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ScienceDirect
Life Sciences 79 (2006) 1661 – 1665
Life Sciences
www.elsevier.com/locate/lifescie

Colorimetric heparinase assay for alternative anti-metastatic activity

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Received 31 October 2005; accepted 25 May 2006

Abstract

Heparanase has been previously associated with the metastatic potential, inflammation, and angiogenesis of tumor cells. Heparanase activity has been detected by means of UV absorption, radiolabeled substrates, electrophoretic migration, and heparan sulfate affinity assays. However, those methods have proven to be somewhat problematic with regards to application to actual biological samples, the accessibility of the immobilized substrates, experimental sensitivity, and the separation of degraded products. Rather than focusing on heparanase activity, then, we have developed a rapid, alternative colorimetric heparinase assay, on the basis of the recent finding that sulfated disaccharides generated from heparin by bacterial heparinase exhibit biological properties comparable to those from heparan sulfate by mammalian heparanase. In this study, the concentrations of porcine heparin and bacterial heparinase I were determined using a Sigma Diagnostics Kit. *Morus alba* was selected as a candidate through this assay system, and an inhibitor, resveratrol, was purified from its methanol extract. Its anti-metastatic effects on the pulmonary metastasis of murine B16 melanoma cells were also evaluated. Our findings suggest that this assay may prove useful as a diagnostic tool for heparinase inhibition, as an alternative anti-metastatic target.

Keywords: Heparanase; Heparinase; Metastasis; *Morus alba*; Resveratrol

Introduction

Heparan sulphate (HS) is a crucial component of the ECM and the vasculature basal lamina. HS consists primarily of clusters of highly sulfated disaccharide units, predominantly *N*-sulfated glucosamine linked 1→4 to α-*L*-iduronic acid residues. The degradation of HS by endoglycosidase or heparanase of invading cells may lead to a disassembly of the ECM and the basal lamina, thereby facilitating the migration of cells, including tumor and inflammatory cells (Parish et al., 2001; Vlodavsky and Friedmann, 2001). Heparanase activity has been previously associated with the metastatic potential, inflammation, and angiogenesis in a variety of tissues and cell homogenates. The cleavage of HS appears to be pivotal with regard to the passage of metastatic tumor cells and leukocytes through the basement membranes (Finkel, 1999; Bame, 2001).

In order to screen selective heparanase inhibitors for use in the development of anti-metastatic and anti-inflammatory drugs, a host of assays have been developed for the detection of heparanase activity. Basically, heparanase activity can be detected by the measurement of UV absorption at a wavelength of 232 nm of the α,β-unsaturated uronides formed during heparin degradation (Linker and Hovingh, 1972). Radiolabeled substrate techniques, including solid-phase assays using radiolabeled HS chains attached to a solid support (Nakajima et al., 1986), as well as direct exposure assays to the radiolabeled ECM after chromatographic analysis (Freeman and Parish, 1997) have also been successfully employed. Another such technique was designed on the basis of observations of changes occurring in the electrophoretic migration pattern of heparin that had been stained with sensitive dyes, including toludine blue, methylene blue, alcian blue, rubipy, or a combination of alcian blue with silver staining (Rozenberg et al., 2001). In addition, the use of glycosaminoglycans without discrete electrophoretic mobility renders the detection of endoglycosidase activity a different proposition.

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0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.05.020

Recently, an assay was developed for the separation of degraded products from the substrate, predicated on the reduced affinity of the heparanase-cleaved products for HS-binding plasma glycoprotein (Ding et al., 2001). However, previous heparanase assay have proven somewhat cumbersome, tedious, and time-consuming, both with regard to the preparation of the radiolabeled substrates and to the separation of the degraded products from the uncleaved substrate through gel filtration. Furthermore, with regard to the enzyme reaction, it remains extremely difficult to prepare pure heparanase, even though the coding genes have previously been cloned (Hulett et al., 1999). Therefore, a simple, rapid, and alternative assay will be required in order to screen candidates for the development of anti-metastatic and anti-inflammatory agents. It has been reported that both sulfated disaccharides (DS) degraded from heparan sulfate by mammalian heparanase and sulphated DS generated from heparin by bacterial heparinase exert similar modulatory effects on the cell-mediated immune reactions, adhesion, and migration of human T cells (Hershkoviz et al., 2000).

This study describes the development of a simple and rapid colorimetric method, which can detect heparinase activity and thus help to identify likely candidates for future anti-metastatic and anti-inflammatory drugs, using bacterial heparinase rather than mammalian heparanase. This novel method for the detection of heparinase inhibitors circumvents the above disadvantages of classical heparanase assays, reducing total handling time to within 1 h.

Materials and methods

Mice and cells

Specific pathogen-free (SPF) C57BL/6 mice (female, 4–6 weeks old) were acquired from the Korea Research Institute of Bioscience and Biotechnology (KRIBB), in Taejon, Korea. The mice were maintained under SPF conditions until use. Sterilized food and water were supplied to the mice ad libitum. B16F10 murine melanoma cells were maintained in RPMI 1640 medium, which had been supplemented with 10% fetal calf serum (HyClone, Kansas, MO).

Plant materials and inhibitor isolation

Root barks samples of *Morus alba* (Sang-Baek-Pi) and all other plants used in the screening of heparinase I inhibitors were purchased from Il-Sin Co., in Taejon, Korea. These were ground and extracted for 7 days with methanol. The methanol extracts were then evaporated and concentrated under reduced pressure. The concentrated extracts were resuspended in the appropriate volumes of methanol, and then subjected to the heparinase I assay system. The inhibitory compound was isolated via activity-guided purification procedures from the methanol (MeOH) extracts of *M. alba* through EtOAc extraction, 1st silica gel, Sephadex LH-20, 2nd silica gel, RP-18 and HPLC.

Heparinase assay

The heparinase activity assay was conducted in accordance with the conventional protocols, with some slight modification (Linhardt et al., 1982). In brief, 0.2 U of heparinase I (Sigma, St Louis, MO) from *Flavobacterium heparinum* were preincubated with the test samples for 5 min, after which the enzyme reaction was initiated via the addition of 100 ng of porcine heparin from intestinal mucosa (low molecular weight, 6.0 kDa) in 25 μl of a buffer containing 14 mM sodium acetate and 1.4 mM $CaCl_2$ (pH 7.0) for the an additional 15 min at room temperature. In order to determine the amount of undigested heparin, 25 μl of antithrombin III was applied for 2 min. Twenty five microliters of Factor Xa were applied for 1 min, and then 25 μl of the Factor Xa substrate was added to the reaction mixture. After 15 min, 20 μl of acetic acid was added in order to quench the reaction. Absorbance was then measured with a microplate reader (Dynatech MR700, Chantilly, VA) at 410 nm (single wavelength, calibration factor = 1.00).

Metastasis assay

B16F10 melanoma cells (5×10^5 cells/mouse) were injected into the tail veins of C57BL/6 mice on day 0. Partially purified SBCM101 and resveratrol obtained from the methanol extract of *M. alba*, or adriamycin (Sigma, St. Louis, MO) as a control, were administered intraperitoneally. Control group mice were given 0.85% NaCl. Ten animals were allocated to each group ($n = 10$). On day 14, the lungs were excised, and the metastatic nodules were counted (Kobayashi et al., 1998; Han et al., 1999).

Statistical analysis

The statistical significance of differences between the control and experimental groups was evaluated for two parallel experiments, using the Student's *t*-test, function in Excel 7.0. All experiments were conducted in duplicate, and standard deviations are indicated with bars.

Results

Optimization of heparinase assay

For the evaluation of heparinase activity, we measured the residual heparin concentrations remaining after the enzyme reaction, which represented a slight modification of the conventional method. When both Factor Xa and antithrombin III are present in excess quantities, a complex is formed from antithrombin III and Factor Xa. Factor Xa then reacts with its chromogenic substrate, the Factor Xa substrate (colored yellow here). In the presence of heparin, an antithrombin inhibitor, Factor Xa activity is inhibited in direct proportion to the heparin concentration (colorless). The administration of heparinase results in the loss of heparin's activity against antithrombin III (yellow color). In order to evaluate heparinase activity, the concentration of porcine heparin was first determined, via a color reaction assay conducted using a Sigma Diagnostics Kit (Accucolor™ Reagents for Heparin Kit). According to the results, the heparin concentrations, from 100 ng/ml and up to 10 μg/ml, were detected via the colorimetric analysis of optical density (Fig. 1, ●). Heparin concentrations were adjusted to 4 μg/ml in the reaction mixtures.