Preliminary Study Showing the Relationship Between Platelet Fibronectin, Sialic Acid, and ADP-induced Aggregation Levels in Coronary Heart Disease

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Summary: Several studies indicate that thrombosis plays an important role in the pathogenesis of coronary heart disease (CHD). Fibronectin is a multifunctional protein in plasma, other body fluids, and cell surface and plays an important role in platelet functions, including mediation of cell-cell and cell-surface interactions. Sialic acid is a regular constituent of glycoproteins and gangliozides in the outer cell membrane of mammalian cells. Therefore, the sialic acid content of platelets, which are characterized by their ability to aggregate with each other, can be important in leading to thrombus formation. In this study, platelet fibronectin, sialic acid-, and adenosine diphosphate (ADP)-induced platelet aggregation levels were determined in patients with CHD. Platelet sialic acid concentrations were determined by Warren's method. Platelet aggregation tests with ADP in platelet-rich plasma (PRP) were analyzed by use of an aggregometer. Platelet homogenate fibronectin levels were determined by ELISA. Total protein levels were determined by Lowry method. Our results indicate that, in patients with no vessel disease (patients with no obstructed vessel but suffering from chest pain, like angina pectoris) platelet fibronectin levels were significantly lower than the total of the other patients (patients with 1, 2, or 3 obstructed coronary vessels) ($p<0.05$). Sialic acid levels in patients with no vessel disease were significantly lower than the total of the patient group ($p<0.05$). There was significant (+) correlation between platelet aggregation, platelet fibronectin, platelet sialic acid, and severity of disease ($p<0.05$). Our preliminary findings suggest that, especially platelet fibronectin levels potentially represent a pathogenic factor for CHD.

Key Words: Platelet aggregation—Coronary heart disease—Sialic acid—Fibronectin.

Coronary heart disease (CHD) is one of the leading causes of death in the Western population and accounts for 50% of mortality in the United States, Europe, and Japan (1). Besides the development of coronary spasms, usually thrombus formation within the arterial lumen is the pathogenic mechanism. Progression of coronary thrombosis appears to be an important determinant of prognosis of patients with acute coronary syndromes (2). The presence of circulating activated platelets preceding massive thrombotic events such as myocardial infarction or stroke may be relevant to the development and progression of atherosclerosis (3). Previously, it was found that platelets of patients with CHD exhibit a higher aggregability when stimulated with agonists like adenosine diphosphate (ADP) and serotonin. Platelets are nuclear discoid cells in the blood that play a central role in hemostasis. Platelets circulate in a quiescent state and do not accelerate blood clotting. When there is an activating stimulus such as exposed

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collagen fibers, adsorbed proteins, or bulk-phase agonists (such as ADP), platelets can become activated. Then they accelerate thrombosis by several modes of action: Secretion of bulk-phase agonists and clotting factors, platelet-platelet aggregation, and acceleration of thrombin generation (4).

Cell surface glycoproteins such as fibronectin are believed to play an important role in platelet functions, including mediation of cell-cell and cell-surface interactions. Vascular endothelial cells are the major source of plasma fibronectin. It is also synthesized by a variety of cells in vitro, including fibroblast, smooth muscle cells and epithelial cells (5). Fibronectin is also found in platelets within the α secretory granules and secreted following platelet stimulation by a variety of agonists (6,7). Avaliable data suggest that expression of platelet fibronectin on the cell surface may indicate a role in platelet aggregation and adhesion to fibrin thrombi and connective tissue (5). Sialic acid (N-acetyl-neuraminic acid), a negatively charged sugar, is a regular constituent of glycoproteins and gangliosides in the outer cell membrane of mammalian cells (8–10). Mostly sialic acid and to a much lesser extent negatively charged amino acids like glutamate and aspartate are responsible for the cells' negative surface charge, which helps cells to keep a distance from each other due to repulsion of the same negative charges (11). Therefore, it is of particular interest to investigate the sialic acid content of platelets, which are characterized by their ability to aggregate with each other, leading to thrombus formation (12–17).

The aim of the present study was determining and correlating the platelet sialic acid-, platelet fibronectin-, and ADP-induced platelet aggregation levels in coronary artery disease (CAD) patient groups.

MATERIALS AND METHODS

Sample Collection and Preparation

Peripheral blood samples of 43 patients with coronary artery disease were collected by venous puncture before a planned heart catheterization, from the Department of Cardiology. CAD was assessed by coronary angiography and stress test (electrocardiography changes), and elevations in serum lactate dehydrogenase and creatine phosphokinase levels. According to the reporting system of the American Heart Association, more than 51% stenosis was considered significant (18). The study was conducted after all institutional ethics recuirements were met.

The patients group consisted of 12 with no-vessel disease (patients with no obstructed vessel but suffering from chest pain, like angina pectoris), and the total of the other patients (8 subjects with single-vessel disease, 8 subjects with double-vessel disease, and 15 subjects with triple-vessel disease) ranging in age from 33 to 77 years.

Methods

Venous blood was withdrawn (9 mL) and anticoagulated with 1 mL of 3.8% trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 1500 rpm for 15 min at 20°C, as described elsewhere (19). The supernatant was drawn into another tube, and the remaining PRP again centrifuged at 2500 rpm for 5 min to obtain platelet-poor plasma (PPP). In addition, platelets were sedimented by further centrifugation of PRP at 10,000xg for 5 min. The platelet pellet was washed with saline and centrifuged again. Briefly, platelets were destroyed by sonication (20kHz, amplitude 8×10^{-3} mm for 30 sec) (20).

The time between blood sampling and aggregation test was 30 min. It is well known that the extent of platelet aggregation varies directly with the interval between blood sampling and aggregation measurement over the first 30 min from sampling, then remains stable for the next 60 min observation (21). After leaving samples for 30 min at 24°C , light permeability was adjusted to 100% by using PPP from the same patient. Then 450 μL of PRP incubated at 37°C for 2 min and stirred again for 2 min in the aggregometer (Chrono-log 500 CA, Haverton, PA, USA). Changes in optical density were recorded for 6 min after the platelets were stimulated with 50 μL ADP (Sigma Diagnostics, St. Louis, MO, USA) at concentrations of 2×10^{-4} mol/L.

A turbidimetry curve, expressing the relation between the amplitude and rate of aggregation, was calculated for each subject's sample. The lowest light transmission at the end of the sixth minute was recorded as the maximal aggregation percentage (%). The time that 50% of maximal aggregation occurred was recorded as the aggregation rate (seconds).

Platelet fibronectin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (Takara Cat No:MK:115, Japan). Sialic acid in platelets was determined by thiobarbituric acid method described by Warren

(22). Before determination of the sialic acid, homogenates were incubated at 80ºC for 1 h in 0.1 N sulfiric acid in order to liberate the bound sialic acid. A calibration curve was obtained using 20, 40, 60, and 80 μg/mL concentrations of standard N-acetylneuraminic acid solutions (Sigma). Serum total-cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol levels were enzymatically determined (Sigma Diagnostics, St. Louis, MO, USA). Serum low-density lipoprotein (LDL) levels were estimated by using the Fridewald formula. Platelet protein was determined by the method of Lowry et al (23).

Statistical Analysis

Conventional methods were used for the calculation of means and standard errors of means. The significance of differences between variables of the patient groups was determined by the Anova/Tukey test. For correlation analysis, Pearson and Spearman correlation was used. Values were expressed as mean ± SEM. P values equal to or less than 0.05 were considered significant. Statistical analyses were done by the statistical program STATA 5.0 (24,25).

RESULTS

Results of determination of platelet fibronectin, ADP-induced platelet aggregation, and platelet sialic acid levels in CAD patient groups are presented Table 1. Our results indicate that, in patients with no-vessel disease (patients with no obstructed vessel but suffering from chest pain, like angina pectoris) platelet fibronectin levels were significantly lower than in the total of the other patients (patients with 1, 2, or 3 obstructed coronary vessels) ($p<0.05$). We also found that sialic acid levels in patients with no-vessel disease were significantly lower than in the total of the patient group ($p<0.05$).

TABLE 1. Platelet Fibronectin-, Sialic Acid-, and ADP-induced Platelet Aggregation Levels in CAD Subgroups (Mean ± SEM)

Group	Platelet Fibronectin (μg/mg Protein)	ADP Rate, sec	ADP Amplitude, %	Platelet Sialic Acid (mg/mg Protein)
Control group (No-vessel disease) (n:12)	3.6 ± 2.6	113.4 ± 21.5	60.5 ± 12.6	0.31 ± 0.15
Total patient group (Double-, single-, triple-vessel disease) (n:31)	7.7 ± 1.3*	101.8 ± 16.6	42.5 ± 22.1**	1.08 ± 0.67*

*P = .001; (total patient group), (control group); (according to Anova/Tukey test).

**P = .005; (total patient group), (control group); (according to Anova/Tukey test).

TABLE 2. Serum Total Cholesterol, Triglyceride, HDL and LDL Cholesterol Levels in CAD Subgroups (Mean ± SEM)

Group	Total Cholesterol, mg/dL	Triglyceride, mg/dL	HDL Cholesterol, mg/dL	LDL Cholesterol, mg/dL
Control group (No-vessel disease) (n:12)	213.0±48.6	138.4±72.5	54.4±16.0*	142.1±39.2
Total patient group (Double-, single-, triple-vessel disease) (n:29)	190.1±44.8	160.5±82.4	39.7±8.1	121.1±31.3

*P<.05; (control group - total patient group); (according to Anova/Tukey test).

Table 2 shows the mean serum total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels in CAD patient groups. In patients with no-vessel disease HDL cholesterol levels were found to be significantly higher than in the total of the patient group.

According to Pearson correlation test, there was a positive correlation between platelet fibronectin levels and severity of disease ($r=0.315$; $p<0.05$) and also a positive correlation between ADP-induced platelet aggregation rate and amplitude ($r=0.482$; $p<0.01$). We have also found a positive but statistically insignificant correlation between ADP-induced platelet aggregation rate/amplitude and platelet fibronectin ($p>0.05$) and a negative but statistically insignificant correlation between ADP-induced platelet aggregation and platelet sialic acid ($p>0.05$). Another positive correlation was found between platelet fibronectin levels and platelet sialic acid content. ($r=0.643$; $p=0.00$)

DISCUSSION

Cardiovascular diseases are the major cause of morbidity and mortality in the elderly. Multiple factors might be responsible for the increased incidence of arterial thromboembolism. Platelets play an important role in arterial thrombosis and acute myocardial infarction. Inhibition of platelet aggregation has become a critical step in preventing thrombotic events that are associated with stroke, heart attack, and peripheral arterial thrombosis (26). Fibronectin is a ligand for platelet integrins and may take a role in platelet adhesion and aggregation (6). Platelet fibronectin is partially released into the surrounding environment after reacting platelets with collagen or thrombin. Little or no fibronectin is found on the unstimulated platelet surface. When platelets are stimulated with thrombin, fibronectin (5). Platelet aggregation can proceed normally in fibronectin-depleted plasma. Platelets themselves contain fibronectin in the α granules, so it is difficult to exclude a role for fibronectin in this process. A monoclonal antibody against platelet fibronectin inhibits platelet aggregation while having no effects on serotonin release (6). Also it has been shown that human monocytes synthesize and express a fibronectin receptor. In vitro monocytes bind specifically to fibronectin.

In our study, we demonstrated that platelet fibronectin levels were significantly higher in the patients group than in the control group. Therefore, elevated fibronectin levels in platelets may increase the binding affinity of platelets for the monocytes and arterial wall fibronectin receptors. This interaction may be of importance in atherogenesis (27). In addition, we have also found a statistically positive correlation between platelet fibronectin levels and severity of disease. Therefore, high platelet fibronectin levels may contribute to platelet aggregation in patients with coronary artery disease. But elevated platelet fibronectin levels cannot be a useful monitoring marker of platelet aggregation in patients with CAD because of insignificant positive correlations between them (Aggregation rate/Platelet fibronectin [$r=0.088$,$p>0.05$]; Aggregation amplitude/Platelet fibronectin [$r=0.162$, $p>0.05$]).

The platelet membrane contains many glycoproteins that play important roles in platelet function (28). N-acetyl neuraminic acid (NANA) is present in platelet proteins (29). NANA is a negatively charged polar molecule and can influence glycoprotein conformation (30). The carbohydrate moiety of glycoproteins plays a major role in its functioning. NANA reportedly inhibits platelet aggregation in vivo (31) and in vitro by different agonists (32). The platelet membrane contains many glycoproteins that play an important role in platelet function. Sialic acid (N-acetyl neuraminic acid), a negatively charged sugar, is a regular constituent of glycoproteins and gangliosides in the outer cell membrane of mammalian cells (10–12).

In our study, we have found in the total patient group higher sialic acid levels in platelets than in the control group. The elevated sialic acid levels in platelets may also be due to the increased rate of synthesis. Platelets will not adhere to normal endothelial cells. They can, however, adhere to injured endothelial cells and to subendothelium. In subendothelium, platelets interact with collagen types III and IV. Elevated sialic acid levels may contribute to binding of platelets for collagen molecules. Also, platelet sialic acid potentially represents a pathogenic factor for thrombus formation and development of myocardial infarction in patients with coronary heart disease. However, further studies will be required to evaluate whether platelet sialic acid could be used as a prognostic marker in CHD.

We conclude that increased platelet fibronectin may partially contribute to platelet aggregation in patients with CAD. Our study can be characterized as a preliminary study, and further

studies will be required to evaluate whether platelet fibronectin or sialic acid could be used as a prognostic marker in CAD.

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