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Heparin Cofactor II in Atherosclerotic Lesions from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Study

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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

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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.

Keywords

Atherosclerosis; immunohistochemistry; heparin cofactor II; thrombin; serpins; PDAY

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; Aihara et al., 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 3,000 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 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; Strong et al., 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 eight minutes in filtered Mayer's hematoxylin (Dako), rinsed in tap water until no more dye was evident and then soaked in tap water for 10 minutes. Slides were then rinsed in distilled and deionized water before being dipped ten times in 95% ethanol. Slides were then submersed for 45 seconds in Eosin Y (Sigma) followed by 95% ethanol for five more minutes.

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 minutes. 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 minutes 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 minutes.

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 three minutes. First, endogenous peroxidase activity was blocked with either HRP-Block (Dako) for five minutes or with 3% hydrogen peroxide for ten minutes. Slides were then incubated for one hour 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 eight minutes. DAB staining was enhanced using a solution of 2.5% cobalt chloride, 2.5% nickel ammonium sulfate for eight minutes and rinsed in water. Slides were counterstained for five minutes with Mayer's Hematoxylin (Dako or Sigma), rinsed in tap water for five minutes 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; Stary et al., 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.

Statistical Analysis

Mean scores for lesion severity and staining intensity were computed for each slide. Spearman correlation coefficients, with their corresponding p-values, were computed to evaluate the correlation between the mean scores for each of the probed proteins and severity of the atherosclerotic lesions. The mean score for each protein decreased the variance of scores, thereby increasing the power for detecting a significant correlation when it did exist. The use of the mean scores had support from assessment of intra-rater reliability with intra-class correlations. In order to maintain an overall type 1 error rate of 0.05 throughout five comparisons between lesion severity and staining intensity, there was adjustment for multiple comparisons with the Bonferroni-Holm method; i.e., the smallest p-value was initially compared to 0.05/5=0.01, if significant, then the second-smallest p-value was compared to 0.05/4=0.0125. If significant, the third-smallest p-value was compared to 0.05/3=0.01667, and so on for each of the five p-values, stopping when a non-significant result was observed.

RESULTS

Ratings of Lesion Severity and Antigen Staining of LAD Coronary Artery Sections

Atherosclerotic lesions were categorized from 1 to 5. No category 6 lesions were observed. Staining of each of the circulating proteins (HCII, (pro)thrombin, AT, albumin, and α_1-protease inhibitor) was observed with intensity range of 0 to 3. Staining intensity for maspin as rated 0 by all reviewers for every section where it was probed despite strong staining on its positive control (tumor in the liver – data not shown). Assessment of inter-rater reliability by inter-class correlation coefficients (ICCs) results in ICCs above 0.5 (Table 2), indicating that there is reliability among raters. This supports the use of the mean scores to assess correlations between plaque severity and staining intensity.

Each of the plasma proteins probed was detected in atheromatous lesions. Maspin was not found in any vessels. Antigen staining was primarily in the lipid rich, necrotic core of the atheromas. Figure 1 shows an example of serial sections of one sample of LAD atherosclerotic coronary artery, immunoprobed for each circulating protein. This atheroma was rated with an average lesion severity of 4.67. Mean staining intensity for the particular atheroma shown in Figure 1 was HCII = 3.00, AT = 1.67, (pro)thrombin = 3.00, α_1-protease inhibitor = 3.00, albumin = 3.00 and goat IgG = 0.

It is evident from Figure 1 that there is protein from all of the circulating proteins found in this atheromatous sample. There is some detectable HCII, AT and albumin in the endothelial region, but not the other proteins. It is of value to note the paucity of staining in the less atherosclerotic/more normal portion of the vessel (seen in the right-most column in Figure 1). No antigen staining was detected in the normal non-atherosclerotic intimal region of this or any of the LAD coronary arteries used in this study. This atheroma represented the pattern of distribution of all of the LAD samples, with staining present predominantly in atherosclerotic core regions and little to no staining perceivable in non-atherosclerotic tunic intima or tunica media.

Relationship Between Plaque Severity and Antigen Staining

Spearman correlation coefficients were determined to evaluate the correlation between lesion severity and staining intensity, using the Bonferroni Holm method to adjust for multiple comparisons. This analysis indicates that HCII staining, (pro)thrombin staining, and AT staining were significantly positively correlated with lesion severity (Table 3). The relationship between plaque severity and (pro)thrombin was the strongest with its Spearman's coefficient = 0.81, that for HCII = 0.63 and for AT = 0.48 (Table 3). α_1-Protease inhibitor and albumin did not appear to be correlated with lesion severity (Table 4), although only the most severe slides were available to be rated for these proteins, thus limiting our ability to detect a correlation with lesion severity for these proteins if one does exist.

DISCUSSION

When beginning the quest to detect the localization of HCII and thrombin in atherosclerotic plaques, we hypothesized that in more severe atherosclerotic plaques there would be low concentrations of detectable HCII and high concentrations of thrombin as compared with less severe atheroscleromas. Somewhat at odds with this hypothesis, we detected more HCII in the more severe atherosclerotic lesions as evidenced by a statistically significant positive Spearman Correlation (Table 3). However, as predicted (pro)thrombin staining exhibited a positive correlation between intensity and lesion severity. Both HCII and (pro)thrombin were detected in the same lipid-rich regions and necrotic cores of the atheromas. One explanation includes that more thrombin present in severe atheromas drives a more severe atherosclerotic process but also provides more ligand for HCII to bind. To effectively inhibit thrombin, a GAG cofactor is necessary. Dermatan sulfate is the predominant GAG in the arterial wall (Tovar et al., 2005) and is specific for HCII. Furthermore, it has been shown that dermatan sulfate found in atheromas has decreased ability to accelerate HCII inhibition of thrombin (Shirk et al., 1996). Therefore, although there is more HCII in severe plaques than compared to less severe plaques, its ability to inhibit thrombin in this arena may be restricted and thus a more severe plaque is evidenced.

The studies that the inhibition thrombin by HCII in atherosclerosis, also indicate that AT, the prominent thrombin inhibitor in coagulation, is unable to fulfill this role (Aihara et al., 2007; Vicente et al., 2007). Therefore, to substantiate the functional specificity of HCII in the atheromas, we probed adjacent sections for the presence of AT, expecting to find little there. To our surprise, AT was detected in the atheromas, in the same location as HCII and (pro) thrombin. The AT staining was somewhat less intense across all of the arterial samples despite comparable staining intensity to HCII and (pro)thrombin in the liver positive controls (data not shown). Due to variability in the quality of antibodies and the requirement to use heat-induction for antigen retrieval for AT, it is irresponsible to compare relative protein concentrations for different proteins in these stained vessels. So while the data hints of less AT than HCII in the atheroma, this cannot be firmly established under these conditions. The results do however demonstrate a positive correlation between staining intensity of AT and plaque severity, albeit not as strong as for (pro)thrombin or HCII. This suggested to us that perhaps the HCII and (pro)thrombin co-localization in the lipid-rich core of the atheromas might not be as specific as we had anticipated.

To further investigate the specificity of the co-localization of these thrombin-inhibiting serpins and (pro)thrombin, we probed additional adjacent LAD coronary artery sections for a non-thrombin-inhibiting plasma serpin, α_1-protease inhibitor (α_1PI); a non-serpin plasma protein, albumin; and a non-circulating serpin, maspin. Although we can find no evidence establishing a role for α_1PI or albumin in the pathophysiology of atherosclerosis, both of these proteins were found localized in the same region as HCII, (pro)thrombin, and AT (Figure 1). While correlations between staining intensity and plaque severity were not significant with albumin

and α_1PI, this could be due to the smaller number of slides probed. It is perhaps more important that these proteins are found in the atheromas while maspin, which is similarly sized and is from the same family, but is not a circulating protein, was not detected.

In an attempt to determine if the (pro)thrombin in atherosclerotic lesions was thrombin or prothrombin and if it was complexed with either the HCII or AT, we examined homogenized aorta by immunoblot analysis (data not shown). Results from these experiments confirmed the presence of HCII, AT, thrombin and prothrombin in atherosclerotic tissue and also in normal vessel. We did not observe thrombin-serpin complexes in either normal or atherosclerotic homogenized aorta. These results do not eliminate the possibility or even probability that thrombin is inhibited by HCII in the atherosclerotic vessel. It is very likely that serpin-thrombin complexes are cleared more quickly from the vessel microenvironment, or that they are somehow less detectable to the antibodies used here.

The presence in atherosclerotic lesions of all of the circulating proteins probed for in this study, but not the non-circulating serpin, maspin, suggests severe atherosclerotic lesions may act as a mire for plasma proteins and that the co-localization of proteins in this region may be non-specific. The positive correlation with plaque severity for HCII, (pro)thrombin and AT bolster this theory especially since the endothelium is known to show increased permeability with atherosclerosis (Feletou and Vanhoutte, 2006; Hirano, 2007; Simionescu, 2007). As the barrier function of the endothelium deteriorates, plasma components are more able to filter into the plaque and become trapped in the fatty debris laden region of atheromas. Many other circulating proteins, in addition to those reported here, have been found in the identical region of human atherosclerotic lesions including the apolipoproteins, apoA-I, apoB and apoE (O'Brien et al., 1998; Wyler von Ballmoos et al., 2006), C-reactive protein (Sun et al., 2005), vonWillebrand factor (Sun et al., 2005), and resistin (Burnett et al., 2005). Sound explanations for the existence and retention of these proteins in atherosclerotic plaques have been presented, just as there appears to be good reason for the presence of thrombin and HCII. One could even make a case for the retention of albumin in the plaque as it is known to bind fatty acids. However, we are unable to rationalize the function of α1-protease inhibitor in the atheroma. This is not to say that none of the proteins found in atherosclerotic lesions influence its progression. Contrarily, we find it likely that many of these proteins play a role in atherosclerotic regulation or advancement. For example, fibrin has been found in atherosclerotic plaques (Kaikita et al., 1999). While it is possible that it had been deposited by infiltrating macrophages, it is just as likely that all of the necessary coagulation factors leak into the plaque and are activated by the presence of tissue factor bearing cells in the atheroma. The result would be fibrin in the atherosclerotic plaque that does not resemble a typical fibrin clot because platelets and red blood cells would be absent from the milieu. This hypothesis also explains how thrombin could be detected in its active form in atherosclerotic plaques. Thus the increased permeability of atherosclerotic lesions may actually be a thrombogenic factor in and of itself. This would explain how increased serum HCII protects against atherosclerosis: as serum levels of HCII increase, more HCII crosses into the intimal layer of a vessel with a increased endothelial permeability and is able to neutralize thrombin that has also crossed the endothelium.

Our study also emphasizes a caveat that must be regarded in immunohistochemical examinations of atherosclerosis. While the presence or co-localization of any circulating protein(s) in atherosclerotic lesion may have functionality, it is important to consider that proteins found in the acellular, lipid-rich region of an atheroma, may simply be the result of increased endothelial permeability and may or may not contribute to atherogenesis and to its regulation.

Conclusions

In this study, we asked is HCII present in atherosclerotic lesions, does it co-localize with thrombin and is there a relationship between these proteins and the severity of the atherosclerosis? The results of immunohistochemical and immunoblot analyses establish the co-localization of (pro)thrombin with HCII in the core of atherosclerotic plaques and demonstrates a positive correlation between the presence of HCII, (pro)thrombin or antithrombin (AT) and lesion severity. Additionally, these results indicate that atheromas may act as a non-specific watershed for plasma proteins and should serve as a warning against assumptions that co-localization of protein in atheromas implies a pathophysiologic role in atherosclerosis.

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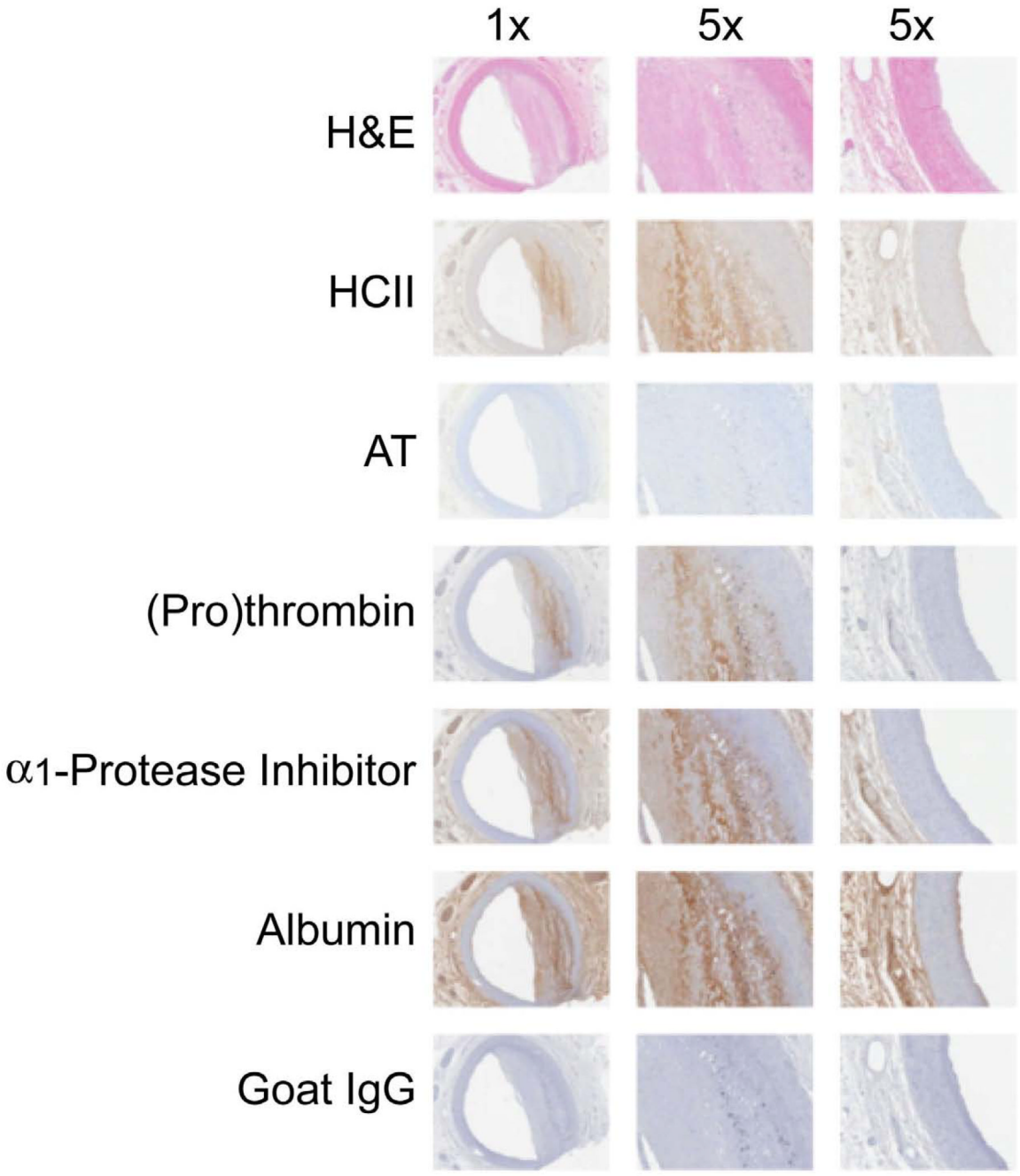

Figure 1. H&E and immunological staining of an example of a left, anterior descending coronary artery with atherosclerotic lesion for various plasma proteins and goat IgG negative control

The left-most column shows a 1× view of the vessel, the middle column shows a 5× view of the atheromatous lipid core and the right-most column a 5× view of the non-diseased region with no visible atherosclerosis.

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* 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

*Note: 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.

Table 2

Intra-class Correlation Coefficients (ICC) to Address Intra-Rater Reliability

Variable	ICC
Severity	0.824
HCII	0.705
(Pro)thrombin	0.719
AT	0.603
α1PI	0.725
Albumin	0.845

Table 3

Spearman Correlation Coefficients to Estimate Correlation of Severity and Antigen Staining.

	HCII	(pro)thrombin	AT	α1PI	albumin
correlation coefficient	0.63378	0.80537	0.48451	0.34403	0.38671
p-value	0.0003	<.0001	0.0090	0.4499	0.1918
N	28	28	28	7	13

Table 4

Breakdown of Tests of Significance Using Bonferroni-Holm Method to Adjust for Multiple Comparisons

Step	Variable	Observed p-value	Compare to	Significant?
1	(Pro)thrombin	<0.0001	0.01	YES
2	HCII	0.0003	0.0125	YES
3	AT	0.0090	0.01667	YES
4	Albumin	0.1918	0.025	NO → Stop after this step.