Study of platelet adhesion in patients with uncomplicated hypertension

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Objective To evaluate platelet function in patients with essential hypertension by sensitive methods investigating platelet adhesion and expression of some platelet glycoproteins (GP), namely GPIIb/IIIa (CD41/$\alpha_2\beta_3$) and GMP-140 (CD62/P-selectin/PADGEM). Other markers of platelet (β-thromboglobulin) and endothelium activation (von Willebrand factor) were also measured.

Methods We studied 21 uncomplicated essential hypertensive patients and 20 healthy normotensive control subjects, non-smokers, matched for age and sex. Resting and stimulated platelet adhesion was performed with a colorimetric method using the activity of platelet acid phosphatase for the determination of the number of platelets adhering to human plasma- or fibrinogen-coated microwells. Platelet activation was characterized by flow cytometric measurement of GPIIb/IIIa and GMP-140 in whole blood and washed platelets suspensions, with antihuman fluorescent monoclonal antibodies.

Results Thrombin-stimulated platelet adhesion to human plasma-coated microwells was significantly higher in hypertensive patients than in control subjects (0.05 U/ml thrombin: 13.4 ± 1.0 versus 7.7 ± 0.6% adhesion; 0.1 U/ml thrombin: 19.4 ± 2.3 versus 12.6 ± 1.8%; means ± SEM), whereas platelet adhesion to fibrinogen-coated wells did not differ in the two groups. Flow-cytometry analysis of whole blood demonstrated a significantly increased expression of GMP-140 in hypertensive patients compared with normal subjects (percentage of $CD62^+$ platelets: 7.3 ± 1.2 versus 3.7 ± 1; means ± SEM), whereas the expression of GPIIb/IIIa did not differ in the two groups (percentage of $CD41a^+$ platelets: 72.5 ± 4.5 versus 70.4 ± 3.9). Moreover, flow cytometry showed an increased size of platelets in hypertensive patients compared with that in control subjects (forwards scattering: 46.5 ± 1.5 versus 38.9 ± 1.1; means ± SEM). Flow-cytometric evaluation of washed platelet suspensions showed no statistically significant differences between the expression of GMP-140 and GPIIb/IIIa in the two groups. β-Thromboglobulin plasma levels were higher in hypertensive patients than they were in normal subjects (36.3 ± 2.0 versus 28.2 ± 1.3 ng/ml; means ± SEM). Von Willebrand factor plasma levels were not significantly different in the two groups (101.2 ± 10.3 versus 86.3 ± 5.6 U/dl).

Conclusions These findings provide further evidence that there is a significant, albeit weak, platelet activation in hypertensive patients compared with normal subjects.

Journal of Hypertension 1996, 14:1215–1221

Keywords: platelets, essential hypertension, adhesion, flow cytometry, β-thromboglobulin, von Willebrand factor

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Received 1 April 1996 Revised 18 June 1996
Accepted 29 July 1996

Introduction

During the last two decades extensive research in experimental and clinical hypertension has described a considerable number of platelet abnormalities [1] and the accumulated data have reported either normal or activated blood platelets from patients with essential hypertension. Some authors have reported enhanced platelet aggregation, at least in patients with more severe hypertension [2,3], increased platelet size [4,5] and a release reaction, determined as increased plasma β-thromboglobulin [5,6], increased intracellular free calcium concentration [7–9], increased dopamine uptake [10], reduced serotonin content and reduced serotonin uptake [11]. However, other studies have not confirmed these findings and the study of the functional response of platelets to adrenaline and other agonists has given contradictory results (see review [12]). This discrepancy could be related to various factors: analytical methods employed, differences in the populations studied (age, duration and severity of the disease) and the presence of other diseases, such as diabetes and atherosclerosis.

Moreover, the conflicting evidence regarding platelet characteristics in human essential hypertension reported by various authors is difficult to interpret, because the conditions of blood sampling, anticoagulation, type of platelet preparation and processing (whole blood, platelet-rich plasma or washed platelets) and type of assay for functional responses used by different investigators may influence

the results greatly. Finally, essential hypertension is a heterogeneous condition in which various pathophysiological mechanisms might identify a large number of phenotypic differences in the patient groups.

The object of this study was to investigate the possibility that platelets of essential, untreated and uncomplicated hypertensive patients could be more activated than those of control subjects. This was carried out using sensitive methods to investigate platelet adhesion and the expression of some platelet glycoproteins, namely glycoproteins (GP) IIb/IIIa (CD41, $\alpha_2\beta_3$) and GMP-140 (CD62, P-selectin/PADGEM).

We proposed recently a new methodology for studying platelet adhesion that proved sensitive and versatile; this could be useful for the study of the complex mechanisms involved in the activation and regulation of platelet functions [13]. Flow cytometry is a rather recent and innovative technology providing accurate, sensitive and rapid measurements of a relatively broad range of cell characteristics that include, among other things, the detection and quantification of cell antigens. Flow-cytometric analysis of human platelet morphology, volume and membrane proteins represents an emerging field in the application of this technology both in investigative and in clinical haematology [14,15]. The recent availability of immunological markers of activation, such as CD62, and the ability of this method to identify small subpopulations of cells allows the assessment of abnormal expression of the platelet membrane glycoproteins.

Patients and methods

Study subjects and eligibility

Twenty-one recently diagnosed patients with mild or moderate hypertension and 20 age- and sex-matched normotensive controls were included in this study. Elevated blood pressure was defined by systolic/diastolic blood pressure levels of more than 160/95 mmHg on at least three determinations. Secondary causes of hypertension were excluded on the basis of clinical examination, routine laboratory tests including plasma renin activity and aldosterone levels, renal scintigraphy and renography. Subjects with diabetes, kidney disease (chronic glomerulonephritis, pyelonephritis, infectious or urological disorders), chronic disease (neoplastic, haematological, hepatic or autoimmune diseases), obesity (body mass index $>30\ kg/m^2$) and other known associated diseases or complications were excluded. Subjects with vascular complications were excluded by standard clinical examinations: electrocardiography, echocardiography, duplex scanning of epi-aortic vessels, fundoscopy (Keith–Wagener–Barker retinopathy stage III–IV). These patients either had never been treated or had stopped their antihypertensive treatment for more than 3 weeks. Moreover, they had not taken drugs affecting platelet function for at least 20 days before the study. All of them were without dietary restrictions. Normotensive controls were enrolled from the clinical staff and from healthy subjects. Blood samples were extracted at 0800 h from all of the subjects after they had fasted overnight. Their informed consent to participate in the study was obtained from all of the subjects.

Isolation of platelets

Platelets were harvested from the blood of hypertensive and control subjects by differential centrifugation. After the first 4 ml blood had been discarded, a final volume of 10 ml blood was drawn by venipuncture into 1.66 ml of anticoagulant solution (15 g/l citric acid, 20 g/l dextrose and 25 g/l sodium citrate) and platelet-rich plasma was obtained by centrifugation at 300 g for 10 min. The platelet-rich plasma was recentrifuged at 700 g for 15 min and platelets were suspended gently (5×10^7/ml) in a buffer composed of 145 mmol/l NaCl, 5 mmol/l KCl, 10 mmol/l HEPES, 0.5 mmol/l Na_2HPO_4, 6 mmol/l glucose and 0.2% human serum albumin, pH 7.4 (buffer A). The recovery of platelets in the isolation procedure was 96–98% of the blood count. The platelet suspension was kept at room temperature and used within 1 h. Ten minutes before their use, platelets were warmed to 37°C.

Assay of adhesion

A colorimetric procedure measuring the activity of acid phosphatase was used for the determination of human platelet adhesion to protein-coated culture microwells [13]. Briefly, 96-well microtitre plates were coated overnight at +4°C by adding 100 µl/well human plasma or human fibrinogen. Plasma was obtained from the same pool of ten healthy subjects, stored at −30°C. It was prepared from acid citrate dextrose-anticoagulated blood, centrifuged at 1300 g for 15 min and assayed for presence of antiplatelet Ab with a negative result; then plasma was diluted 1:1 in Dulbecco's phosphate-buffered saline (PBS; Gibco Ltd, Paisley, UK). Human fibrinogen was diluted in PBS (0.2 mg/ml). The plates were then washed twice with physiological saline. Immediately after the coating and washing, the wells were supplemented with 25 µl test agonist (3 × the final desired concentration, in buffer A with 3 mmol/l $CaCl_2$ and 3 mmol/l $MgSO_4$). Low doses of thrombin (0.01, 0.05 and 0.1 U/ml) were used to activate platelets. Platelets were then brought to 37°C and 50 µl platelet suspension (2.5×10^8 platelets), prewarmed at 37°C, added to each well using a multichannel pipette. The incubation was carried out for a further 60 min, then plates were transferred to the automatic washer (Easy Washer 2; SLT Labs Instruments, Strasbourg-Schiltigheim, France) and subjected to two washing cycles with PBS at room temperature. After washing, the wells containing adherent platelets were rapidly supplemented with 150 µl 0.1 mol/l citrate buffer, pH 5.4, containing 5 mmol/l *p*-nitrophenyl-phosphate and 0.1% Triton X-100. After incubation at room temperature for 60 min, the reaction was stopped and the colour was developed by the addition of 100 µl 2 mol/l NaOH. The *p*-nitrophenol produced by the reaction was measured with a microplate

reader (Reader 400; SLT Labs Instruments) at 405 nm against a platelet-free blank. The percentage of adherent cells was calculated on the basis of a standard curve obtained with a defined number of platelets of the same donor.

Flow cytometry

Measurements of the expression of GPIIb/IIIa (CD41) and GMP-140 (CD62) in the platelets were performed by fluorescence-activated flow cytometry. Whole blood or washed platelet suspensions (2.5×10^6 platelets/ml) were incubated for 10 min at 37°C with buffer A (supplemented with 1 mmol/l $CaCl_2$ and 1 mmol/l $MgSO_4$) or thrombin at the indicated concentrations, in an end volume equal to 200 µl. The incubation was stopped with 1 ml ice-cold buffer A at +4°C and each sample was divided into two 60 µl aliquots, one of which was incubated with 6 µl antihuman CD41 mAb (FITC) and antihuman CD62 mAb (phycoerythrin) (Immunotech S.A., Birmingham, UK), while the other was incubated with a corresponding amount of fluoresceinated and phycoerythrinated control immunoglobulin G_1 mAbs (Immunotech S.A.). The antihuman CD 41 mAb is by clone P2 (hybridoma SP2/O-Ag.14 myeloma X Balb/C spleen cells) and reacts with glycoproteins IIb and IIIa in complex inhibiting platelet aggregation induced by thrombin, collagen and ADP. The antihuman CD 62 mAb is by clone CLB-thromb/6 (hybridoma SP2/O X Ag 1.4 X Balb/c x AJ spleen cells) and recognizes the GMP-140 expressed on the activated platelets. Incubation with mAbs was carried out for 20 min at +4°C. This temperature was selected on the basis of preliminary experiments showing a minimal platelet activation under this condition (percentage of $CD62^+$ platelets with incubation at +4°C: 3.7 ± 0.9; percentage of $CD62^+$ platelets with incubation at room temperature: 6.4 ± 1.1; n = 6). Finally, the samples were diluted with 1 ml ice-cold buffer A and analysed immediately [16] by a FACScan flow cytometer (Becton Dickinson, San Jose, California, USA) equipped with a 5 W argon laser and operated at 200 mW power at a wavelength of 488 nm. Fluorescein fluorescence was detected using a 530/30 mm band pass filter and phycoerythrin fluorescence was detected with a 585/42 mm filter (Becton Dickinson). The instrument was aligned daily with 2 µm Calibrite beads (Becton Dickinson) to calibrate the light-scattering and fluorescence parameters. The analyses were performed for 10 000 events in each sample, using the software Lysis-II (Becton Dickinson). Samples were passed through the laser beam through a 70 µm nozzle at a flow rate of 300–600 blood cells/s. Logarithmic amplification was used for the fluorescence signals and linear amplification for light-scattering signals. The platelet population was identified on the basis of the forwards and sideways scattering characteristics and the identity was confirmed by the use of a monoclonal antibody to a platelet antigen such as GPIIb/IIIa and P-selectin. The size distribution, calculated as forwards scattering, was the same in blood and in washed platelets.

Table 1 General characteristics of the populations studied

	Normotensives (n = 20)	Hypertensives (n = 21)
Sex (male/female)	10/10	14/7
Age (years)	46.1 ± 5.4 (28–66)	51.3 ± 4.9 (37–63)
Body mass index (kg/m²)	24.2 ± 0.8	23.5 ± 1.0
Blood pressure (mmHg)	122.4 ± 8.3/77.6 ± 8.1	178.1 ± 11.1/106.6 ± 6.3
Glucose (mmol/l)	3.99 ± 0.38	4.21 ± 0.33
Cholesterol (mmol/l)	4.53 ± 0.16	4.72 ± 0.10
HDL cholesterol (mmol/l)	1.02 ± 0.20	1.01 ± 0.21
LDL cholesterol (mmol/l)	3.18 ± 0.31	3.33 ± 0.27
Triglycerides (mmol/l)	1.51 ± 0.24	1.54 ± 0.30
Fibrinogen (mg/dl)	281 ± 39	302 ± 51
Platelet count ($\times 10^3$/µl)	230 ± 40	243 ± 31

Values are expressed as means ± SEM (ranges for age only). HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Other laboratory investigations

The quantitative determinations of von Willebrand factor (vWf) and β-thromboglobulin (β-TG) were performed by enzyme-immunoassays (Boehringer Mannheim Italia, Milan, Italy). For these determinations, blood was collected without venous occlusion, to avoid stasis and anoxia, in chilled tubes containing acid citrate dextrose and processed immediately for removal of platelets by high-speed centrifugation. All other parameters documented were determined with routine methods.

Statistical analysis

Differences between two means were compared by unpaired Student's t-test. Variables were tested to ascertain a normal distribution and changes within and between the groups by using Student's t-test and, when applicable, with non-parametrical statistical methods (the Wilcoxon test). To evaluate correlations between variables, Pearson's r correlation test was performed. The reproducibility of the assays was determined by calculation of the interassay and intra-assay coefficients of variation. $P<0.05$ was considered statistically significant. Values are presented as means ± SEM.

Results

Baseline characteristics

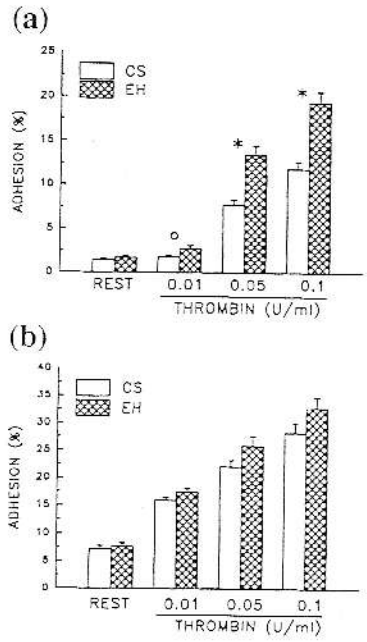
The most relevant characteristics of the hypertensive patients and control subjects studied and their plasma parameters are summarized in Table 1. No significant differences were observed between the two groups, except for systemic blood pressure. Metabolic indices and body mass index were remarkably similar in the two groups. There was a difference in sex distribution. However, platelet activation did not differ between men and women in both groups.

Platelet adhesion

Nine preliminary samples were used for the determination of intra-assay and interassay coefficients of variation. These were found to be 5.8 and 9.2%, respectively.

Figure 1(A) shows platelet adhesion to human plasma-coated wells under resting conditions and in terms of the dependence on increasing doses of thrombin (0.01–0.1 U/

Fig. 1



Platelet adhesion to human plasma-coated wells (A) and to fibrinogen-coated wells (B). CS, control subjects; EH, hypertensive patients. Values are expressed as means ± SEM. °$P<0.02$, *$P<0.01$.

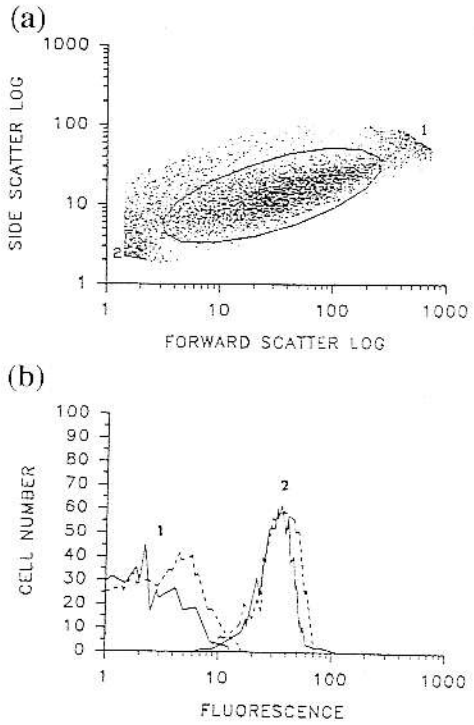
ml). Figure 1(B) shows platelet adhesion to fibrinogen-coated wells under the same conditions. In both cases unstimulated platelets showed a minimal adhesion to the coating surface; moreover, in the absence of stimulants and in the presence of low doses of thrombin (0.01 U/ml), adhesion was higher for platelets both from hypertensive and from control subjects when the assay was performed on fibrinogen-coated plates than when it was performed in plasma-coated wells. Thrombin-stimulated adhesion to microtitre plates coated with human plasma was significantly higher for platelets from hypertensive patients than it was for those from control subjects (0.05 U/ml thrombin: 13.4 ± 1.0 versus 7.7 ± 0.6% adhesion; 0.1 U/ml thrombin: 19.4 ± 2.3 versus 12.6 ± 1.8%; $P<0.01$). Thrombin-stimulated adhesion to fibrinogen-coated wells was also higher for platelets from hypertensives, but the difference was not statistically significant.

Flow-cytometric evaluation of platelets

For flow-cytometric assay, nine preliminary samples were used for the determination of intra-assay and interassay coefficients of variation. These were found to be 5.2 and 9.8%, respectively.

The behaviour and membrane properties of platelets on whole-blood flow-cytometric analysis are shown in Figure 2. Figure 2(A) shows the graphic presentation of analysed cells in an unstimulated sample from a normal subject, discriminated in terms of their forwards scattering (size) on the x axis and sideways scattering (granularity) on

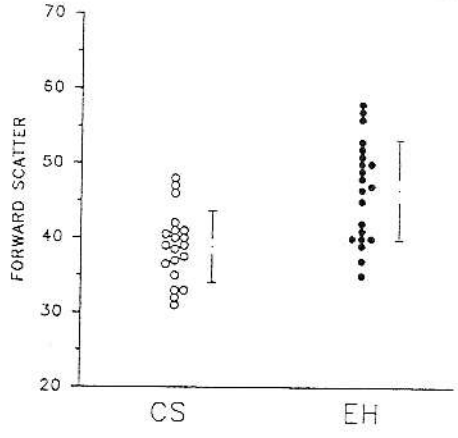
Fig. 2.



Whole-blood flow-cytometric analysis of platelets. (A) Cells are separated by their size (forwards scattering, logarithmic scale) on the x axis and granularity (sideways scattering, logarithmic scale) on the y axis. Platelets are contained in the circled region, enclosed by an electronic bit map; the area marked 1 defines erythrocytes; area 2 corresponds to machine noise and cellular debris. In the bit map, 73% of particles was positive for glycoproteins (GP)IIb/IIIa. (B) A representative fluorescence histogram of GMP-140 (marked 1) and GPIIb/IIIa (marked 2) expressions on platelets in unstimulated blood samples of a control and of a hypertensive subject. Solid lines, control subject; dotted lines, hypertensive patient.

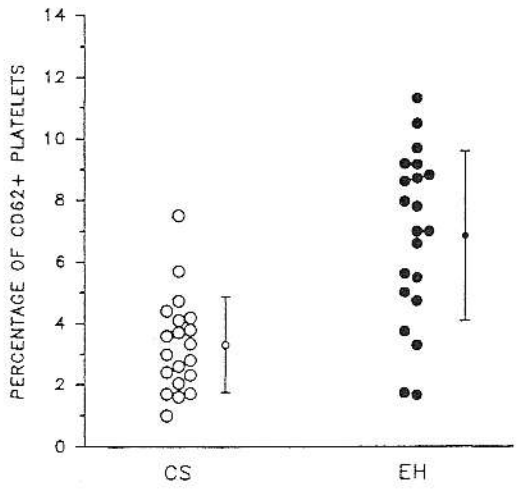
the y axis. Figure 2(B) is a representative fluorescence histogram of platelet populations obtained in whole-blood samples of hypertensive and control subjects. Figure 3

Fig. 3.



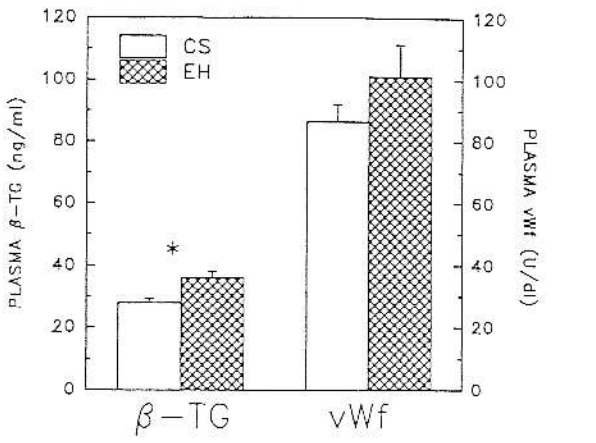
Whole-blood flow-cytometric evaluation of platelets: analysis of size parameter as forwards scattering. Values are expressed as means ± SEM. $P<0.05$. CS, normotensive; EH, hypertensive.

Fig. 4.



Whole-blood flow-cytometric evaluation of platelet GMP-140 expression: mean results of the experiments. CS, normotensives; EH, hypertensives. Values are expressed as means ± SD. $P<0.01$.

Fig. 5.



Quantitative determinations of β-thromboglobulin (β-TG) and von Willebrand factor (vWf) antigen plasma levels. CS, normotensives; EH, hypertensives. Values are expressed as means ± SEM. *$P<0.05$.

summarizes the results of scattering analysis of platelets from our patients and control groups. Platelets in whole blood from hypertensives showed a significant increase in size parameter measured as forwards scattering (forwards scattering 46.5 ± 1.5 versus 38.9 ± 1.1; $P<0.05$); the sideways scattering did not differ in the two groups (31.6 ± 2.5 versus 29.8 ± 1.9). Figure 4 summarizes the immunocytofluorimetric evaluation of platelet GMP-140 expression in whole blood. P-selectin expression was significantly higher in patients than it was in control subjects (percentage of $CD62^+$ platelets: 7.3 ± 1.2 versus 3.7 ± 1, means ± SEM; $P<0.01$). The expression of GPIIb/IIIa did not differ significantly in the two groups (percentage of $CD41a^-$ platelets: 72.5 ± 4.5 versus 70.4 ± 3.9). Flow-cytometric evaluation of purified platelet suspensions both under resting conditions and after thrombin stimulation showed no statistical differences between the expression of GMP-140 and GPIIb/IIIa in hypertensives and in normal subjects (Table 2).

β-TG and vWf

Figure 5 reports the results of the quantitative determination of β-TG and vWf. Plasma β-TG levels were significantly higher in hypertensive patients than they were in control subjects (36.3 ± 2.0 versus 28.2 ± 1.3 ng/ml; $P<0.05$), whereas vWf levels increased only slightly and were not significantly different in patients from those in normal subjects (101.2 ± 10.3 versus 86.3 ± 5.6 U/dl).

Correlations between platelet activation markers and characteristics of the patients

No significant correlation was found between the platelet activation markers (adhesion, GMP-140, β-TG and platelet size) and clinical or laboratory characteristics of hypertensive patients (sex, age, body mass index, systolic and diastolic blood pressures, and the laboratory parameters tested: glucose, cholesterol, platelet count and so on). Moreover, no correlation was detectable between platelet adhesion and membrane expression of GPIIb/IIIa and of GMP-140, respectively. Neither was a correlation between β-TG and flow-cytometric markers of platelet activation detected.

Discussion

The first step of platelet functional response is adhesion to the vessel wall, a complex event involving a series of plasma and subendothelial tissue components (collagen, fibrinogen, fibronectin and von Willebrand factor) which bind themselves specifically to several different platelet membrane glycoproteins. A critical event in platelet adhesion is the induction of a change in the disposition of

Table 2 **Immunocytofluorimetry evaluation of washed platelet suspensions and of whole blood**

	Normotensives (n = 20)		Hypertensives (n = 21)	
Washed platelets	Glycoproteins IIb/IIIa	GMP-140	Glycoproteins IIb/IIIa	GMP-140
Resting	71.4 ± 3.9(12.10 ± 1.92)	8 ± 0.5 (4.66 ± 0.03)	68.2 ± 1.8(11.58 ± 0.94)	7.9 ± 1(4.64 ± 0.3)
0.01 U/ml Thrombin	82.1 ± 3.9(22.17 ± 1.92)	30 ± 1.7(12.95 ± 0.93)	78.1 ± 2.9(19.16 ± 1.47)	36 ± 5.2(15.64 ± 2.29)
0.05 U/ml Thrombin	89 ± 6.7(42.23 ± 4.13)	69.2 ± 3.8(43.73 ± 1.88)	86 ± 6.4(39.71 ± 3.98)	71.1 ± 4.9(46.33 ± 2.02)
0.1 U/ml Thrombin	93 ± 5.5(44.69 ± 3.48)	78.8 ± 2.7(49.80 ± 1.23)	91.1 ± 6.1(41.99 ± 3.55)	81.5 ± 3.8 (52.28 ± 1.84)
Whole blood	70.4 ± 3.5(11.2 ± 1.3)	3.7 ± 1 (3.1 ± 0.2)	72.5 ± 3.9(13.2 ± 1.9)	7.3 ± 1.2* (4.4 ± 0.4)

Values are expressed as means ± SEM percentages of $CD41a^-$ and $CD62^+$ platelets (means of fluorescence intensities). **$P<0.01$, versus normotensives.

the surface membrane glycoproteins, which acquire the capacity to bind fibrinogen, von Willebrand factor, fibronectin and vitronectin. The activation of platelets is also associated with the expression of GMP-140, a selectin-type α-granule protein, which occurs within seconds on the platelet surface.

The classic measurement of ex-vivo platelet aggregatory response to a series of agonists is relatively insensitive as a marker of platelet activation [17]. In recent years, the use of fluorescence-labelled antibodies and flow cytometry has allowed the detection of membrane antigens and of activation antigens on individual platelets [14,15]. Whole-blood flow-cytometric techniques minimize artefactual platelet activation, which can be caused *in vitro* by centrifugation or other procedures used to separate platelets and, above all, it can detect hyporesponsive or hyperresponsive platelet subpopulations [15].

The objective of the present study was to examine the function and the properties of platelets from a group of carefully selected hypertensive patients by investigating platelet adhesion, the expression of some platelet glycoproteins, namely GPIIb/IIIa and GMP-140, and β-TG release as indices of platelet activation *in vitro*. Our results show that thrombin-stimulated platelet adhesion is stronger in hypertensive patients than it is in control subjects: this difference is significant only when the cells are left to adhere to a plasma-coated surface. We have also observed that resting platelet adhesion to plasma- and to fibrinogen-coated surfaces is very small and this allows us to exclude activations resulting from the platelet preparation. Both under basal conditions (i.e. without agonist) and in the presence of low doses of thrombin, the adhesion to fibrinogen is stronger than that observed using microtitre plates coated with plasma. It is, therefore, conceivable that, when using fibrinogen as a coating surface, platelets both of hypertensive and of control subjects show maximal adhesion because the surface interaction is facilitated by the high availability of binding sites. Moreover, the binding to fibrinogen further activates platelets and this may lead eventually to platelet aggregation. We cannot exclude that a partial aggregation may occur also in our experimental setting, in which low platelet concentration and stationary conditions were used to minimize aggregation. On the basis of these observations, the use of fibrinogen as a binding surface might therefore not be suitable to assay ex-vivo platelet adhesion. On the contrary, in the presence of plasma there is competition between adhesion substrates, such as von Willebrand factor, fibrinogen and fibronectin, and factors like albumin, which hinder adhesion [13]. Platelets of hypertensive patients do not exhibit an increase in basal adhesion, but appear to be primed to the response to thrombin. This primer state may be unmasked by specific assay conditions. Only in this case can the difference between the two groups be detected.

In order to characterize the possible molecular mechanism of this enhanced platelet adhesion in hypertensives further, we measured, by immunocytofluorimetry, the complex GPIIb/IIIa on platelets and a specific marker of platelet activation (GMP-140). The fact that the expression of GPIIb/IIIa, the most important platelet receptor for fibrinogen [18], is not significantly different in the hypertensive and in the normotensive subjects indicates that the platelet activation found in hypertensives is probably weak, which would be consistent with the low differences in adhesion to fibrinogen. It is possible that the anti-GPIIb/IIIa mAb which we used does not allow one to detect the small difference between hypertensive patients and control subjects because it is not specific for epitopes unmasked by activation, even if it is specific for the GPIIb/IIIa complex which is more strongly expressed on the surface after a stimulus [19].

In the present investigation, we showed that platelets of hypertensive patients have a significantly increased expression of GMP-140 compared with those of control subjects, when tested in whole blood. However, the increased expression of GMP-140 in whole blood cannot be observed with washed platelets and is not correlated with the increased adhesion. These results demonstrate that the detection of the primed state of platelets in hypertensive patients is strongly dependent on experimental conditions.

The aim of this study was not to investigate the mechanistic basis of platelet activation but only to gain better understanding of whether this abnormality is actually present or not. This was achieved by using two new methods: platelet adhesion to protein-coated microwells and adhesion glycoprotein expression.

The evidence of increased thrombin-stimulated adhesion and the increased expression of GMP-140 are in keeping with other abnormalities, such as the increased platelet volume and plasma concentration of β-TG. These abnormalities are consistent with previous reports from our laboratory [9] and others [7,8], which show that levels of cytosolic free Ca^{2+} ions are significantly higher in platelets of hypertensive subjects. These observations are of obvious importance since the $[Ca^{2+}]_i$ plays a pivotal role in platelet activation.

Regarding the increased platelet size measured by forwards scattering which we have noted, a possible objection is that it might have resulted from an artefact of platelet microaggregation rather than being a real increase in size parameter. We think that this possibility is extremely unlikely since the cytofluorimetric assay system used was designed to study platelet function under conditions in which aggregation is highly discouraged. In fact, the platelet concentration in the final incubation was much too low to allow platelets to aggregate. Moreover, the incubations were performed under stationary conditions and aggregation is facilitated by stirring [20].

These data support the hypothesis that the platelet hyperresponsiveness plays a role in the long-term consequences of hypertension. Increased platelet volume is considered a non-specific marker of platelet activation since it has been established that large platelets are more reactive per unit volume than are small platelets and release more prothrombotic factors, such as serotonin and β-TG [21]. Besides in essential hypertension, increased platelet volume has been reported in other diseases (i.e. myocardial infarction [22], cerebral infarction [23] and pre-eclampsia [24]).

We were not able to identify any correlation between platelet abnormalities and clinical profiles (i.e. age, duration of hypertension and blood pressure). Therefore, there is no evidence from these data that platelet activation is linked directly to the severity of the disease.

The increased platelet activation observed in hypertensives might be secondary to a defect of endothelial function or to an intrinsic platelet abnormality involving the transduction mechanism. Endothelial dysfunction has been postulated to contribute significantly to the pathogenesis of essential hypertension through several mechanisms. In particular, there is growing evidence that the synthesis of NO, which has anti-aggregating and vasodilatory properties, by vascular endothelium [25,26] and by platelets [27] may be reduced in essential hypertension. An impaired endothelial function has recently been reported in uncomplicated hypertensive patients also by measuring circulating vWf antigen [28,29] and consequently we used it as a marker of endothelial function. In this study, however, we found no difference between vWf plasma levels in uncomplicated hypertensives and in control subjects. Pedrinelli *et al.* [30] found normal vWf plasma levels in hypertensive patients without microalbuminuria.

In conclusion, this study provides further evidence that there is a significant, albeit weak, platelet activation in essential hypertension. This abnormality may contribute to the increased vascular risk of hypertensive patients. Whether this hypertensive platelet activation is an expression of a primary rather than a secondary defect could be addressed in future studies.

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