Abstract

Program Number 600.3

The purpose of this study was to determine if heparin competed with THP for binding to proteins that interact with both THP and heparin (i.e. C1q and fH), and to screen other heparin-binding proteins for previously unidentified interactions with THP. Dot blots were performed by immobilizing 1 µg of C1q, H, and bovine serum albumin (BSA) to 4 strips of nitrocellulose (NC). These strips showed that THP bound strongly to C1q and H, and that this binding was inhibited by heparin. Heparin also inhibited the background binding of the antisera to C1q. ELISAs were used to quantify the ability of heparin to inhibit C1q (15 µg/ml) binding to immobilized THP (40 µg/ml). A steep inhibition curve was obtained, with no detectable binding of immobilized THP occurring with 0.01 units/ml heparin, 50% inhibition of binding occurring between 0.04 and 0.05 units heparin/ml, and 90% inhibition occurring with 0.1 units/ml heparin (170 units/mg heparin). Control wells showed that heparin's inhibition of C1q binding to THP was not due to inhibition by heparin of the antisera's binding to C1q. Additionally, dot blots were used to evaluate binding of soluble THP to ATIII, IL-10, and FGF2, three of the many proteins that heparin has been shown to bind. Protein-protein interactions between THP and FGF2 and IL-10, that were sensitive to heparin inhibition, were discovered. Heparin and THP coexist in the urine, especially after intravenous heparin administration or when heparin is instilled into the bladder as a therapy for interstitial nephritis.

ELISA Methods

The dot blot results showing that heparin inhibited binding between THP and C1q were investigated further using an ELISA format (see figure to the left). All samples were added to 96 well microtiter plates (Falcon, Pro-Bind, Becton Dickinson, Lincoln Park, NJ), and unless stated otherwise, were added at 50 µl/well and incubated overnight at 4°C. Between incubation steps, wells were washed with 20mM Tris/20mM NaCl/0.05% Tween 20 buffer. Initially, wells were coated with 40 µg/ml THP (purified from one healthy adult male) or with 0.5 µg/ml C1q (Complement Technology, Tyler, TX) in 0.05 M NaCO3 pH 9.6 buffer for control wells. The next day, 5 µg/ml C1q was added to THP-coated wells in 1% BSA/Tris buffer with one of 11 different concentrations of heparin ranging from 0 to 0.5 units/ml (170 units/mg heparin). Bound C1q was detected with goat anti-human C1q (Complement Technology, Tyler, TX) diluted in BSA/Tris buffer and then with rabbit anti-goat IgG alkaline phosphatase (Southern Biotech, Birmingham, AL) for one hour at 37°C. Alkaline phosphatase activity in wells was detected using 4 mM pNPP/1mM MgCl2/0.05 M NaCO3 pH 9.6.

Results of THP/C1q Heparin Competition ELISA

ELISAs showed that there was no significant inhibition of C1q binding to immobilized THP at heparin concentrations of 0.01, 0.02, or 0.03 units/ml. However, as shown in the graphs below, the binding of C1q to THP was very sensitive to heparin concentrations above 0.03 units/ml with 90% inhibition of binding occurring with between 0.04 and 0.05 units heparin/ml, and 90% inhibition occurring with 0.1 units/ml heparin. Control wells showed that heparin's inhibition of C1q binding to THP was not due to inhibition by heparin of the antisera's binding to C1q.

Novel THP-Protein Interactions Detected with Dot Blot

Since THP and heparin appear to bind proteins similarly, dot blots were performed to see if THP binds other heparin-binding proteins (ATIII, FGF2, and IL-10). BSA was included as a negative control. As seen below, THP bound FGF2 strongly and IL-10 weakly. Both of these interaction were inhibited by heparin. THP did not bind ATIII.

Compete Between Tamm-Horsfall Protein and Heparin in Ligand Interactions

Matthew Haskell and Diana C. J. Rhodes, DVM, PhD

Department of Anatomy, Pacific Northwest University of Health Sciences, Yakima, WA

Introduction

Tamm-Horsfall protein (THP), also called uromodulin, is an 86kD, acidic (pI ~ 3), single-chain, N-linked glycoprotein produced in the kidney and excreted in milligram quantities into the urine daily, making it the most abundant protein in normal human urine1. THP binds various molecules of the immune system including immunoglobulins, complement 1q (C1q), and factor H (fH)2, 3, 4. Heparan sulfate and heparin interactions with THP/ATIII, IL-10, and FGF2, three of the many proteins that heparin has been shown to bind. Protein-protein interactions between THP and FGF2 and IL-10, that were sensitive to heparin inhibition, were discovered. Heparin and THP coexist in the urine, especially after intravenous heparin administration or when heparin is instilled into the bladder as a therapy for interstitial nephritis. Mast cells are prominent in the wall of the urinary bladder in interstitial cystitis, so competition of heparin and THP to bind to other proteins may be important in this disease.

Dot Blot Testing Ability of Heparin to Inhibit THP Binding to C1q and Factor H

Dot blots were performed by immobilizing C1q and Factor H (H) (each at 1 µg/dot) to 4 strips of nitrocellulose (NC). One NC strip was stained with amido black and the other 3 NC strips were first blocked with 10 µg/ml BSA in 10 mM NaPO4/10 mM NaCl/1 mM MgCl2 and then incubated with either THP (10 µg/ml)/heparin (20 µg/ml) (sodium salt from porcine intestine, Calbiochem), or no THP/heparin in NaPO4 buffer with 0.05% Tween 20, before incubation with sheep anti-THP antiseraum and then donkey anti-sheep IgG - HRP. The dots were developed with 0.05% 4-chloro-naphthol.

This dot blot showed that THP strongly bound both C1q and H (THP + 1 µg/2 panel) and that this binding was effectively inhibited by heparin (THP + heparin + 1 µg/2 panel). It also appeared that C1q's binding to the antisera (in 1 + 2 panel) was inhibited by heparin as well (THP + heparin + 1 µg/2 panel).

Contact Information

Matthew Haskell
Osteopathic Medical Student | Pacific Northwest University of Health Sciences
mhaskell@pnw.edu 801.471.8443

Diana C.J. Rhodes, DVM, PhD
Professor and Chair of Anatomy | Pacific Northwest University of Health Sciences
drhodes@pnw.edu

References


Acknowledgements

This work was supported by PNWU’s Research Seed Program. James A. Rhodes, Anita Cleafly, and Mary Giovanni were very helpful in this study.