Following electrophoresis, proteins were transferred to PVDF membrane (Bio-Rad) and reacted with the appropriate antibody followed by HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescent substrate (Pierce, Rockford, IL), as described [35–37].

Heparanase activity assay. Preparation of ECM-coated 35 mm dishes and determination of heparanase activity were performed as described in detail elsewhere [27,34,36]. To evaluate heparanase activity in tissue extracts, fresh liver and lung tissues derived from heparanase over-expressing transgenic (*hpa*-tg) mice [40] and control mice were homogenized, as described above, and the supernatant fractions were incubated (4 h, 37 °C, pH 6.0) with ^{35}S-labeled ECM. The incubation medium (1 ml) containing sulfate labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity was counted in a β-scintillation counter. Degradation fragments of HS side chains are eluted at $0.5 < K_{av} < 0.8$ (peak II, fractions 15–30) and represent heparanase generated degradation products [33,37].

Statistics. Data are presented as means ± SE. Statistical significance was analyzed by two-tailed Student's t test. The value of $P < 0.05$ is considered as significant.

Results

Establishment of quantitative ELISA

Heparanase upregulation in several pathological settings, including cancer, and the secreted nature of the enzyme predict elevated heparanase levels in patients' body fluids. In order to detect and quantify heparanase in multiple samples, we set to establish an ELISA suitable for the detection of low protein levels. Several anti-heparanase antibodies from different species (mouse, rabbit, and goat) were tested for their ability to detect heparanase in a sandwich ELISA. The antibodies were tested reciprocally, and the best pair selected for further characterization was monoclonal antibody 1E1, applied for coating, and polyclonal antibody 1453 used for detection of the bound enzyme. Similar to several other classes of enzymes, heparanase is first synthesized as a latent enzyme that appears as a 65 kDa protein when analyzed by SDS–PAGE [1,2]. The protein undergoes proteolytic processing yielding an 8 kDa peptide at the N-terminus and a 50 kDa polypeptide at the C-terminus that heterodimerize to form the active heparanase enzyme [36,38,39]. Thus, we first compared the sensitivity of our assay for the 65 kDa latent heparanase vs. the 8 + 50 kDa active heterodimer. As demonstrated in Fig. 1A, ELISA reactivity was significantly higher with the 8 + 50 kDa active enzyme than with the 65 kDa proenzyme. Applying increasing amounts of the 8 + 50 kDa heparanase further revealed the linearity of the assay, up to 30 ng/ml. Plotting the data in a semilogarithmic scale revealed sensitivity of 200 pg/ml (Fig. 1B), providing a linearity range exceeding 2 orders of magnitude.