Platelet Adhesion and Aggregate Formation in Type I Diabetes Under Flow Conditions

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To study platelet activation as a phenomenon that may precede development of angiopathy in diabetes mellitus, we compared platelet adhesion and thrombus formation in a flow system with blood from insulin-dependent (type I) diabetic subjects with and without macroangiopathy and age- and sex-matched control subjects. Adhesion and thrombus formation on matrix of cultured human endothelial cells (ECM) and adhesion on matrix of human fibroblasts (FBM) were studied after exposure to flowing blood at shear rates of 300 and 1300 s^{-1} and exposure times of 1, 3, 5, and 10 min (and 20 min in adhesion experiments). Blood was anticoagulated with trisodium citrate (1:10 vol/vol, 110 mM) or low-molecular-weight heparin ([LMWH] 20 U/ml). Endothelial cell cultures were either unstimulated or stimulated with 4β-phorbol 12-myristate 13-acetate (PMA) 16 h before isolating their matrix. Platelet adhesion on ECM and FBM in citrated and LMWH-anticoagulated blood was identical in diabetic patients and control subjects, with comparable increases of adhesion with increasing perfusion times. Platelet aggregate formation on ECM of PMA-stimulated cells with LMWH-anticoagulated blood was similar in diabetic patients, whether macroangiopathy was present, compared with control subjects. Fibrin deposition and fibrinopeptide A generation during perfusion were comparable in diabetic and control subjects. Platelet thromboxane B_2 formation after stimulation with arachidonic acid was increased in diabetic patients without macroangiopathy compared with age- and sex-matched control subjects. In the perfusion system, the patterns of platelet adhesion and aggregate formation on extracellular matrix in flowing blood of diabetic patients (with or without macroangiopathy), and healthy age- and sex-matched control subjects followed a similar pattern. An increase in platelet–vessel wall interaction in diabetes mellitus was not detected, even in the presence of macroangiopathy.
***Diabetes* 40:1410–17, 1991**

Macrovascular disease in diabetes is histologically identical to atherosclerosis in general, but it is more diffusely present in the vasculature and progresses more rapidly than arterial disease in nondiabetic subjects. One explanation for rapid progression of arterial disease in diabetic subjects is provided by a suggested increased reactivity of platelets, but evidence for this is mainly derived from experiments in vitro with isolated platelets. Moreover, there is as much circumstantial evidence supporting the refutation of this hypothesis (1,2). To explore this question in a setting that approximates the in vivo situation, i.e., with platelets in flowing blood, we used a modification (3) of a well-characterized perfusion system (4). Perfusion experiments were carried out to establish whether enhanced platelet-platelet and platelet–vessel wall interaction is detectable in flowing blood from subjects with diabetes.

To study different aspects of platelet–vessel wall interaction, various conditions of anticoagulation and various adhering surfaces were used. First, fibroblast matrix (FBM), which does not contain von Willebrand factor (vWF), was used for the study of the role of exogenous vWF in platelet adhesion in citrated blood (3). Second, extracellular matrix of human umbilical artery endothelial cells (ECM) was used to study platelet adhesion in citrated blood. In this model, vWF and fibronectin, present in the matrix, are involved in platelet adhesion (5). Third, tissue factor-dependent thrombus formation (platelet-platelet interaction) in flowing blood anticoagulated with low-molecular-weight heparin (LMWH) was studied with the ECM that were stimulated for 16 h with

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Received for publication 28 February 1990 and accepted in revised form 12 April 1991.

TABLE 1
Clinical characteristics of diabetic patients

	Sex	Age (yr)	Duration (yr)	Retinopathy	Nephropathy	Microproteinuria	Neuropathy	Cardiopathy	Vascular	Hypertension
Group 1 (adhesion experiments on fibroblast matrix)										
Subject										
1	M	38	6	+	−	−	−	−	−	−
2	M	33	18	−	−	−	−	−	−	−
3	M	42	15	−	−	−	−	−	−	−
4	M	49	34	−	−	−	−	−	−	−
5	M	23	9	−	−	−	−	−	−	−
Group 2 (adhesion experiments on endothelial cell matrix)										
Subject										
6	F	22	2	−	−	−	−	−	−	−
7	M	23	8	−	−	−	−	−	−	−
8	M	27	5	−	−	+	−	−	−	−
9	F	24	23	+ +	−	+	−	−	−	+
10	F	32	15	−	−	−	−	−	−	−
11	M	20	10	+	−	+ +	−	−	−	+
Group 3 (adhesion, thrombus forming on stimulated endothelial cell matrix, without macrovascular disease)										
Subject										
12	F	40	28	+ +	−	+ +	+	−	−	−
13	M	28	10	+	−	+	−	−	−	−
14	M	22	5	−	−	+	+	−	−	−
15	M	25	12	−	−	−	−	−	−	−
16	M	22	4	−	−	−	−	−		
17	F	21	11	−	−	+	−	−	−	−
Group 4 (adhesion, thrombus forming on stimulated endothelial cell matrix, with macrovascular disease)										
Subject										
18	F	46	20	+	−	−	+	−	+	+
19	M	54	26	+	−	−	+	+	+	−
20	M	72	8	+	−	+ +	+	−	+	−
21	F	66	40	+ +	−	+ + +	+	+	+	+
22	M	51	40	+ +	+	+ + +	+	+	+	+
23	F	59	45	+	−	−	+	−	+	−

+, −, Presence or absence of complications, respectively. For retinopathy, + is background retinopathy, and + + is situation after coagulation treatment. Nephropathy was defined as elevated serum creatinine levels. For microproteinuria, + is normal (up to 30 mg/day), + + is incipient (30–300 mg/day), and + + + is albuminuria >300 mg/day. Peripheral neuropathy was assessed by pedal temperature and vibration threshold testing. Vascular peripheral macrovascular disease detected by vascular laboratory testing and documented by contrast arteriography. Hypertension was defined as systolic value >150 mmHg and/or diastolic value >95 mmHg.

phorbol ester before removing the cells from their matrix as perfusion surface. In this model, blood from patients without and with overt macrovascular disease was used. Thus, aspects of potential differences in platelet–vessel wall interaction between diabetic and nondiabetic subjects could be studied in three different experimental setups.

RESEARCH DESIGN AND METHODS

For all experiments, blood samples were obtained from patients and control subjects after overnight fasting. Hematocrit (Hct) (Coulter counter, model S, Harpenden, UK) and platelet count (Platelet Analyzer 810, Baker Diagnostics, Bethlehem, PA) were determined from EDTA-anticoagulated blood. Bleeding time was determined with a Simplate-II device (General Diagnostics, Morris Plains, NJ). Plasma fibrinogen level was determined according to Clauss (6). von Willebrand Factor (vWF:Ag) was determined by electroimmunoassay (7) and vWF:RiCoF with formalin-fixed platelets and 1 mg/ml ristocetin (8). Thromboxane B_2 (TXB_2) production in response to exogenous arachidonic acid (1.5 mM for 1 or 10 min) or exogenous collagen (1 μg/ml for 5 min) was determined by radioimmunoassay (Du Pont-NEN, Boston, MA). Levels of β-thromboglobulin (β-THG) and platelet factor 4 (PF4) in plasma were determined with radioimmunoassays (β-THG, Amersham, Aylesbury, UK; PF4, Abbot, North Chicago, IL). Plasma levels of cholesterol were determined by enzyme-linked immunosorbent assay (Boehringer Mannheim, Mannheim, Germany) and of triacylglycerol (TG) by kinetic enzymatic assay (Boehringer Mannheim). Plasma high-density lipoprotein cholesterol (HDL-chol) was determined in the supernatant after precipitation of triacylglycerolrich particles (9). Plasma concentrations of apolipoproteins A and B (apoA and apoB) were determined nephelometrically (10).

Human umbilical vein endothelial cells and fibroblasts from human fetal lung tissue were isolated and cultured on glass or Thermanox cover glasses (Miles, Naperville, IL) as previously described (3). Endothelial cells of the 3rd passage and fibroblasts from the 10th to 16th passages were used and cultured on coverglasses. This enables the introduction of coverglasses with extracellular matrix into the perfusion chamber. For thrombus-forming conditions (see below), confluent endothelial cells were treated with 4β-phorbol 12-myristate 13-acetate (PMA; 20 ng/ml culture medium) 16 h before isolating the matrix.

Perfusion experiments were performed as follows. Blood was standardized for platelet count (180,000 platelets/μl) and Hct (40%) with components of autologous blood. The

TABLE 2
Hematological and biochemical determinations in 23 diabetic patients and 23 nondiabetic control subjects

Variable	Diabetic			Control			P
	Mean ± SD	Min	Max	Mean ± SD	Min	Max	
Age (yr)	35 ± 14	20	72	35 ± 12	22	66	0.98
Duration of diabetes (yr)	15 ± 11	2	45				
Hematocrit (%)	44 ± 6	35	56	44 ± 4	37	54	0.68
Platelets (10^9/L)	282 ± 63	191	457	252 ± 51	163	350	0.08
Bleeding time 1	4.36 ± 1.48	1	8.20	5.12 ± 1.40	1.25	8.20	0.21
Bleeding time 2	4.48 ± 1.40	2.15	8.25	5.20 ± 1.55	1.30	10.10	0.22
Fibrinogen (g/L)	2.48 ± 0.75	1.45	5.20	2.18 ± 0.48	1.30	3.40	0.03
vWF:Ag (%)	117 ± 64	22	314	90 ± 28	50	185	0.03
vWF:RiCof (U)	137 ± 46	55	220	113 ± 58	44	280	0.02
PT (s)	13.1 ± 1	11.7	15.1	12.9 ± 1	11.4	14.9	0.26
patt (s)	38.7 ± 7	30	61.2	38.9 ± 4	31.6	47.9	0.88
β-THG (ng/ml)	82 ± 65	10	258	54 ± 55	16	222	0.08
Pf4 (ng/ml)	37 ± 43	3	181	28 ± 39	1	138	0.60
TXB_2/AA_1	549 ± 273	8	1290	450 ± 236	41	1130	0.12
TXB_2/AA_{10}	1142 ± 473	178	2100	931 ± 378	74	1830	0.05
TXB_2/col_5	184 ± 172	7	844	162 ± 88	11	340	0.46
Glucose (mM/L)	12.7 ± 4.4	4.6	20.5	4.1 ± 0.5	2.7	5.0	0.0001
HbA_1 (%)	11.2 ± 2.7	5.9	18.9	5.3 ± 0.9	4.1	8.1	0.0001
TG (mM/L)	1.55 ± 1.2	0.40	5.0	1.06 ± 0.6	0.5	3.4	0.05
Total cholesterol (mM/L)	5.37 ± 1.30	3.3	8.6	5.03 ± 0.80	3.3	6.8	0.15
HDL-chol (mM/L)	1.09 ± 0.3	0.10	1.71	1.23 ± 0.3	0.42	2.27	0.10
ApoA (g/L)	1.52 ± 0.27	0.98	2.08	1.55 ± 0.21	1.21	2.00	0.56
ApoB (g/L)	0.79 ± 0.26	0.34	1.40	0.70 ± 0.15	0.46	0.98	0.11

Table gives pooled data from 4 experiments. vW, von Willebrandt factor; PT, prothrombin time; patt, partial thromboplastin time; β-THG, β-thromboglobulin; PF4, platelet factor 4; TXB_2/AA_1 and TXB_2/AA_{10}, production of thromboxane B_2 by platelets after stimulation with 1.5 mM exogenous arachidonic acid for 1 and 10 min, respectively; TXB_2/col_5, platelet TXB_2 production in response to exogenous collagen (1 μg/ml for 5 min); TG, triacylglycerol; HDL-chol, high-density-lipoprotein cholesterol; Apo, apolipoprotein.

perfusion experiments were carried out in duplicate with blood flowing through a double rectangular perfusion chamber as previously described (3). Shear rates of 300 and 1300 s^{-1} and perfusion times of 1, 3, 5, and 10 min (also 20 min in experiments 1 and 2) were used.

Four sets of perfusion experiments were performed to study platelet adhesion on FBM and ECM, respectively, both with blood anticoagulated with 1:10 vol/vol 110 mM trisodium citrate (3).

After perfusion, the coverglasses were removed from the perfusion chambers and fixed, stained, and embedded for evaluation *en face* with a light microscope connected to an image analyser (40-10 Image Analyser, Analytical Measuring, Shirehill, UK) as described before (5).

The third and fourth sets of experiments served to study tissue-dependent thrombus formation on ECM of PMA-stimulated endothelial cells (11) in blood of diabetic patients with and without macroangiopathy. Blood was anticoagulated with 20 U/ml LMWH (Fragmin, Kabi Vitrum, Stockholm), which inhibits thrombin generation in the perfusate (11).

After perfusion, the coverglasses were removed from the perfusion chambers and fixed, embedded in Epon, cut, and stained as described (3). For light-microscopic evaluation of 0.8-μm cross sections, the nomenclature according to Baumgartner was used (12). Total adhesion to the matrix was defined as the percentage of the surface covered by platelets. Platelet adhesion was subdivided into contact-and-spread platelets and spread platelets covered with aggregates. Aggregate formation with a minimum height of 2 μm was subdivided into aggregates <5, between 5 and 10, and >10 μm in height. As a measure of platelet-platelet interaction, the percentage of spread platelets covered with aggregated platelets was calculated.

To determine thrombus class, cross sections of the fixed extracellular matrix surfaces were evaluated for thrombus formation with an image analyser (AMS 40-10) by scanning each of the surfaces at 2000 successive points at 1-μm intersections. Thrombus area was then sorted in classes with increments of 10 μm^2 and expressed as relative percentages of total thrombus area.

Concentrations of fibrinopeptide A (FPA) in the perfusate before and after perfusion were determined by radioimmunoassay (Mallinckrodt, St. Louis, MO), and the difference in concentration was taken as a measure for fibrin generation (13).

Twenty-three patients with insulin-dependent (type I) diabetes mellitus cooperated in this study and were matched by age and sex with 23 healthy control subjects. The clinical characteristics and possible complications of the individual patients are given in Table 1.

The presence or absence of complications was on record from all patients as part of the surveillance program of the University Hospital Diabetes Clinic and included physical examination; ophthalmological examination with fluorescence angiography of the retina; renal function tests, including a test for microalbuminuria; foot temperature and vibration threshold testing; electrocardiography; and vascular laboratory tests (segmental systolic Doppler pressures, toe pressures). In the six patients with peripheral vascular disease (macroangiopathy, as diagnosed by physical and

TABLE 3
Hematological and biochemical determinations in diabetic patients and age- and sex-matched control subjects

Variable	Group	Diabetic	Control	Paired *t* test *P*	Variable	Group	Diabetic	Control	*P*
Hematocrit (%)	1	45 ± 5	44 ± 2	0.56	Glucose (mM/L)	1	11.4 ± 2.8	4.3 ± 0.3	0.005
	2	44 ± 7	39 ± 10	0.38		2	13.0 ± 5.4	3.7 ± 0.5	0.01
	3	44 ± 5	47 ± 4	0.24		3	14.8 ± 4.1	4.3 ± 0.4	0.001
	4	39 ± 6	41 ± 3	0.58		4	12.5 ± 3.7	4.4 ± 0.4	0.005
Platelets ($\times 10^9$/L)	1	285 ± 64	256 ± 37	0.13	HbA_{1c} (%)	1	11.7 ± 2.9	5.6 ± 1.4	0.03
	2	244 ± 16	245 ± 56	0.95		2	12.3 ± 2.2	5.3 ± 0.6	0.001
	3	313 ± 51	283 ± 50	0.04		3	11.1 ± 4.1	5.4 ± 0.5	0.02
	4	281 ± 84	249 ± 65	0.59		4	9.6 ± 2.4	5.7 ± 1.1	0.02
Bleeding time (s)	1	235 ± 57	345 ± 126	0.09	TG (mM/L)	1	1.1 ± 0.3	0.8 ± 0.2	0.23
	2	244 ± 66	352 ± 164	0.07		2	0.8 ± 0.5	1.4 ± 1.1	0.24
	3	373 ± 65	332 ± 87	0.27		3	1.2 ± 0.4	1.2 ± 0.4	0.85
	4	359 ± 143	294 ± 70	0.64		4	2.7 ± 1.7	1.0 ± 0.3	0.05
Fibrinogen (g/L)	1	2.35 ± 0.4	2.24 ± 0.2	0.60	Cholesterol (mM/L)	1	4.8 ± 0.6	4.9 ± 0.4	0.86
	2	2.5 ± 0.5	2.16 ± 0.5	0.21		2	4.7 ± 0.7	4.7 ± 0.6	0.96
	3	2.65 ± 0.7	2.43 ± 0.5	0.47		3	5.3 ± 1.1	4.8 ± 0.8	0.31
	4	3.07 ± 1.2	2.4 ± 0.5	0.30		4	6.7 ± 1.1	5.8 ± 0.7	0.13
vWF:Ag (%)	1	70 ± 40	104 ± 18	0.18	HDL-chol (mM/L)	1	1.2 ± 0.2	1.2 ± 0.2	0.95
	2	111 ± 50	73 ± 12	0.10		2	1.1 ± 0.4	1.1 ± 0.4	0.98
	3	103 ± 37	99 ± 22	0.79		3	1.1 ± 0.3	1.1 ± 0.3	0.68
	4	221 ± 80	108 ± 52	0.02		4	1.2 ± 0.3	1.3 ± 0.5	0.73
vWF:RiCof (%)	1	126 ± 16	108 ± 79	0.58	ApoA (g/L)	1	1.4 ± 0.2	1.5 ± 0.1	0.78
	2	161 ± 53	92 ± 36	0.03		2	1.6 ± 0.3	1.6 ± 0.2	0.37
	3	112 ± 40	101 ± 19	0.40		3	1.5 ± 0.2	1.5 ± 0.3	0.80
	4	187 ± 18	177 ± 74	0.78		4	1.7 ± 0.2	1.6 ± 0.2	0.90
β-THG (ng/ml)	1	84 ± 76	19 ± 5	0.29	ApoB (g/L)	1	0.7 ± 0.1	0.7 ± 0.1	0.96
	2	109 ± 92	74 ± 74	0.15		2	0.7 ± 0.2	0.7 ± 0.2	0.68
	3	71 ± 42	56 ± 54	0.56		3	0.8 ± 0.2	0.7 ± 0.1	0.43
	4	91 ± 53	29 ± 9	0.18		4	1.0 ± 0.3	0.8 ± 0.2	0.13
PF4 (ng/ml)	1	32 ± 46	7 ± 1	0.35	TXB_2/col_5 (nM/cl)	1	247 ± 339	194 ± 99	0.66
	2	55 ± 68	34 ± 44	0.12		2	181 ± 116	153 ± 55	0.51
	3	19 ± 9	22 ± 27	0.67		3	137 ± 49	113 ± 92	0.37
	4	38 ± 46	16 ± 26	0.63		4			
TXB_2/AA_1 (nM/cl)	1	525 ± 265	497 ± 200	0.90	TXB_2/AA_{10} (nM/cl)	1	865 ± 416	887 ± 380	0.95
	2	594 ± 438	590 ± 314	0.97		2	1283 ± 709	1140 ± 431	0.46
	3	510 ± 88	345 ± 161	0.04		3	1183 ± 323	835 ± 39	0.01
	4					4			
TXB_2 (serum) (nM/cl)	4	131 ± 87	61 ± 40	0.08					

Values are means ± SD given as group values for 4 experiments. Groups 1, 2, and 3, patients without macroangiopathy; group 4, patients with macroangiopathy; TG, triacylglycerol; vWF, von Willebrand factor; HDL-chol, high-density lipoprotein cholesterol; apo, apolipoprotein; β-THG, β-thromboglobulin; PF4, platelet factor 4; TXB_2/AA_1 and TXB_2/AA_{10}, production of thromboxane B_2 by platelets after stimulation with 1.5 mM exogenous arachidonic acid for 1 and 10 min, respectively; TXB_2/col_5, platelet TXB_2 production in response to exogenous collagen (1 μg/ml for 5 min).

vascular laboratory examination), the extent of vascular disease was also established by contrast arteriography.

Each patient and matched control subject participated in one of the four experiments as described previously (Table 1).

Paired *t* tests were performed on untransformed results of hematological and biochemical determinations for experiments 1, 2, 3, and 4 (group 3, no macroangiopathy; group 4, macroangiopathy) separately and for the pooled data of these experiments. Paired *t* tests were also carried out on *en face* evaluations of platelet coverage in experiments 1 and 2 and on fibrin deposition and FPA generation in experiments 3 and 4.

Results for perfusion experiments with groups 3 and 4 (pooled) were analyzed by repeated measure analysis of variance (ANOVA; 14) after square-root transformation to approximate normal distribution. ANOVA was performed on data of spread platelets, platelet aggregates, and total coverage for both wall shear rates. ANOVA was used to detect differences between diabetic patients and control subjects, common time effects in patients and control subjects, differences in time dependence of platelet deposition between patients and control subjects (interaction), and a potential shift in platelet deposition from smaller to larger aggregates in patients versus control subjects.

RESULTS

Mean ± SD group values for hematological and biochemical tests are given in Tables 2 (pooled data) and 3 (separate groups), with paired *t* tests for differences between patients and control subjects. Plasma glucose and HbA_{1c} levels (Tables 2 and 3) were elevated in all groups of patients compared with control subjects. Triglyceride levels in patients with macrovascular disease were elevated, consistent with the presence of macroangiopathy. The mean plasma cholesterol level in group 4 was higher in both patients (having

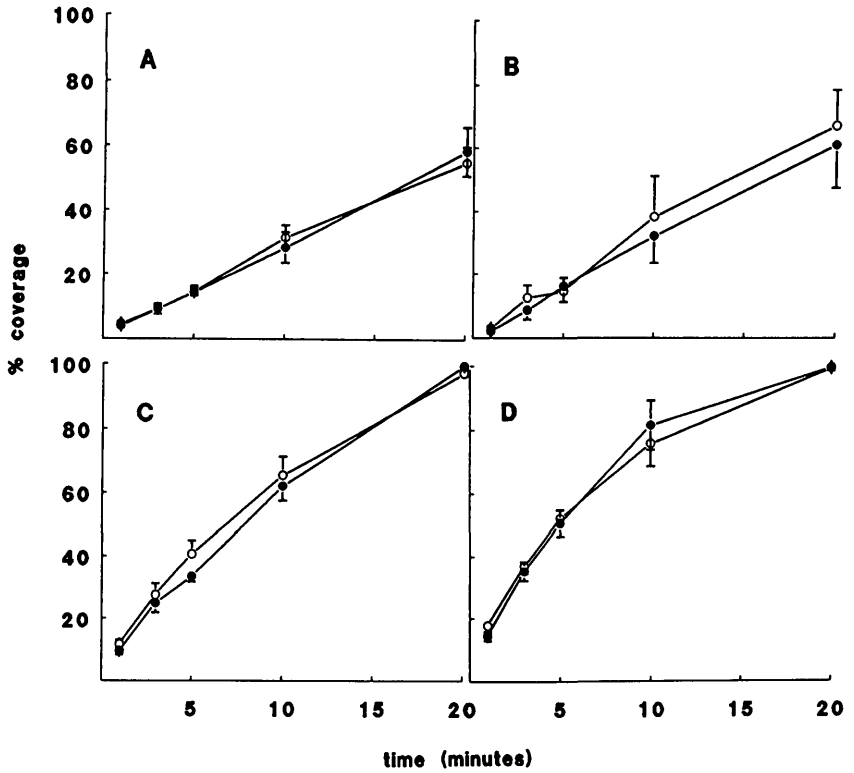

FIG. 1. Experiments 1 (*A* and *B*) and 2 (*C* and *D*). Platelet deposition under adhesion conditions at 2 shear rates (300 s^{-1} [*A* and *C*]; 1300 s^{-1} [*B* and *D*]) after perfusion times of 1, 3, 5, 10, and 20 min. Experiment 1, platelet coverage on fibroblast matrix (wall shear rates 300 and 1300 s^{-1}, respectively [n = 5 diabetic patient–control subject pairs]). Experiment 2, platelet coverage on endothelial cell matrix (wall shear rates 300 and 1300 s^{-1}, respectively [n = 6 diabetic patient–control subject pairs]). Blood was anticoagulated with 1:10 vol/vol 110 mM trisodium citrate, and perfusates were standardized for platelet count (180,000 platelets/μl) and hematocrit (40%) by computer-guided reconstitution with components of autologous blood. ●, Diabetic patients; ○, nondiabetic control subjects. Values are means ± SE.

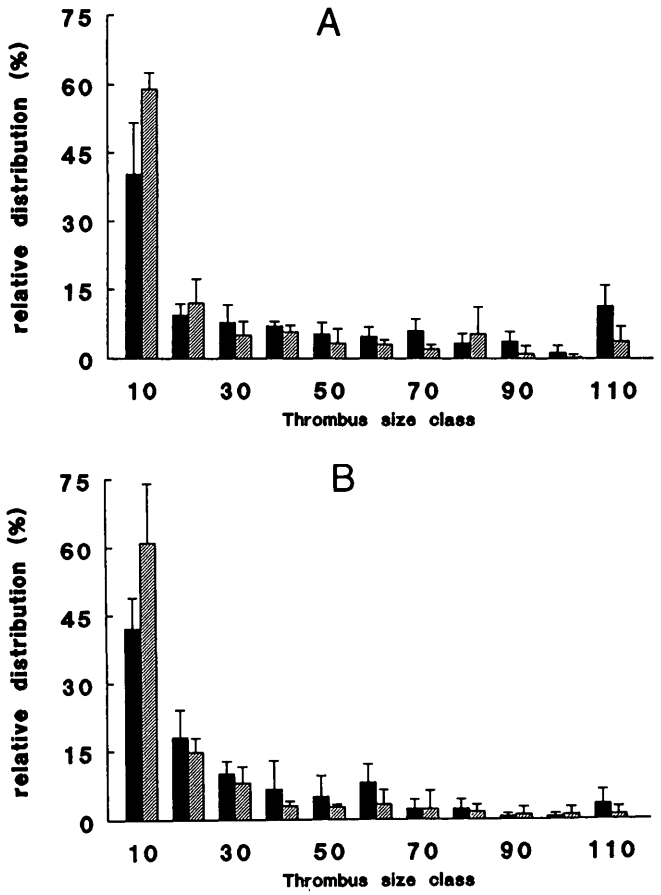

FIG. 2. Thrombus size relative distribution in 3 diabetic patients (*solid bars*) versus matched nondiabetic control subjects (*hatched bars*) at shear rates of 300 s^{-1} (*A*) and 1300 s^{-1} (*B*). Thrombus areas were evaluated en face and were sorted in sizes with increments of 10 μm^2. Values are means ± SD.

macroangiopathy) and control subjects, consistent with the higher average age in this group. Platelet counts were elevated in patients without macroangiopathy (313 ± 51 vs. 283.10^9/L ± 50, P = 0.04). vWF:Ag levels were elevated in diabetic patients with macroangiopathy (221 ± 80% in patients vs. 108 ± 52% in control subjects, P = 0.02). vWF:RiCof was elevated in group 2 diabetic patients (161 ± 53 vs. 92 ± 36%, P = 0.03). Platelet release of TXB_2 after stimulation with 1.5 mM arachidonic acid after 1 and 10 min (TXB_2/AA_1 and TXB_2/AA_{10}) was elevated in group 3 patients compared with control subjects (510 ± 88 vs. 345 ± 161 nmol/cl after 1 min, P = 0.04; 1183 ± 323 vs. 835 ± 39 nmol/cl after 10 min, P = 0.01), but platelet TXB_2 production after stimulation with exogenous collagen for 5 min was not enhanced. In group 4, no values of TXB_2 after stimulation were obtained, but serum TXB_2 levels (unstimulated) tended to be higher in the patients with macrovascular disease than in control subjects. For all other determinations, there were no differences between diabetic patients and matched control subjects in the four groups (by paired t tests).

Evaluation en face of platelet coverage (groups 1 and 2) revealed that total coverage with spread platelets on FBM and on ECM in citrated blood at subsequent perfusion times from 1 to 20 min was statistically indiscernable in diabetic patients and control subjects (Fig. 1).

Regarding cross-sectional evaluation of adhesion and aggregation (groups 3 and 4), under thrombus-forming conditions (LMWH-anticoagulated blood), total platelet coverage, spreading of platelets, and aggregate formation on PMA-stimulated ECM were similar in diabetic patients and control subjects, at subsequent perfusion times from 1 to 10 min (paired t tests; Table 4). As analyzed by ANOVA, the increases in platelet deposition in patients and control sub-

TABLE 4
Platelet deposition and thrombus formation on matrix of phorbol ester–stimulated endothelial cells.

Shear rate	Perfusion duration (min)	Perfusate	Contact and spread	Aggregates (μm)			Total coverage
				<5	5–10	>10	
Group 3							
300 s^{-1}	1	Diabetic	2.1 ± 0.6	13.8 ± 3.8	0.4 ± 0.2	0	16.3 ± 3.8
		Control	7.7 ± 2.7	7.6 ± 1.2	0.3 ± 0.2	0	15.6 ± 3.2
	3	Diabetic	1.7 ± 0.3	21.8 ± 3.3	1.4 ± 0.6	0.1 ± 0.1	25.0 ± 3.8
		Control	6.5 ± 2.5	18.4 ± 4.1	1.1 ± 0.5	0.1 ± 0.1	26.0 ± 5.0
	5	Diabetic	2.1 ± 0.6	30.4 ± 3.3	3.5 ± 1.2	0.4 ± 0.2	36.5 ± 4.0
		Control	2.8 ± 1.1	28.8 ± 3.6	2.4 ± 0.5	0.1 ± 0.1	34.1 ± 4.0
	10	Diabetic	1.7 ± 0.8	38.8 ± 3.9	6.1 ± 1.3	0.9 ± 0.4	47.5 ± 3.5
		Control	3.1 ± 0.9	43.4 ± 5.3	3.7 ± 0.8	0.1 ± 0.1	50.2 ± 5.4
1300 s^{-1}	1	Diabetic	8.8 ± 4.4	10.7 ± 1.7	0.5 ± 0.3	0	20.1 ± 4.8
		Control	7.1 ± 3.8	13.8 ± 3.5	0.2 ± 0.1	0.1 ± 0.1	21.2 ± 3.5
	3	Diabetic	4.9 ± 1.4	25.1 ± 5.4	4.6 ± 2.4	0.2 ± 0.1	34.9 ± 7.1
		Control	3.5 ± 1.1	35.9 ± 5.6	0.7 ± 0.2	0.1 ± 0.1	40.2 ± 6.0
	5	Diabetic	2.2 ± 0.5	34.2 ± 7.0	6.2 ± 3.7	0.9 ± 0.8	43.3 ± 7.9
		Control	6.4 ± 2.4	39.2 ± 9.1	2.2 ± 1.1	0.2 ± 0.2	48.0 ± 9.0
	10	Diabetic	6.6 ± 2.8	44.7 ± 6.4	4.8 ± 2.3	0	56.1 ± 6.2
		Control	8.7 ± 5.4	49.2 ± 8.0	2.3 ± 1.2	0	60.2 ± 8.5
Group 4							
300 s^{-1}	1	Diabetic (4)	5.0 ± 2.9	7.0 ± 3.3	0.3 ± 0.3	0	12.2 ± 4.8
		Control (5)	1.2 ± 0.4	7.5 ± 0.9	0.2 ± 0.1	0	9.1 ± 0.6
	3	Diabetic (4)	1.8 ± 0.7	12.6 ± 1.4	2.1 ± 0.7	0	16.5 ± 1.8
		Control	1.5 ± 0.4	12.3 ± 1.2	2.1 ± 0.6	0.5 ± 0.4	16.4 ± 2.1
	5	Diabetic	4.2 ± 2.5	15.6 ± 1.7	1.0 ± 0.3	0.2 ± 0.2	21.0 ± 3.4
		Control	2.9 ± 1.5	13.1 ± 4.2	4.1 ± 1.9	0.6 ± 0.4	20.7 ± 5.4
	10	Diabetic	6.6 ± 2.7	17.9 ± 3.2	4.0 ± 1.2	0.4 ± 0.3	28.9 ± 9.5
		Control	3.6 ± 1.7	10.8 ± 4.3	6.5 ± 2.4	0.0 ± 0.5	30.6 ± 4.7
1300 s^{-1}	1	Diabetic	4.4 ± 0.9	10.7 ± 4.8	0	0	15.1 ± 4.2
		Control	2.5 ± 0.2	9.0 ± 2.0	1.0 ± 0.3	0	13.1 ± 2.0
	3	Diabetic	8.0 ± 3.8	15.1 ± 0.8	1.6 ± 0.9	0	24.7 ± 4.9
		Control	4.4 ± 0.9	18.4 ± 3.1	2.0 ± 0.8	0.2 ± 0.2	25.0 ± 3.8
	5	Diabetic	9.5 ± 1.3	24.1 ± 3.3	1.0 ± 0.6	0.2 ± 0.2	34.7 ± 3.2
		Control	7.8 ± 2.1	23.5 ± 2.3	4.9 ± 1.4	0	36.2 ± 3.0
	10	Diabetic	10.4 ± 3	38.8 ± 2.7	1.4 ± 0.8	0	50.6 ± 1.3
		Control	10.3 ± 2.2	33.7 ± 4.6	10.6 ± 5.2	0.1 ± 0.1	54.7 ± 6.0

Values are means ± SE. Groups 3 and 4, diabetic patients without and with macroangiopathy, respectively, and their age- and sex-matched control subjects (n = 6 except as indicated in parentheses). Platelet deposition was measured on endothelial cell matrix in flowing blood after incubation of endothelial cells with 4β-phorbol 12-myristate 13-acetate (PMA) for 16 h before harvesting. Blood was anticoagulated with 20 U/ml low-molecular-weight heparin, and perfusates were standardized (180,000 platelets/ml, hematocrit 40%). Platelet deposition is described as contact-and-spread platelets (mainly spread platelets), aggregates <5 μm in height, aggregates 5–10 μm in height, aggregates >10 μm in height, and total coverage.

jects paralleled each other and did not differ in time dependence (Table 5). Larger aggregates (aggregates >5 μm in height and <10 μm in height and aggregates >10 μm in height) were present in very small percentages, and observed differences reached statistical significance by Wilcoxon's signed-rank test only occasionally, so that these differences do not represent a consistent pattern. Moreover, between all diabetic patients and control subjects, there was a similar distribution of thrombus size classes at all perfusion times and a parallel shift from smaller to larger thrombus size classes with increasing perfusion times, as scanned by image analyzer (ANOVA) (Fig. 2).

FPA generation was comparable for patients and control subjects in all perfusion experiments, with increasing values over longer perfusion times, mean values 10–20 ng/ml after 1 min and 70–180 ng/ml after 10 min (Table 6).

The comparable increases in plasma FPA levels before and after circulation of blood in the perfusion chamber suggest that fibrinogen activation in blood from diabetic subjects is not different from that in healthy control subjects under these perfusion conditions.

DISCUSSION

Vascular complications largely determine the life expectancy of patients with diabetes mellitus. It is therefore crucial to discern elements in the pathogenesis of diabetic vascular disease that can be prevented or corrected. Enhanced platelet reactivity is one potential mechanism involved in accelerated atherosclerosis in diabetes, although the meaning of this remains controversial (1,2).

We attempted to detect an increased platelet–vessel wall interaction in diabetes under flow conditions. For this purpose, we studied platelet–vessel wall interaction in blood from patients with diabetes and age- and sex-matched control subjects in a well-characterized perfusion system (3,4). The wall shear rates used (300 and 1300 s^{-1}) represented flow in larger arteries and arterioles, respectively (15). Primary platelet adhesion was studied in citrated blood with FBM or unstimulated ECM as adhering surface (16), and aggregate formation was studied with LMWH-anticoagulated blood flowing along ECM stimulated with PMA 16 h before isolating the matrix as adhering surface. The latter model provides a setting to study tissue factor-dependent thrombin

TABLE 5
Analysis of variance of platelet deposition on 4β-phorbol 12-myristate β-acetate–stimulated endothelial cell matrix in flowing blood

			Type of platelet deposition			
Shear rate	Effect	Group	Contact and spread	Aggregates <5 μm	Aggregates 5–10 μm	Total coverage
300 s^{-1}	PORC	3	0.166	0.009	0.465	0.860
		4	0.531	0.771		0.729
		3 + 4	0.469	0.021	0.948	0.640
	Time	3	0.026	0.010	0.006	0.002
		4	0.186	0.035		0.001
		3 + 4	0.553	0	0.009	0
	Case	3	0.517	0	0.222	0
		4	0.281	0.016		0.151
		3 + 4	0.440	0	0.279	0
	Case by time	3	0.064	0.973	0.900	0.384
		4	0.718	0.503		0.255
		3 + 4	0.185	0.761	0.895	0.418
	PORC by time	3	0.154	0.553	0.703	0.327
		4	0.526	0.849		0.549
		3 + 4	0.853	0.320	0.625	0.712
1300 s^{-1}	PORC	3	0.522	0.120	0.134	0.399
		4	0.760	0.843		0.675
		3 + 4	0.961	0.390	0.850	0.690
	Time	3	0.107	0.004	0.078	0.008
		4	0.113	0.014		0.007
		3 + 4	0.630	0.001	0.009	0
	Case	3	0.077	0.015	0.305	0.002
		4	0.797	0.943		0.635
		3 + 4	0.171	0.040	0.540	0.001
	Case by time	3	0.289	0.445	0.754	0.699
		4	0.268	0.467		0.920
		3 + 4	0.120	0.400	0.700	0.600
	PORC by time	3	0.360	0.527	0.547	0.706
		4	0.436	0.511		0.745
		3 + 4	0.230	0.190	0.580	0.500

P values were derived with analysis of variance of paired data of diabetic patients and matched nondiabetic control subjects in the perfusion experiments under thrombus-forming conditions. Platelet deposition is described as contact-and-spread platelets (mainly spread platelets), aggregates <5 μm in height, aggregates 5–10 μm in height, aggregates >10 μm in height, and total coverage. PORC, patient or control test for treatment difference between diabetic patients and matched control subjects, assuming a constant diabetic patient–control subject difference for all matched pairs; time, test for time effect for both patients and control subjects; case, test for differences between matched pairs (cases). A low *P* value attests for large interpair differences. Case by time, test for difference in time effects between matched pairs (cases). PORC by time, test for differences in time effects between patients and control subjects. Groups 3 and 4, patients without and with macroangiopathy, respectively. In group 3, more small aggregates (<5 μm in height) are formed in blood from patients but only at a shear rate of 300 s^{-1}. Variation between cases in deposition of aggregates <5 μm in height is found in both group 3 (at 1300 s^{-1}) and 4 (at 300 s^{-1}). There is uniformity in time effects between diabetic patients and control subjects and between diabetic patient–control subject pairs (cases) at both shear rates.

formation and subsequent platelet adhesion and aggregation on extracellular matrix (11). Primary adherence of platelets in citrated or LMWH-anticoagulated blood to ECM or FBM was identical for diabetic patients and control subjects, with identical increases in percentage of spread platelets with increasing perfusion times. Platelet aggregate formation in diabetic patients without or with macroangiopathy was also similar.

Thus, in the perfusion experiments performed, we found no evidence for an increased platelet adhesion or platelet-platelet interaction in diabetes whether macroangiopathy was present. The results of the hematological and biochemical determinations generally support this conclusion, with a few exceptions. The elevated serum TXB_2 levels in the patients with vascular disease, although the difference did not reach statistical significance, may point to platelet activation by macroangiopathy in itself.

In group 3, the results of in vitro stimulation of platelet TXB_2 production by arachidonic acid (TXB_2/AA_1 and TXB_2/AA_{10}) may suggest but do not prove an increased activity of enzymes involved in the conversion of arachidonic acid to TXA_2, inducing an arterial thrombosis tendency in this group of persons with diabetes. The increased TXB_2 production by platelets after stimulation with arachidonic acid was found in the group with slightly but significantly elevated platelet counts. The findings could not be correlated with the presence of complications.

Determinations of FPA were included as an indicator of thrombin formation in the perfusate. The FPA generation after perfusion was comparable in patients and control subjects and increased further in a parallel fashion with longer perfusion times, indicating a comparable degree of thrombin formation in blood from patients with or without macroangiopathy and control subjects (Table 6).

Plasma levels of glucose and HbA_1 were clearly elevated in diabetic patients compared with control subjects. Total triglyceride levels were elevated in group 4, consistent with the presence of macrovascular disease. The plasma total

TABLE 6
Fibrinopeptide A generation under thrombus-forming conditions

Wall shear rate	Perfusion duration (min)	No vascular disease		Vascular disease	
		Diabetic	Control	Diabetic	Control
300 s^{-1}	1	27.4 ± 9.2	21.4 ± 7.2	12.4 ± 5.2	8.8 ± 1.9
	3	83.1 ± 14.6	65.9 ± 18.7	74.4 ± 33.7	66.4 ± 24.4
	5	132.5 ± 23.6	122.2 ± 40.7	67.7 ± 18.5	82.8 ± 28.2
	10	178.2 ± 53.0	155.6 ± 71.2	81.4 ± 17.5	98.8 ± 31.7
1300 s^{-1}	1	15.7 ± 3.7	21.0 ± 8.4	11.3 ± 4.4	10.5 ± 3.2
	3	91.5 ± 17.4	108.6 ± 50.4	54.3 ± 24.5	44.8 ± 25.0
	5	142.7 ± 38.9	148.3 ± 53.4	62.7 ± 18.2	65.4 ± 19.6
	10	170.9 ± 42.7	153.7 ± 49.9	69.6 ± 16.4	98.7 ± 17.9

Values are means ± SE (n = 6).
Fibrinopeptide A generation is expressed in nanograms per milliliter of plasma after perfusion. Perfusions were for 1–10 min at 300 s^{-1} with blood anticoagulated with 20 U/ml low-molecular-weight heparin, and perfusates were standardized for platelet count (180,000 platelets/μl) and hematocrit (40%) performed with matrix of endothelial cells stimulated with 4β-phorbol 12-myristate 13-acetate 16 h before harvesting. Blood was obtained from diabetic patients with and without macrovascular disease and age- and sex-matched non-diabetic control subjects.

cholesterol levels found in this same group in both diabetic patients and control subjects are in accordance with the plasma cholesterol levels in the Dutch population, which increase slightly with advancing age. For nearly all other determinations of clinical chemistry, hematology, and coagulation, no conclusive pattern of differences between diabetic patients and control subjects were detected. All of these variables have been reported as elevated in diabetes, with tendency to normalization of these values after normoglycemia with adequate insulin treatment (17–20). The elevated vWF:Ag levels in the group with macrovascular disease are in accordance with the assumption of the presence of perturbed endothelial cells in vascular disease, producing more vWF. There is no clear explanation for the increase in vWF:RiCoF in group 2.

FBM was included as perfusion surface (group 1) to study plasma vWF-dependent platelet–vessel wall interaction (10), but the patterns of platelet adhesion on FBM were similar in diabetic patients and control subjects, suggesting that the binding of plasma vWF to FBM was not impaired in diabetes.

In these perfusion experiments, in which we studied primary platelet adhesion and platelet-platelet interaction, together with fibrinogen activation and fibrin deposition, under flow conditions, platelet reactivity toward the vessel wall in diabetic patients did not differ from control subjects, whether macroangiopathy was present. These findings suggest that the accelerated development of vascular disease in diabetes is not enhanced by a chronic state of increased platelet reactivity.

ACKNOWLEDGMENTS

This work was supported by Grant 589/1165 from the Dutch Diabetes Foundation.

We are indebted to the patients, internists, nurses, and secretaries of the Utrecht University Hospital Diabetes Clinic for cooperation with this study.

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