High-Dose Aspirin Is Required to Influence Plasma Fibrin Network Structure in Patients With Type 1 Diabetes

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OBJECTIVE—Patients with type 1 diabetes form a less permeable fibrin network, which could contribute to their increased risk of cardiovascular disease (CVD). Low-dose aspirin treatment is the standard in the management of CVD; however, the effect seems reduced in patients with diabetes. We investigated the effects of low- and high-dose aspirin treatment on fibrin network formation in patients with type 1 diabetes (primary aim) and the possible interaction between the treatment effects of aspirin on fibrin network permeability and glycemic control in these patients (secondary aim).

RESEARCH DESIGN AND METHODS—Forty-eight patients (24 subjects with good [HbA_{1c} <7.4%] and 24 subjects with poor [HbA_{1c} >8.4%] glycemic control) were randomly assigned to treatment with 75 or 320 mg/day aspirin during 4 weeks in a crossover fashion. A 4-week washout period separated the treatment periods. The plasma fibrin network was assessed by determination of the permeability coefficient (K_s).

RESULTS—Treatment with 75 mg aspirin did not influence fibrin network permeability (K_s). However, K_s increased significantly during treatment with 320 mg aspirin ($P = 0.004$), and a significant treatment effect was seen compared with treatment with 75 mg aspirin ($P = 0.009$). The increase in K_s during high-dose aspirin treatment was significant in patients with poor glycemic control ($P = 0.02$), whereas K_s only tended to increase in patients with good glycemic control ($P = 0.06$).

CONCLUSIONS—A high dose of aspirin is required to influence fibrin network permeability in patients with type 1 diabetes. The observed lack of effect with low-dose aspirin may contribute to aspirin treatment failure in diabetes.

Diabetes is associated with increased platelet activation (1), elevated plasma fibrinogen levels (2), and impaired fibrinolysis (3), factors that may contribute to the elevated risk of cardiovascular disease (CVD) in these patients. Increased platelet activation in patients with diabetes is reflected by elevated levels of platelet microparticles, which are small circulating procoagulant vesicles shed from the platelet membrane upon activation (4). Low-dose aspirin therapy is one of the cornerstones in the management of CVD; however, the preventive effect seems reduced in patients with diabetes (5).

Aspirin inhibits platelet function by irreversibly acetylating a serine residue in cyclooxygenase-1, thereby grossly reducing the production of the platelet-activating and vasoconstrictive compound thromboxane A2. This is the most accepted effect of aspirin in terms of cardiovascular protection. However, aspirin also may influence coagulation through effects on thrombin generation, factor XIII activation, and fibrin network formation (6). The fibrin network is an important part of the arterial thrombus, and its structure may influence the predisposition to atherothrombotic events (7). During thrombin activation, fibrinopeptides are released from fibrinogen, which polymerize and, in the presence of factor XIII, form a cross-linked fibrin network. The structure of the fibrin network is influenced by the environment in which it is formed and affects the fibrinolytic rate (8). A tighter and less permeable fibrin network, which is less susceptible to fibrinolysis, is formed in patients with manifest CVD or conditions associated with increased risk of atherothrombotic complications (7–10). In previous studies, we have shown that patients with type 1 diabetes have reduced fibrin network permeability and that improved metabolic control is associated with increased fibrin network permeability (11,12). The altered fibrin network in patients with type 1 diabetes may in part be attributed to increased fibrinogen glycation, as shown in studies on fibrinogen purified from diabetic patients (13,14). Treatment with aspirin increases fibrin network permeability in nondiabetic subjects, possibly through acetylation of lysine residues on plasma fibrinogen (15–17). However, the effect of aspirin on fibrin network permeability in patients with diabetes is unclear. Possible competition between acetylation and glycation on lysine residues in the fibrinogen molecule might contribute to the reduced preventive effect of aspirin in the management of CVD in patients with diabetes, and higher doses of aspirin might be required in these patients.

The primary aim of the current study was to investigate the effects of low- and high-dose aspirin treatment on fibrin network permeability in patients with type 1 diabetes. The secondary aim was to investigate the possible interaction between the treatment effects of aspirin on fibrin

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network permeability and glycemic control in these patients. Because platelet microparticles may influence the fibrin formation (18,19), and because aspirin has well-known effects on platelet function, we also measured plasma concentrations of platelet microparticles.

RESEARCH DESIGN AND METHODS

—A crossover study with randomization to receive a daily dose of 75 or 320 mg aspirin (Trombyl; Pfizer) was designed. The 4-week treatment periods were separated by a 4-week washout period. Investigations were performed at the start and the end of each treatment period. Compliance was checked through tablet counts.

A total of 24 patients (12 women) with good glycemic control (HbA_{1c} <7.4%) and 24 patients (12 women) with poor glycemic control (HbA_{1c} >8.4%) were recruited from the Division of Medicine, Danderyd Hospital, Stockholm, Sweden. Eligible for the study were patients with type 1 diabetes between the ages of 30 and 70 years, without previous aspirin treatment, ongoing nonsteroidal anti-inflammatory or anticoagulant treatment, or a history of macrovascular events. Data on retinopathy status was based on fundoscopic findings. All patients arrived at the laboratory in the morning after a 10-h fast. Clinical signs of peripheral neuropathy were investigated with tests of vibration and superficial sensation using a vibration fork (128 Hz) and monofilament (Semmes-Weinstein 5.07), respectively. Prevalence of albuminuria was assessed by urinary dipstick tests (Clinitek; Bayer HealthCare).

Fibrin network

The fibrin network structure was studied in citrated plasma samples by measurement of the permeability coefficient (K_s), as described in detail elsewhere (20). In brief, plasma samples were dialyzed and supplemented with $CaCl_2$ and thrombin to final concentrations of 20 mmol/L and 0.2 NIH/mL, respectively. The fibrin network permeability coefficient (K_s) was determined following percolation of a TRIS buffer through the formed fibrin clots at five different hydrostatic pressures. Low levels of K_s indicate reduced fibrin network permeability. The interassay coefficient of variation was 9.5%.

Platelet microparticles

Previously frozen platelet poor plasma was thawed and centrifuged at 2,000*g* for 20 min at room temperature. The supernatant was then recentrifuged at 13,000*g* for 2 min. Twenty-microliter samples were incubated for 20 min with Phalloidin-Alexa 660 (Invitrogen, Paisley, U.K.), lactadherin–fluorescein isothiocyanate (phosphatedylserine exposure; Hematologic Technologies, Essex Junction, VT), and CD42a-PE (glycoprotein IX; Abcam, Cambridge, U.K.). Microparticles were measured by flow cytometry on a Beckman Gallios instrument (Beckman Coulter, Brea, CA) according to a modified method (21). The microparticle gate was determined using Megamix beads (BioCytex, Marseille, France), which is a mix of 0.5-, 0.9-, and 3-μm diameter beads. Appropriate conjugate isotype-matched immunoglobulin with no reactivity against human antigens was used as a negative control to define the background noise of the cytometric analysis. The mean concentration of microparticles was calculated as follows: (microparticle counts × standard beads/liter)/standard beads counted (FlowCount; Beckman Coulter).

Biochemical analyses

HbA_{1c} levels were analyzed by the Mono S method using high-performance liquid chromatography (Variant II; Bio-Rad Laboratories, Hercules, CA) and are expressed in National Glycohemoglobin Standardization Program equivalent values. Lipoproteins were assayed enzymatically with reagents from Synchron LX System(s) (Beckman Coulter, Galway, Ireland). C-reactive protein (CRP) was analyzed using immunonephelometry (Dade Behring, Marburg, Germany). Total plasma fibrinogen was analyzed by means of a Fibri-Prest Automate method (von Clauss method) from Diagnostica Stago.

Statistical analysis

Sample size calculations based on previous results on fibrin network permeability in patients with type 1 diabetes indicated that 14 patients were required in each group (12). In the current study, we included 24 patients in each group to compensate for dropouts. The Shapiro-Wilk W test was used to assess conformity with a normal distribution. Differences between and within the different doses of aspirin treatments and the patients with good and poor glycemic control, respectively, were investigated by means of two-way repeated-measures ANOVA. Data are presented as mean values ± SD, 95% CIs for the mean, the median with lower- to upper-quartile values in parentheses, or as numbers. A probability (P) of <0.05 was considered statistically significant.

Ethical considerations

The protocol of this trial was approved by the local ethics committee of Karolinska Hospital and the Medical Products Agency. Written informed consent was obtained from all patients.

RESULTS

—Seven patients did not complete the study (i.e., three patients discontinued because of the possible mild side effects of aspirin, two patients were excluded because of failure with analyzing procedures, one patient was excluded because of poor compliance, and one patient proved not to meet the inclusion criteria). Baseline characteristics of 41 patients who completed the study are summarized in Table 1. There were no significant differences between the patients with good or poor glycemic control regarding mean age, diabetes complications, and antihypertension and statin treatments. The patients with good glycemic control had, compared with the group with poor glycemic control, longer diabetes duration (30 years [19–43] vs. 15 years [10–29], medians with lower-upper quartiles in brackets; P = 0.01), better lipid profile, and lower plasma fibrinogen levels (2.5 ± 0.4 vs. 2.9 ± 0.7 g/L; P = 0.02). No significant differences between the groups

Table 1—*Baseline characteristics*

Characteristics	
n (women/men)	41 (19/22)
Age (years)	51 ± 12
Diabetes duration (years)	21 (13–33)
Smokers	4 (10)
BMI (kg/m^2)	25 ± 3
Systolic blood pressure (mmHg)	132 ± 18
Diastolic blood pressure (mmHg)	75 ± 7
Antihypertensive treatment	17 (41)
Statin treatment	21 (51)
Proliferative retinopathy	12 (29)
Peripheral neuropathy	1 (2)
Microalbuminuria	10 (24)
S-Creatinine (μmol/L)	75 (63–84)
K_s (cm^2 × 10^{-9})	10.1 ± 3.4
Fibrinogen (g/L)*	2.7 ± 0.7
CRP (mg/L)	1.0 (1.0–2.0)
Total cholesterol (mmol/L)	4.5 ± 0.7
LDL cholesterol (mmol/L)	2.5 ± 0.6
HDL cholesterol (mmol/L)	1.6 ± 0.4
Triglycerides (mmol/L)	0.7 (0.5–1.0)

Data are means ± SD, median (lower to upper quartiles), or *n* (%). K_s, fibrin network permeability coefficient. *Reference range 2–4 g/L.

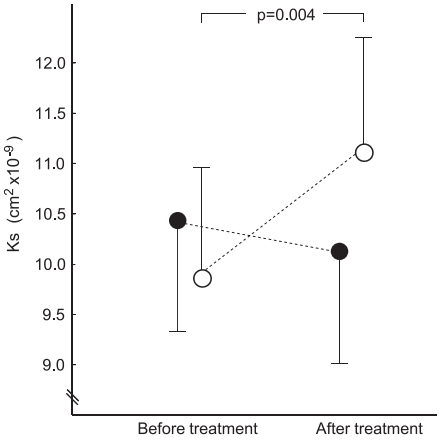

Figure 1—*Fibrin network permeability coefficient* (K_s) *during treatment with 75 and 320 mg aspirin. Data are presented as means and 95% CIs* (n = *41*). K_s *increased during treatment with 320 mg aspirin* (○), *and a significant treatment effect was seen compared with treatment with 75 mg aspirin* (●) (P = *0.009*).

were found regarding baseline concentrations of platelet microparticles or CRP.

Fibrin network permeability (K_s) was not changed during treatment with 75 mg aspirin, whereas K_s increased from 9.8 ± 3.3 to 11.0 ± 3.4 $cm^2 \times 10^{-9}$ during treatment with 320 mg aspirin (P = 0.004). A significant difference in treatment effects was found when the two treatments were compared (P = 0.009; two-way repeated-measures ANOVA) (Fig. 1). K_s was inversely correlated to fibrinogen levels at all four time points of blood sampling (r = 0.47–0.71, r^2 = 0.22–0.50; $P <$ 0.01). K_s did not correlate with diabetes duration or levels of HbA_{1c}, lipids, or CRP. No carry-over effects were found.

A tendency to lower K_s levels at baseline was seen in the patients with poor compared with patients with good glycemic control (Fig. 2 and Table 2). During treatment with high-dose aspirin (320 mg), levels of K_s increased significantly in patients with poor glycemic control (P = 0.02), whereas K_s tended to increase in patients with good glycemic control (P = 0.06) (Fig. 2).

There were no significant changes in platelet microparticles, plasma fibrinogen, CRP, HbA_{1c}, or lipid levels during treatment with 75 or 320 mg aspirin (Table 2).

CONCLUSIONS—In the current study, no effect on fibrin network permeability was found during low-dose aspirin (75 mg) treatment in patients with type 1 diabetes, whereas treatment with a high dose of aspirin (320 mg) was associated with significantly increased fibrin network permeability. Furthermore, we found that the effect of high-dose aspirin on fibrin network permeability was more pronounced in patients with poor metabolic control.

The fibrin network structure seems important in the development of atherothrombotic events, because individuals at high risk of CVD, including patients with type 1 diabetes and nephrotic syndrome, as well as patients with manifest CVD, have a tighter and less permeable fibrin network structure (7,9–11). Our previous studies in patients with type 1 diabetes have shown increased fibrin network permeability during treatments with subcutaneous continuous insulin infusion, statins, and dalteparin, respectively (12,18,22). These studies indicate that fibrin network permeability (K_s) is a functional variable suitable for intervention studies.

Aspirin alters the fibrin/fibrinogen properties and thereby influences the fibrin network structure, possibly through acetylation of the lysine residues in the fibrinogen molecule involved in cross-linking of fibrin (15–17). This cyclooxygenase-independent effect of aspirin may add to the antithrombotic effects of aspirin. Increased fibrin network permeability during aspirin treatment has been shown in healthy individuals at daily doses of 37.5, 75, and 320 mg (15,16). In the current study, no effect of low-dose aspirin treatment (75 mg) was found on fibrin network permeability (K_s) in patients with type 1 diabetes, whereas treatment with 320 mg aspirin increased K_s levels significantly. Our results thus indicate that patients with type 1 diabetes may require higher doses of aspirin to influence the fibrin network structure. This finding supports the hypothesis that diabetes is associated with a reduced response to aspirin compared with nondiabetic subjects and that higher aspirin doses, rather than the recommended low-dose treatment (defined as 75–162 mg/day, according to the American Diabetes Association), are required in the management of CVD in patients with diabetes.

One plausible explanation behind the observed dose-dependent effect of aspirin on fibrin network permeability in

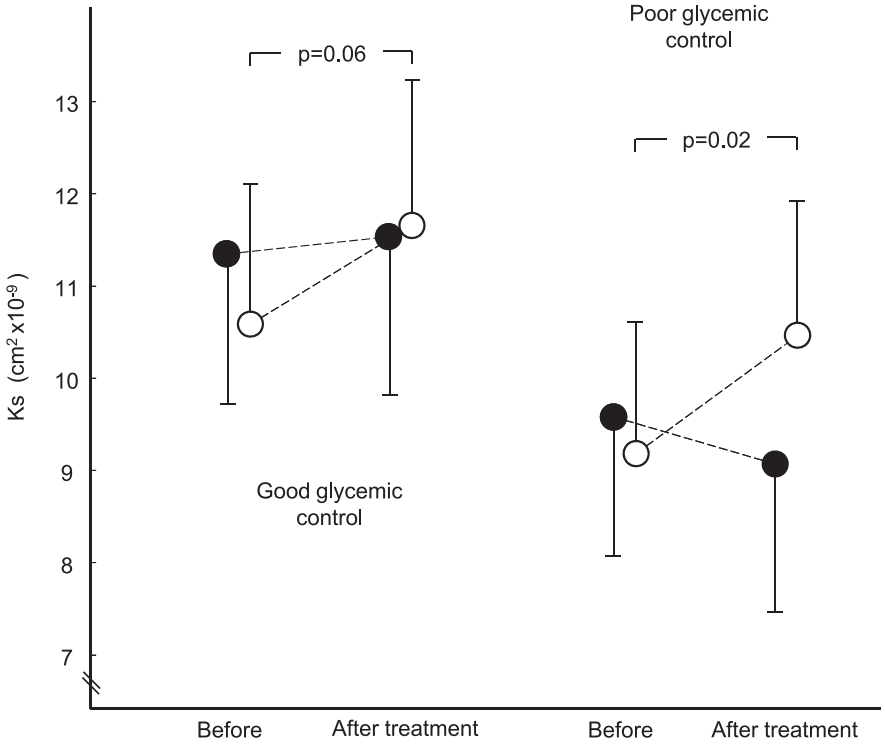

Figure 2—*Fibrin network permeability coefficient* (K_s) *during treatment with 75 and 320 mg aspirin in patients with good and poor glycemic control, respectively. Data are presented as means and 95% CIs* (n = *41*). *No significant treatment effects were seen in patients with good glycemic control.* K_s *increased during treatment with 320 mg aspirin* (○) *in patients with poor glycemic control* (P = *0.02*), *and a significant treatment effect was seen compared with treatment with 75 mg aspirin* (●) (P = *0.01*).

Table 2—*Treatment effects of 75 and 320 mg aspirin in patients with good and poor glycemic control, respectively*

	Good glycemic control (n = 19 [11 male])			Poor glycemic control (n = 22 [11 male])			
	75 mg aspirin	320 mg aspirin	P	75 mg aspirin	320 mg aspirin	P	P‡
K_s ($cm^2 \times 10^{-9}$)	11.4 ± 3.6→11.5 ± 2.7	10.8 ± 3.0→11.9 ± 2.9 *	0.22	9.4 ± 3.9→8.7 ± 4.1	9.1 ± 3.6→10.4 ± 3.9 †	0.01	0.36
Fibrinogen (g/L)	2.5 ± 0.4→2.5 ± 0.3	2.4 ± 0.5→2.5 ± 0.5	0.94	3.0 ± 0.8→3.0 ± 0.9	2.9 ± 0.9→2.8 ± 0.8	0.49	0.62
Platelet microparticles (10^6/L)	12.310 ± 1.830→12.030 ± 1.680	11.700 ± 1.510→11.780 ± 1.880	0.45	12.180 ± 1.970→12.460 ± 1.400	12.110 ± 1.740→12.090 ± 1.570	0.51	0.32
HbA_{1c} (%)	6.7 ± 0.4→6.8 ± 0.6	6.8 ± 0.5→6.8 ± 0.6	0.57	9.3 ± 1.0→9.2 ± 1.2	9.2 ± 0.9→9.2 ± 1.0	0.47	0.37
LDL cholesterol (mmol/L)	2.4 ± 0.6→2.2 ± 0.5	2.3 ± 0.6→2.3 ± 0.6	0.11	2.7 ± 0.6→2.6 ± 0.7	2.6 ± 0.6→2.5 ± 0.5	0.96	0.25

Data are means ± SD. K_s, fibrin network permeability coefficient; the arrows indicate changes during the treatment periods. *P = 0.06. †P = 0.02. ‡P represents the interaction between treatment effects of 75 and 320 mg aspirin in patients with good and poor glycemic control, respectively.

diabetes is increased glycation of fibrinogen molecules in plasma (13,14). It has been suggested that glycated fibrinogen may have reduced susceptibility to acetylation by aspirin, and competition between glycation and acetylation of fibrinogen amino groups has been observed in vitro (23). In support of this theory, interaction between acetylation and glycosylation in lysine residues has also been indicated in studies on plasma albumin, as the glycosylation rate of lysine residues was inhibited during simultaneous incubation with aspirin and glucose (24). In the current study, however, subgroup analyses assessing the possible influence of glycemic control on the treatment effects of aspirin on fibrin network permeability did not support the theory of competitive inhibition between acetylation and glycation on plasma fibrinogen, since the effect of high-dose aspirin treatment was more pronounced in patients with poor glycemic control. Of note, the baseline K_s levels tended to be higher in patients with good glycemic control compared with patients with poor glycemic control (Table 2 and Fig. 2). Thus, aspirin might be too weak a modifier of the fibrin network to have a substantial impact on fibrin network permeability in diabetic patients with relatively high baseline K_s values, as in the patients with good glycemic control in the current study.

Plasma fibrinogen concentration, per se, also has a significant impact on the fibrin network structure, and, accordingly, the fibrinogen levels in the current study were inversely related to fibrin network permeability coefficient (K_s) levels at all investigation time points. Increased fibrinogen levels are an independent risk marker for CVD (25), and elevated levels are common in patients with diabetes (2). In line with earlier studies, we found significantly higher levels of plasma fibrinogen in patients with poor compared with good glycemic control.

Because platelet microparticles may influence the fibrin network formation (18,19), and because aspirin has well-known effects on platelet function, we measured the plasma concentration of platelet microparticles in the current study. However, no effect of 75 or 320 mg aspirin was found on plasma concentrations of platelet microparticles. Thus, it is unlikely that the aspirin-induced increments of fibrin network permeability were mediated through effects on platelet microparticles.

We conclude that treatment with high-dose aspirin (320 mg) is required to increase fibrin network permeability in patients with type 1 diabetes. The observed lack of effect with low-dose aspirin may contribute to aspirin treatment failure in diabetes.

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No potential conflicts of interest relevant to this article were reported.

S.T. assembled and analyzed the data, performed the statistical analyses, and wrote the manuscript. A.A. edited the manuscript. F.M. and K.M. acquired the data and edited the manuscript. P.-E.L., U.A., H.N.W., and G.J. designed the study, handled the funding, and edited the manuscript. S.T. is the guarantor of this article.

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