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CLINICAL STUDY

Plasma hyaluronidase activity as an indicator of atherosclerosis in patients with coronary artery disease

Mine Kucur[1], Bilgehan Karadag[2], Ferruh K Isman[3], Yusup Ataev[2], Dursun Duman[4], Nalan Karadag[2], Zeki Ongen[2], Vural A Vural[2]

Istanbul University, Fikret Biyal Research Laboratory, Cerrahpasa School of Medicine, Istanbul.
josephatayev@yahoo.com

Abstract: *Background and objective:* Recent information has highlightened the impact of HA metabolism alterations in vascular permeability through its actions on endothelial glycocalyx and the importance of HA-cell interactions in cell behavior of arterial endothelial and smooth muscle cells. Therefore hyaluronan is thought to involve in pathophysiology of atherosclerosis. The aim of this study is to investigate the association of plasma hyaluronidase activity with atherosclerosis in non-diabetic patients with stable coronary artery disease.
Methods: In the present study we used plasma hyaluronidase measurement as an indicator of hyaluronan metabolism and activity. A total of 162 subjects undergoing to coronary angiography were divided into two groups according to presence or absence of coronary artery disease, and their serum hyaluronidase activity were measured.
Results: Serum hyaluronidase activities were 3797±670.62 mU/L and 2838±417.67 mU/L for patients with CAD (n:109) and control patients without CAD (n:53), respectively. Serum hyaluronidase activity in patients with coronary artery disease (CAD) were significantly higher than control subjects without CAD ($p<0.001$).
Conclusion: In the present study hyaluronidase activity was found to be associated with coronary artery disease reflecting the role of hyaluronan in atherosclerosis. We believe that the demonstration of relationship between serum hyaluronidase activity and atherosclerosis represents a remarkable finding highlighting the potential role of hyaluronan in pathophysiology of atherosclerosis *(Tab. 2, Fig. 3, Ref. 28).* Full Text (Free, PDF) *www.bmj.sk.*
Key words: hyaluronidase, hyaluronan, atherosclerosis, coronary artery disease.

A consistent feature of atherosclerosis is the excessive accumulation of extracellular matrix. Hyaluronan appears to be of particular interest among these extracellular matrix components. Recent information reveals that all stages of human atherosclerotic lesions are characterized by marked changes in the content and distribution of hyaluronan (1–3). Hyaluronan, (HA, also known as hyaluronic acid or hyaluronate) a glycosaminoglycan composed of repeating disaccharide units, is a multifunctional protein that is involved in water and protein homeostasis of extracellular matrix, cell proliferation and migration (1). HA is also a principal constituent of endothelial glycocalyx, which is a dynamic structure enveloping the vascular endothelial cells. Endothelial glycocalyx is known as a principal determinant of vascular permeability barrier (4–6). A Loss of glycocalyx integration in experimental models leads to a wide spectrum of vascular abnormalities, which are known to be pro-atherogenic incidents (7, 8). The removal of hyaluronan-rich glycocalyx with hyaluronidase is shown to be associated with increased vascular permeability leading to atherogenic insults. (5–6). Increased plasma hyaluronan and hyaluronidase levels are found to be associated with endothelial glycocalyx damage, presence of microvascular disease and increased carotid intima-media thickness in diabetic subjects (9, 10).

The atherogenic character of HA is probably not solely attributable to its biophysical properties that contribute to extracellular matrix structure. In addition to function of HA metabolism in endothelial glycocalyx, the recent work has also highlightened the equally important role of HA in behavior of resident cells of vasculature. There are studies suggesting that HA may influence arterial endothelial and smooth muscle cell proliferation and migration in the development of atherosclerosis (2, 11, 12). Hyaluronan may also serve as a substrate for migration of inflammatory cells as monocytes and lymphocytes into the atherosclerotic lesion as a part of an inflammatory reaction (13).

There is no clinical data about the correlation between hyaluronan metabolism and atherosclerosis in humans. To our knowledge, this is the first study assessing the relation of hyaluronan metabolism with atherosclerosis in humans with documented stable coronary artery disease. Moreover, since stud-

[1]Istanbul University, Fikret Biyal Research Laboratory, Cerrahpasa School of Medicine, Istanbul, [2]Istanbul University, Department of Cardiology, Cerrahpasa School of Medicine, Istanbul, [3]Taksim Training and Research Hospital, Department of Clinical Biochemistry, Istanbul, and [4]Haydarpasa Training and Research Hospital, Department of Cardiology, Istanbul

Address for correspondence: Yusup Ataev, MD, Division of Cardiology, Istanbul University, Cerrahpasa School of Medicine, 34098 Istanbul, Turkey.
Phone: +90.212.4143000-21645, Fax: +90.212.4143070

ies investigating HA-vascular disease relationship were predominantly focused on diabetes, there is no information about hyaluronan activity in subjects without diabetes. In the present study, we used plasma hyaluronidase measurement as an indicator of hyaluronan metabolism and activity. The aim of this study is to investigate the association of plasma hyaluronidase activity with the presence and extent of atherosclerosis in non-diabetic patients with stable coronary artery disease.

Methods

Study design and subjects

The study was conducted in accordance with the Helsinki Declaration of 1975. The study protocol was approved by the Ethics Committee at Istanbul University, Cerrahpasa School of Medicine, and written informed consent was obtained from all patients. Plasma hyaluronidase activity and hsCRP were measured in 162 patients with suspected stable coronary artery disease who underwent coronary angiography for routine diagnostic purposes. Subjects had either a cardiac history or symptoms sufficient to warrant angiography. The indications included the history of coronary artery disease, clinical evidence of angina pectoris, and suspect chest pain. All patients with myocardial infarction within 30 days, those suffering from unstable angina with anginal pain at rest within 30 days and those with history of prior coronary revascularization were excluded. None of the subjects included in this study showed any evidence of ongoing systemic or cardiac inflammatory disease. Patients with diabetes mellitus, cases with history of recent clinical infection and those with concurrent renal, hepatic or malignant diseases were also excluded. Chronic liver disease is known to be associated with increased plasma hyaluronan levels that may also indirectly lead to elevated plasma hyaluronidase activity (14). Therefore, patients with elevated hepatic function markers as ASAT and ALAT (aspartate aminotransferase, alanine aminotransferase) are excluded. The study population was composed of 129 men and 33 women who underwent elective coronary angiography for suspected CAD at Istanbul University Cerrahpasa School of Medicine Cardiac Catheterization Laboratory. A total of 162 subjects are classified into two groups, namely the control group (n=53) composed of subjects with strictly normal coronary angiograms, and coronary artery disease group composed of 109 patients. Coronary artery disease was defined as at least one coronary artery having 50 % luminal diameter stenosis. Subjects with minor irregularities of the coronary vasculature or a moderate diameter reduction (<50 %) were excluded from the study. Serum lipid parameters were also determined.

Protocol for coronary angiography

Selective coronary angiography was performed by the Judkins technique through the femoral approach with 6 F catheters. Stenosis severity was determined by visual estimation (in ≥2 orthogonal views) and angiographic findings were assessed by experienced cardiologists in our centre. Operators reading the angiograms were blinded to the results of all laboratory analyses. The number, location, and severity of lesions on each arterial segment were recorded.

Blood Sampling

In patients undergoing the coronary angiography, blood samples were drawn after an overnight fasting at the time of catheterization, before administration of contrast agent or medications. Blood samples were left on the clot, and serum was separated from cellular elements by centrifugation within two hours after blood sampling. All serum samples were stored at -70 °C until analysis.

Biochemical analysis

Hyaluronidase Assay: 10 L serum is incubated with 250 µL of buffered substrate solution (0.10 mol/L sodium formate, pH 3.9 containing 0.1 mol/L sodium chloride, 250 mg/L hyaluronan and 1.5 mmol/L saccharic acid 1,4-lactone) for 4 h at 37 °C. The enzyme reaction was specifically terminated by addition of 50 µL of 0.8 mol/L potassium tetraborate at pH 9.1 to each sample. The tubes were heated for 3 min in a boiling water bath and cooled in tap water. The p-dimethylaminobenzaldehyde reagent (1.5 mL), made as described, was added to each sample. The samples were then vortexed, heated at 37 °C for 20 min, briefly centrifuged and read at 585 nm (15). Consequently, the „reducing N-acetylglucosamine termini“ reaction product was determined. Blanks for the reaction consisted of tubes in which the buffered substrate was incubated for 4 h at 37 °C in the absence of serum, and which subsequently recieved potassium tetraborate, then serum, and then treated as mentioned above. A standard curve was formed by using known concentrations of N-acetylglucosamine. In this method, a unit of hyaluronidase activity was defined as the production of a micromole of reaction product (reducing terminal N-acetylglucosamine) per min at 37 °C (16).

Statistical methods

The statistical analyses were performed using the version 12.0 of SPSS statistical program (SPSS, Chicago, IL). All values are expressed as mean±SD unless stated otherwise in text. Quantitative variables were compared with ANOVA one factor adjusted for age and sex. Multiple comparisons of hyaluronidase activity and number of diseased vessels were done by ANOVA one factor and the Bonferroni adjustment. A value of $p<0.05$ was considered statistically significant for all above analyses.

Results

Description of subjects

The study population included a total of 162 subjects (129 men and 33 women) undergoing the coronary angiography, who were classified into two groups according to the presence or absence of coronary artery disease: 109 patients with coronary artery disease constituted the CAD group and the other 53 had strictly normal angiograms and constituted the CAD-free control group. The characteristics and the laboratory findings of the groups are detailed in Table 1. No statistical differences between

Tab. 1. Baseline characteristics and laboratory findings of study subjects.

	Control group	CAD group	p value*
Subjects	53 (32.7%)	109 (67.3%)	NS
Male gender	41 (77.3%)	88 (80.7%)	NS
Age, yrs	55.7±8.2	59.6±9.1	NS
Current smokers	5 (9.4%)	19 (17.4%)	NS
TC (mg/dl)	146±5.6	135±6.7	NS
HDL-C (mg/dl)	40±2.9	34±1.87	NS
LDL-C (mg/dl)	79±3.56	83±5.66	NS
TG (mg/dl)	128±10.9	108±8.32	NS

Values are mean±SD (range) or percentage of total subjects in each group. p value indicates significance levels between groups.
NS – notsignificant (p>0.05)
Control group – subjects without coronary artery disease; CAD group – subjects with coronary artery disease. HDL-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; TC – total cholesterol; TG – triglycerides, *p<0.05 indicates statistical significance

the groups in relation to age, total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol and Total cholesterol/HDL ratio were found.

Serum hyaluronidase activity

In Figure 1, serum hyaluronidase activities are presented as box whisker plots for each of the groups. Plasma hyaluronidase activity for CAD group and control group were 3797±670.62 mU/L and 2838±417.67 mU/L, respectively (Tab. 2). Plasma hyaluronidase activity in coronary artery disease group was found to be significantly higher in comparison to control group with normal coronary arteries (p<0.001) (Fig. 1). The variables of total cholesterol, LDL-C, HDL-C, TG, and age did not correlate with hyaluronidase activity.

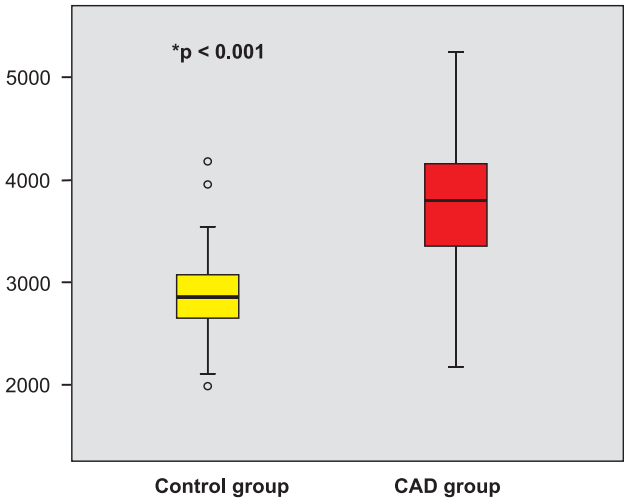

Fig. 1. Association of plasma hyaluronidase activity with presence of coronary artery disease. Plasma hyaluronidase activities are presented as box-whisker plots in the studied groups. The whiskers are lines that extend from the box to the highest and lower values, excluding outliers. A line across the box indicates the median. * – indicates the presence of statistically significant difference (p<0.001) between coronary artery disease group and the control group (composed of subjects without coronary artery disease).

Tab. 2. Plasma hyaluronidase activity and hsCRP concentrations in control subjects (subjects without coronary artery disease) and in patients with coronary artery disease (CAD).

	Control group	CAD group	p value*
Hya. Actv.(mU/L)	2838±417.67	3797±670.62	<0.001
hsCRP (mg/l)	2.97±0.31	6.82±1.23	<0.05

Values are means±SD. Hya. Actv. – plasma hyaluronidase activity, hsCRP – high sensitivity C-reactive protein, *p<0.05 indicates statistical significance

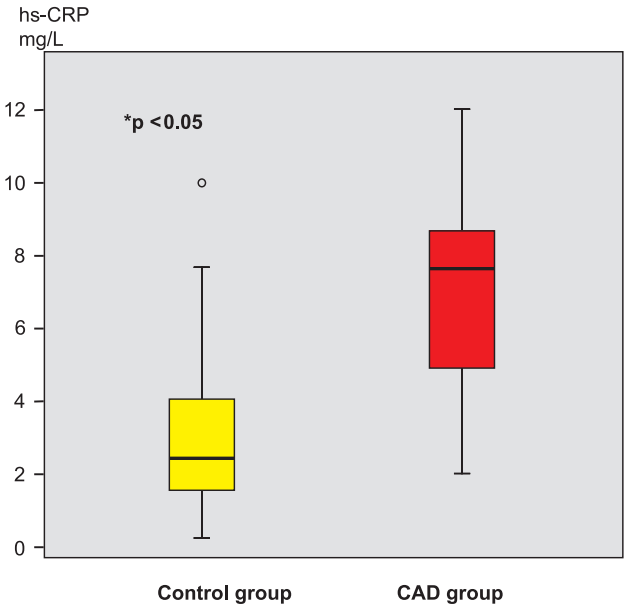

Fig. 2. Association of plasma hsCRP levels with the presence of coronary artery disease. Plasma concentrations of hsCRP are presented as box-whisker plots in the studied groups. The whiskers are lines that extend from the box to the highest and lower values. A line across the box indicates the median. * – indicates the presence of statistically significant difference (p<0.05) between coronary artery disease (CAD) group and the control group (composed of subjects without coronary artery disease). hsCRP – high sensitivity C-reactive protein.

High-sensitivity C-reactive protein (hsCRP)

In Figure 2, serum concentrations of hsCRP are presented as box whisker plots for each of the groups. Plasma hsCRP levels for CAD group and control group were 6.82±1.23 mg/l and 2.97±0.31 mg/l, respectively (Tab. 2). Plasma hsCRP level in coronary artery disease group was found to be significantly higher in comparison to control group with normal coronary arteries (p<0.05) (Fig. 2). The variables of total cholesterol, LDL-C, HDL-C, TG and age did not correlate with hsCRP concentrations. Backward linear regression analysis also showed a significant correlation between plasma hyaluronidase activity and hsCRP values (p<0.001) (Fig. 3).

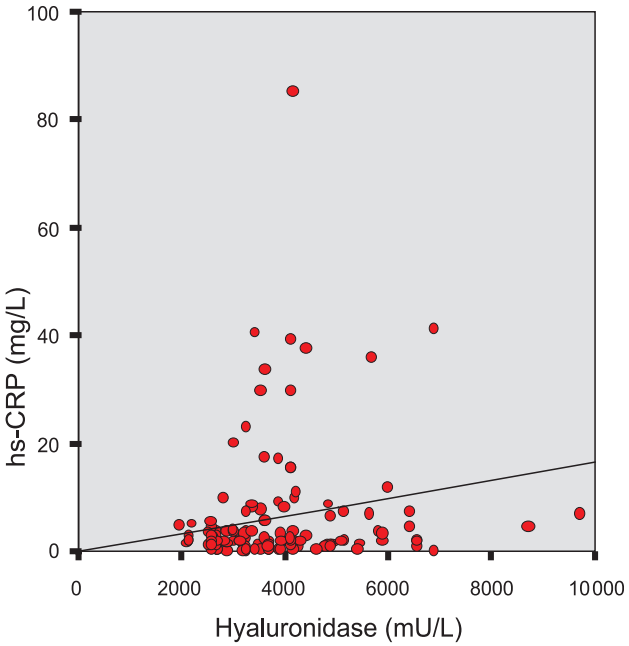

Fig. 3. Backward linear regression analysis showed a significant correlation between plasma hyaluronidase activity and plasma hsCRP levels in study groups (r=0.72, p<0.001).

Discussion

The knowledge on association of HA and vascular disease has advanced rapidly over the past several years. The efforts in this area have highlightened the impact of HA metabolism alterations in vascular permeability through its actions on endothelial glycocalyx and the importance of HA-cell interactions in cell behavior of arterial endothelial and smooth muscle cells (10, 11, 17).

Hyaluronan, a component of extracellular matrix, is a multifunctional protein, which is involved in water and protein homeostasis of extracellular matrix, cell proliferation and migration through interactions with its receptors as CD44 and RHAMM (Receptor for HA-Mediated Mobility) (1). HA is synthesized by the resident cells of the arterial wall; endothelial cells, smooth muscle cells and adventitial fibroblasts. Intima, media and adventitia layers of blood vessels are formed by cells embedded in a mixture of HA and other molecules of extracellular matrix (1).

HA is synthesized at the plasma membrane and immediately extruded out of the cell and into the extracellular matrix. Tissue HA is mainly removed by lymph nodes but liver also participates in HA degradation process. Hyaluronidase is the enzyme that is involved in the degradation of hyaluronan. Six hyaluronidase-like enzymes have been defined in human genome. Hyaluronidase-1, also known as plasma hyaluronidase, is unique since it is the only one to be found in human circulation (18). In addition to its role as an indicator of HA metabolism, hyaluronidase itself is also proposed to have implications in the cellular events that occur in vascular wall (19). There seems to exist a deliberately regulated balance between the production and the removal of HA that controls its biological functions during normal conditions. It has been hypothesized that when this balance is disturbed, disease may develop (20).

Several mechanisms are likely to play a role in HA mediated augmentation of atherosclerosis;

First, alterations in HA metabolism are shown to damage the integration of endothelial glycocalyx. Since endothelial glycocalyx in known to be the principal determinant of vascular permeability barrier, damage to glycocalyx leads to a wide spectrum of vascular abnormalities which are presumably atherogenic (4–6). These vascular abnormalities include increased vascular permeability for macromolecules as lipoproteins, adhesion of mononuclear cells and platelet agents to the endothelial surface and attenuated nitric oxide availability (7, 8). The removal of hyaluronan-rich glycocalyx with hyaluronidase is shown to be associated with increased vascular permeability leading to atherogenic insults (5–6). Nieuwdorp et al showed a decrease in systemic glycocalyx volume and an increase in human plasma HA and hyaluronidase levels in patients with microvessel disease in diabetes. These findings are believed to reflect the presence of vascular damage in consequence of disturbed endothelial glycocalyx structure (10).

Second, there are studies suggesting that HA may influence the arterial smooth muscle cell proliferation and migration in the development of atherosclerosis (12, 17). Tissues enriched in HA may undergo an expansion due to the ability of the molecule to bind large amounts of water, thus creating a loose hydrated microenvironment that facilitates cell migration and proliferation which are two critical events in atherogenesis. Beside its biophysical properties that contribute to extracellular matrix structure in atherosclerosis pathophysiology, there is also remarkable evidence about the equally important receptor-mediated role of HA in behavior of resident cells of vasculature. In atherogenesis, smooth muscle cells are transformed from the contractile, quiescent phenotype to the proliferative migratory phenotype that secretes abundant extracellular matrix (21). This change in smooth muscle phenotype and the migration and proliferation of smooth muscle cells seem to be regulated by receptor-mediated HA activity. The interference with the binding of HA to CD44 receptor either by the use of competing HA oligosaccharides or blocking antibodies to the receptors has been shown to block formation of pericellular matrices and the proliferation and migration of smooth muscle cells (17, 22).

Third, HA also influences the behavior of the vascular endothelial cells. HA is shown to stimulate CD44 mediated endothelial migration, proliferation and extracellular matrix synthesis, which is associated with new blood vessel formation in vitro (11).

Fourth, it is possible that lipoprotein transport may be altered due to modifications in the arterial wall, and the binding of HA to lipoproteins may accelerate the atherogenesis. In vitro studies have shown that HA does interact with phospholipids through hydrophilic interactions (23). HA is also present in areas of atherosclerotic lesions that contain extracellular lipid deposits, and lipoprotein-HA complexes have been isolated from human atherosclerotic lesions (1, 3, 23, 24).

Finally, HA is also present in regions of atherosclerotic lesions that contain inflammatory cells such as macrophages and lymphocytes (3, 24). Consistent with this, the extravasations of leukocytes from blood into the vascular wall involves hyaluronan anchored to surface of endothelial cells by DD 44 or RHAMM (1). These findings place HA to the beginning of the inflammatory response that is thought to be the critical step in the formation of atherosclerotic lesions (21). HA is not only important in the initial stages of leukocyte extravasations, but its accumulation in the early lesions may also promote inflammatory cell retention by serving as a substrate for cells. (1). The presence of HA in macrophage-rich regions of the plaque supports this possibility (3, 24). Mine S et al showed a correlation between peripheral blood level of hyaluronan and hsCRP, which is a well-documented inflammatory reference marker of atherosclerosis (25, 26). They also documented that both hsCRP and plasma hyaluronan levels were associated with diabetic angiopathy. In the present study, in accordance with the findings of Mine et al, plasma hyaluronidase activity also showed a significant correlation with hsCRP, and both were found to be correlated with coronary artery disease (Fig. 3). Since atherosclerosis is regarded as an inflammatory disease, the production and metabolism of HA reflected as serum hyaluronidase activity can also be expected to increase in patients with atherosclerotic disease.

A number of studies investigated the relationship of hyaluronan and its metabolism with diabetes on experimental models or in patients with uncomplicated diabetes (26, 9, 10). The knowledge about the association of HA with atherosclerosis is derived solely from experimental models or human autopsy studies. (17, 27, 28) and there is no information on the correlation between hyaluronan metabolism and atherosclerosis in humans on the clinical ground. To our knowledge, this is the first study assessing the relation of hyaluronan metabolism with atherosclerosis in humans with documented coronary artery disease. In the present study, we used plasma hyaluronidase activity as an indicator of hyaluronan metabolism. Although there is information suggesting the usefulness of serum HA level as a marker of diabetic angiopathy, there is no information about plasma HA activity in subjects without diabetes (26). Our work is also unique in the fact that our study subjects are composed of non-diabetic population.

In conclusion, the present study found hyaluronidase activity to be associated with atherosclerosis. Plasma hyaluronidase activity elevations were related to the presence of coronary artery disease. Although our findings require further confirmation, we believe that the demonstration of the relationship between plasma hyaluronidase activity and atherosclerosis represents a remarkable finding highlighting the potential role of hyaluronan in pathophysiology of atherosclerosis.

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