Experimental and Molecular Pathology 87 (2009) 178–183

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

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

Heparin cofactor II in atherosclerotic lesions from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study

Jill C. Rau [a], Carolyn Deans [b], Maureane R. Hoffman [a,c], David B. Thomas [a], Gray T. Malcom [d], Arthur W. Zieske [d], Jack P. Strong [d], Gary G. Koch [b], Frank C. Church [a,e,f,g,*]

[a] Department of Pathology and Laboratory Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[b] Department of Biostatistics, School of Public Health, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[c] Pathology and Laboratory Medicine Service, Durham Veterans Affairs, Durham, NC 27710, USA
[d] Department of Pathology, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA
[e] Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[f] Department of Pharmacology, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
[g] Carolina Cardiovascular Biology Center, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

ARTICLE INFO

Article history:
Received 20 August 2009
Available online 9 September 2009

Keywords:
Atherosclerosis
Immunohistochemistry
Heparin cofactor II
Thrombin
Serpins
PDAY

ABSTRACT

Heparin cofactor II (HCII) is a serine protease inhibitor (serpin) that has been shown to be a predictor of decreased atherosclerosis in the elderly and protective against atherosclerosis in mice. HCII inhibits thrombin *in vitro* and HCII–thrombin complexes have been detected in human plasma. Moreover, the mechanism of protection against atherosclerosis in mice was determined to be the inhibition of thrombin. Despite this evidence, the presence of HCII in human atherosclerotic tissue has not been reported. In this study, using samples of coronary arteries obtained from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study, we explore the local relationship between HCII and (pro)thrombin in atherosclerosis. We found that HCII and (pro)thrombin are co-localized in the lipid-rich necrotic core of atheromas. A significant positive correlation between each protein and the severity of the atherosclerotic lesion was present. These results suggest that HCII is in a position to inhibit thrombin in atherosclerotic lesions where thrombin can exert a proatherogenic inflammatory response. However, these results should be tempered by the additional findings from this, and other studies, that indicate the presence of other plasma proteins (antithrombin, albumin, and α_1-protease inhibitor) in the same localized region of the atheroma.

Introduction

Heparin cofactor II (HCII) is a serine protease inhibitor (serpin) that has been strongly implicated in the inhibition of atherosclerosis (Aihara et al., 2007, 2004; Huang et al., 2007; Tollefsen, 2007; Vicente et al., 2007). Individuals with high levels of HCII have been shown to have less atherosclerosis than their counterparts (Aihara et al., 2004), and HCII deficient mice show increased atherosclerotic plaque formation (Aihara et al., 2007; Vicente et al., 2007). Thrombin exhibits mitogenic and chemotactic activities that contribute to the chronic inflammatory processes of atherosclerosis (Baykal et al., 1995; Harker et al., 1995). Studies that examined restenosis after arterial stent placement showed that elevated blood concentrations of HCII were associated with decreased incidence of restenosis (Schillinger et al., 2004; Takamori et al., 2004); the processes that dominate restenosis, smooth muscle cell proliferation and migration, are also important processes in atherogenesis and can be induced by thrombin. HCII–thrombin complexes have been detected in human plasma (Liu et al., 1995) and, thrombin has been found in its active form in atherosclerotic lesions (Stoop et al., 2000). HCII inhibits thrombin at physiologically relevant rates only in the presence of glycosaminoglycans (GAGs) (Rau et al., 2007). Dermatan sulfate is the predominant antithrombotic GAG in the artery wall (Tovar et al., 2005). It specifically accelerates HCII inhibition of thrombin and has been shown to have decreased activity in atherosclerotic lesions as compared to normal tissue (Shirk et al., 2000). Although HCII is presumed to regulate thrombin in atherosclerosis, the presence of HCII in atherosclerotic lesions has not been reported.

In this study we utilized atherosclerotic lesions of coronary arteries sampled from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) research program. PDAY was established in 1985 to quantitatively assess the risk factors for coronary heart disease. Data and arterial samples from over 3000 individuals from the ages of 15–34 who died of external causes (accidents, homicides, suicides) were collected by fifteen cooperating centers across the United States and managed by the Department of Pathology at Louisiana State University Health Science Center. The data published

* Corresponding author. Campus Box 7035, 932 Mary Ellen Jones Building, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7035, USA. Fax: +1 919 966 7639.
E-mail address: fchurch@email.unc.edu (F.C. Church).

0014-4800/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2009.09.003

using PDAY has greatly enhanced our understanding of atherosclerosis and its associated risk factors (see (McGill et al., 2008) for recent review).

We probed the atherosclerotic plaques for HCII, (pro)thrombin and several other proteins with the hypothesis that we would find decreased levels of HCII and increased levels of thrombin in more severe atherosclerotic plaques, reasoning that less HCII would result in decreased thrombin inhibition and therefore more severe atheromas.

Materials and methods

Histological samples

Samples of human left anterior descending (LAD) coronary artery were collected, formalin-fixed, paraffin-embedded, serially sectioned and mounted on glass slides by PDAY (Cornhill et al., 1995; Strong et al., 1999, 1997; Wissler, 1994). Twenty-eight cases with varying severity of atherosclerotic lesions were analyzed in this study.

Sections of formalin-fixed, paraffin-embedded liver were obtained from the McLendon Clinical Laboratory at the University of North Carolina Hospitals, cut into 4 μm serial sections and mounted on glass slides. These served as positive controls slides for immunohistological staining as almost all of the proteins that were probed are of hepatic origin. The exception is maspin, a non-plasma serpin of mamillary epithelial origin involved in tumor suppression. Maspin is not synthesized in the liver and thus served as a negative control. A liver section with a maspin-positive tumor served as the maspin-positive control.

Slide preparation, mounting and staining with hematoxylin and eosin

Before staining, paraffin was dissolved and slides were rehydrated using standard techniques. After staining and dehydration all slides were coverslipped using 1 drop of Permount Mounting Media (Fisher Scientific) and air dried overnight. For staining with hematoxylin and eosin, rehydrated slides were immersed first for 8 min in filtered Mayer's hematoxylin (Dako), rinsed in tap water until no more dye was evident and then soaked in tap water for 10 min. Slides were then rinsed in distilled and deionized water before being dipped ten times in 95% ethanol. Slides were then submersed for 45 s in Eosin Y (Sigma) followed by 95% ethanol for 5 min more.

Antigen retrieval

Heat-induced antigen retrieval was employed for probing the AT antigen only. Six slides at a time were placed into plastic Copeland jars with a large hold drilled in the lid filled with Tissue Unmasking Fluid (Invitrogen). These jars were placed into 400 mL glass beaker in 180 mL of distilled, deionized water and heated on highest power in a Samsung Classic Collection Microcooking microwave for 2.5 min. The water was then exchanged for 180 mL of fresh water and the beaker containing the Copeland jar with slides was heated again on highest power for 2.5 min to bring the internal temperature to 90 °C. The water was exchanged again for 180 mL of tap water and everything was permitted to cool for 20 min.

Immunohistochemical staining

Slides were laid flat on a rack and incubated in 460 μL per slide of each successive solution. To remove solution, slides were rinsed with Dulbecco's phosphate buffered saline (PBS) (Gibco) containing 1% Tween 20 (Sigma) ($PBStw_{1\%}$). Before and between incubation with each solution, slides were washed in $PBStw_{1\%}$ for 3 min. First, endogenous peroxidase activity was blocked with either HRP-Block (Dako) for 5 min or with 3% hydrogen peroxide for 10 min. Slides were then incubated for 1 h in the dark with primary antibody diluted in PBS with either 1% ovalbumin or 1% bovine serum albumin. Table 1 describes the antibodies used, their dilutions and incubation times. Next, slides were incubated in the dark with peroxidase-conjugated secondary antibody appropriately matched to primary antibody either HRP-conjugated donkey anti-goat IgG (Serotec) or HRP labeled polymer (Dako). Secondary antibodies were diluted in the same solution as their primary antibodies. Slides were then covered with nine drops per slide of diaminobenzidine solution (DAB) (DAKO) and let sit for 8 min. DAB staining was enhanced using a solution of 2.5% cobalt chloride, 2.5% nickel ammonium sulfate for 8 min and rinsed in water. Slides were counterstained for 5 min with Mayer's Hematoxylin (Dako or Sigma), rinsed in tap water for 5 min before dehydrating and coverslipping.

Lesion classification

One serial section from each case was stained with hematoxylin and eosin. Using this slide set, the severity of each atherosclerotic lesion was classified separately by three trained individuals. Plaque severity was rated from I (least severe) to VI (complicated lesions) according to the American Heart Association classification system (Stary et al., 1995, 1994).

Staining intensity

The intensity of staining for each probed antigen was also independently ranked on a scale of 0 to 3 with 0 indicating no staining, 1 indicating weak staining, 2 indicating intermediate staining and 3 indicating strong staining. Raters were unaware of the specific antigen probed when they were rating staining intensity. Additionally, raters were asked to consider staining intensity only in the tunica intima and tunica media as these are the portions of the vessel susceptible to atherosclerosis. Images were created using the Aperio ScanScope and Aperio ImageScope software version 9.0. Slides were scanned using factory settings for immunohistochemistry.

Table 1
Description of antibody, dilutions and incubation times used in immunohistological staining.

1° Antibody	Clonal	Raised in	1° Ab dilution	1° Ab time (min)	2° Ab dilution	2° Ab time (min)
Anti-human HCII IgG—affinity purified (Affinity Biologicals)	poly	goat	1:1000	60	1:100	30
Anti-human antithrombin IgG (Diasorin)	poly	goat	1:500	60	1:200	20
Anti-human (pro)thrombin[a] IgG (Dako)	poly	rabbit	1:2000	60	None	10
Anti-human α1-protease inhibitor IgG (Fitzgerald Industries)	poly	goat	1:10,000	60	1:100	10
Anti-human albumin IgG—affinity purified (Antibodies Inc)	poly	goat	1:1000	60	1:1000	10
Anti-human maspin IgG (Pharmingen)	poly	goat	1:100	60	1:100	30
Goat IgG		goat	1:1000	60	1:100	30

[a] Antibodies that distinguish between human prothrombin and human thrombin are not commercially available. Therefore, under circumstances such as fixed-tissue probing when molecular weight is not ascertainable, the two are indistinguishable by antigen staining.