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◆ ORIGINAL ARTICLE ◆

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Platelet morphology and plasma indices of platelet activation in essential hypertension: effects of amlodipine-based antihypertensive therapy

Sunil Nadar, Andrew D Blann and Gregory YH Lip

BACKGROUND AND AIM. Platelet abnormalities have been described in hypertension, especially in the presence of target organ damage. Our aim was to study the differences in morphology and indices of platelet activation in treatment-naive patients with essential hypertension as compared to normotensive controls and secondly, to study the effects of amlodipine-based antihypertensive therapy on these indices.

METHODS. We recruited 42 previously untreated, newly diagnosed hypertensive patients (25 men; mean age 53 years) for the cross-sectional study, where data were compared with those from 30 normotensive controls (20 men; mean age 57 years). Of the 42 untreated hypertensive patients who were recruited, 27 patients successfully completed, the six-month treatment phase with amlodipine-based antihypertensive therapy. Platelet morphology (volume and mass) was quantified, and plasma markers of platelet activation (βTG and sPsel) measured in citrated plasma. The mass of P-selectin in each platelet (pPsel) was determined by lysing a fixed number of platelets and then determining the levels of P-selectin in the lysate.

RESULTS. Hypertensive patients had significantly higher platelet volume ($P = 0.01$) and mass ($P = 0.003$), plasma βTG and sPsel, and pPsel levels (all $P < 0.001$) compared to the controls. After a mean treatment time of 6 months, there was a decrease in platelet volume ($P < 0.001$) and mass ($P = 0.02$), with lower pPsel, sPsel and BTG levels (all $P < 0.001$) compared to the untreated state.

CONCLUSION. Treatment of uncomplicated essential hypertension using amlodipine-based anti-hypertensive therapy results in a reversal of the platelet morphology abnormalities and indices of platelet activation. This may contribute to a reduction in thrombosis-related complications seen in those whose blood pressure lowering is effective.

Keywords: calcium channel blockers; hypertension; platelet activation

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Introduction

Paradoxically, hypertension is associated with an increased risk of thrombotic, as opposed to haemorrhagic, complications such as stroke and myocardial infarction (1). This may be due, at least in part, to the prothrombotic state associated with inappropriate platelet activation (such as increased aggregation *ex-vivo* and release of soluble P-selectin (sP-selectin)) and increased plasma levels of coagulation proteins, such as fibrinogen and von Willebrand factor (1, 2). Furthermore, pharmacological reduction of blood pressure is associated with reversal in these changes in platelet activation (3, 4), showing that the effects of some classes of anti-hypertensive drugs extend beyond that of simple blood pressure reduction alone. The calcium channel blockers are one such group of drugs that appear to act directly on the platelets, as well as bringing about changes that are seemingly independent of blood pressure lowering (4). Indeed, platelet activation (by whatever cause) results in the increase in intracellular calcium ions, and interfering with the entry of calcium into the cells could be a way of minimising blocking platelet (5, 6).

Recognised plasma markers of platelet activation include beta-thromboglobulin (βTG), platelet factor 4, and sP-selectin (7–11). Platelet size is also increased in hypertension (11), and we have also shown that the total mass of P-selectin within each platelet (as determined by measuring levels of P-selectin in the lysate formed by solubilising a fixed number of these cells) are increased in atrial fibrillation, a condition often associated with hypertension (12). Several

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(4, 13–15), but not all (16) intervention studies indicate that increased levels of plasma markers can be reduced by blood-pressure lowering.

In the present study, we hypothesised the following: 1) that abnormalities in platelet morphology (mass, volume) and platelet P-selectin levels exist in treatment-naïve patients with essential hypertension; and 2) that amlodipine-based antihypertensive therapy would normalise these abnormalities. To test these hypotheses, we performed a cross-sectional study of previously untreated newly diagnosed patients with hypertension, who were compared to normotensive controls. The hypertensive patients were then treated with amlodipine-based antihypertensive therapy, to achieve blood pressure control. Levels of sP-selectin and βTG were also measured as additional markers of platelet activation previously investigated in hypertension (3).

Key messages

- There is evidence of platelet activation in hypertension
- Platelets from hypertensive patients are larger and heavier than those from normotensive controls
- Platelets from hypertensive patients have higher levels of the adhesion molecule P-selectin than normotensive patients
- With the treatment of hypertension using amlodipine-based antihypertensive therapy, the morphological changes as well as the levels of P-selectin both in the plasma and within the platelets revert to within normal limits.

Patients and methods

Forty-two newly diagnosed and never treated patients with essential hypertension were recruited. Exclusion criteria were women of child bearing potential in whom pregnancy could not be ruled out, secondary hypertension, coexisting medical conditions such as diabetes, coronary artery disease, peripheral vascular disease, previous stroke or myocardial infarction, previous malignant hypertension or congestive cardiac failure. Patients who were already on an antiplatelet therapy (aspirin or clopidogrel) were also excluded. Of these 42 patients, 27 were started on amlodipine 5 mg, and the dose was up-titrated as required to achieve a blood pressure goal of less than 140/80 mmHg. Fifteen patients needed the dose to be up-titrated to 10 mg. However, in 7 cases another class of antihypertensive drug was required to achieve target blood pressures: 4 patients put on an Angiotensin Converting Enzyme (ACE)-inhibitor and 3 started on a beta-blocker. A second blood sample was obtained 6 months after starting treatment.

We also recruited 30 healthy normal subjects from hospital staff, relatives of the patients or those attending the hospital for routine cataract or hernia surgery. The subjects were not taking any regular medication and were non-smokers with no clinical evidence of vascular, metabolic, neoplastic or inflammatory disease by careful history, examination and routine laboratory tests. These subjects were normotensive (<140/90 mm Hg) and in sinus rhythm. Informed consent was given by all the study participants. The study was approved by the local ethics committee of the hospital.

Laboratory

Blood was drawn from an antecubital vein with atraumatic venipuncture. Blood was collected in vacutainers containing citrate, theophylline, adenosine and dipyridamole (CTAD) for estimating β-thromboglobulin, and into citrated vacutainers for the collection of samples for estimating pP-selectin and sP-selectin. The blood samples collected in the CTAD tubes were immediately placed on ice and plasma separated within 1 hour of collection. Aliquots were stored at −70°C to facilitate batch analysis. β-thromboglobulin levels were determined by enzyme linked immunosorbent assay (ELISA) (Asserachrom β-thromboglobulin, Diagnostica Stago, Asniere sur Seine, France). P-selectin in citrated plasma and platelet lysate was also determined by ELISA (R&D Systems, Abingdon, UK). Inter- and intra-assay coefficients of variation for all assays were 5% and 10% respectively.

pP-selectin levels were measured by a lysate technique, as previously described (12). Briefly, platelet-rich plasma was obtained by centrifugation of venous blood at 1000 rpm (200 × g) for 10 minutes. From this, 10^8 platelets were pelleted by centrifugation at 2500 rpm (1000 × g) for 20 minutes, washed in phosphate buffered saline, and lysed by a small volume (250 μL) of 0.1% Triton X-100 (Sigma, Fancy Rd, Poole, UK). The total amount of P-selectin in an aliquot of this lysate was determined by ELISA (as for sP-selectin), and, after adjustment, was reported as ng/cell. Platelet parameters total platelet count, mean platelet volume (MPV) and mean platelet mass (MPM) were measured using by flow cytometry on an ADVIA Haematology 2000 system (Bayer, Newbury, Berks, UK).

Power calculation

Based on our previous experience with P-selectin

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Table 1. Demographic features of the study population.

	Controls ($n = 30$)	Hypertensive ($n = 42$)	*P*-value
Age	57 ± 10	53 ± 14	0.10
Sex (M:F)	20:10	25:17	0.35
Ethnic			
Caucasian	19 (63%)	28 (66%)	
Asian	8 (26%)	11 (26%)	0.9
Afro-Caribbean	3 (11%)	3 (8%)	
SBP (mmHg)	127 ± 9	167 ± 12	<0.001
DBP (mmHg)	75 ± 9	96 ± 11	<0.001
DM	0	4 (9%)	
Smokers	0	1 (2%)	
'Add-on' drugs during treatment phase			
ACE-I		4 (9%)	
Beta blocker		3 (7%)	

M:F = male to female ratio; SBP = systolic blood pressure; DBP = diastolic blood pressure; DM = diabetes mellitus; ACE-I = angiotensin converting enzyme inhibitor. Values are mean ± standard deviation (SD) or number (percentage), analysed by Student's *t* test or the chi-squared test.

(10, 12, 15), in our cross-sectional study we hypothesised an increase of half of a standard deviation in the patients compared to the controls. To achieve this at $P < 0.05$ and $1-\beta = 0.8$, at least 26 cases and 26 controls are needed. Therefore, for additional confidence, we recruited in excess of this figure. In our intervention study, we hypothesised that the effect of treatment would be to reduce P-selectin by half a standard deviation, and for $P < 0.05$, again, at $1-\beta = 0.8$, paired data from a minimum of 26 cases are required.

Statistics

Data continuously variable were subjected to the Anderson-Darling test to define its distribution. Results are expressed as mean with standard deviation (SD) or as median with inter-quartile range (IQR) for the normally distributed data and skewed data respectively. Data between patients and controls were analysed by the unpaired *t* test or Mann-Whitney U test, as appropriate. Correlations were performed by Spearman's correlation method (where necessary, on logged data). All statistical calculations were performed on a microcomputer using a commercially available statistical package (Minitab release 12, Minitab Inc, PA, USA). A *P*-value <0.05 was considered statistically significant

Results

We studied 42 previously untreated newly diagnosed patients with hypertension, and compared them with 30 normotensive controls who were matched for age, sex and ethnicity (Table 1). We found raised sP-selectin ($P = 0.01$), βTG ($P < 0.001$), and platelet volume ($P = 0.01$) among the hypertensive patients. We also found increased platelet mass ($P = 0.003$) and increased platelet P-selectin ($P < 0.001$). The difference in mean platelet count was not statistically different (Table 2).

Effects of treatment

Of the 42 untreated hypertensive patients who were recruited, 9 patients did not consent to the follow-up study, and after treatment with amlodipine, 6 patients discontinued their drug due to side effects (mainly dependant pedal oedema). Thus, 27 patients successfully completed, the six-month treatment phase with amlodipine-based therapy (average dose of amlodipine was 7.8 mg, with 12 patients taking 5 mg and 15 taking 10 mg). As expected (Table 3), there was a significant reduction in blood pressure, βTG and sP-selectin from the pre-treatment level. Hypertensive patients also demonstrated a reduction in platelet volume ($P < 0.001$), platelet mass ($P = 0.02$) and platelet P-selectin ($P < 0.001$), compared to the untreated state (Figs 1 and 2). There was no significant change in the platelet count with treatment. There were no significant correlations between the reductions in blood pressure and the changes in any of the research parameters (data not shown).

Seven patients had additional antihypertensive agents added in order to treat the blood pressure to target; however, even when these 7 patients were

Table 2. Platelet count, morphology, beta-thromboglobulin and soluble and platelet P-selectin in controls and untreated hypertensives.

	Controls ($n = 30$)	Hypertensives ($n = 42$)	*P*-value
Platelet count ($\times 10^6$/cu mm)	240 ± 50	266 ± 65	0.057
MPM (pg)	1.75 ± 0.15	1.89 ± 0.2	0.003
MPV(fl)	6.4 ± 0.9	7.09 ± 1.07	0.01
sPsel (ng /ml)	70 (65–112)	90 (80–112)	0.01
pPsel (pg/10^{-6} platelet)	100 (73–120)	210 (127–350)	<0.001
βTG (IU/ml)	90 (60–152)	155 (150–170)	<0.001

MPM = mean platelet mass; MPV = mean platelet volume; sPsel = soluble P-selectin; pPsel = platelet P-selectin; βTG = beta thromboglobulin. Values are mean ± standard deviation or median (inter-quartile range). Data analysed by Student's *t* test or Mann-Whitney U test, as appropriate.

Table 3. Effect of treatment on blood pressure, platelet count and morphology, beta-thromboglobulin and soluble and platelet P-selectin in 27 patients with essential hypertension.

	Pre-treatment	Post-treatment	*P*-value
SBP (mmHg)	169 ± 12	137 ± 10	<0.001
DBP (mmHg)	97 ± 10	83 ± 8	<0.001
Platelets (× 10^6/cu mm)	273 ± 71	257 ± 55	0.13
MPM (pg)	1.87 ± 0.2	1.76 ± 0.15	0.02
MPV (fl)	7.16 ± 1.04	5.61 ± 0.58	<0.001
sPsel (μg /ml)	100 (83–170)	50 (47–55)	<0.001
pPsel (pg/10^{-6} platelet)	210 (135–350)	70 (68–80)	<0.001
βTG(IU/ml)	160 (150–170)	130 (127–145)	<0.001

SBP = systolic blood pressure; DBP = diastolic blood pressure; MPM = mean platelet mass; MPV = mean platelet volume; sPsel = soluble P-selectin; pPsel = platelet P-selectin; βTG = beta thromboglobulin. Values are mean ± standard deviation or median (inter-quartile range). Data analysed by Student's *t* test or Mann-Whitney U test, as appropriate.

excluded from the analysis, the changes in the platelet markers remained significant (data not shown). As the numbers were small (and underpowered), we did not analyse whether these patients on more than one antihypertensive agent had greater changes in the markers of platelet activation.

Discussion

In the present study of treatment-naïve uncomplicated essential hypertensive patients, we have confirmed previous observations of platelet abnormalities in hypertension (4, 7–11, 13–16). The novel contributions of the present work are as follows: 1) the finding of increased platelet mass and increased platelet P-selectin in uncomplicated essential hypertensive patients; and 2) the observation that the use of (largely) amlodipine-based anti-hypertensive therapy leads to a reversal of these platelet morphology abnormalities and indices of platelet activation.

Increased platelet volume in hypertension has previously been taken to be a marker of platelet activation, and carries with it a poor prognosis in ischaemic heart disease (11, 17–20), although not all studies concur with this view (21). However, little is known of the aetiology or significance of increased platelet mass, although it intuitively seems likely to be deleterious (22, 23). There is however, no conclusive evidence that these changes are initiated in the bone marrow itself, although there is a single report (24) of increased DNA content in the megakaryocytes of hypertensive patients as compared to normotensive controls. However, the numbers in this study were small ($n = 12$) and the differences were minor. Nevertheless, the fact that both platelet morphology indices (and the plasma markers), but not platelet count, are improved by treatment provides an alternative route by which reducing blood pressure protects against thrombotic events. This theme is continued with platelet P-selectin. In the present study, we report (for the first time) that raised levels are reduced by treatment, again supporting the evidence for benefits of anti-hypertensive treatment that seem to extend beyond those of simply lowering blood pressure alone. Indeed, there were no significant correlations between the level of reductions in blood pressure and the changes in the platelet functions or the platelet indices. This suggests that perhaps there are alter-

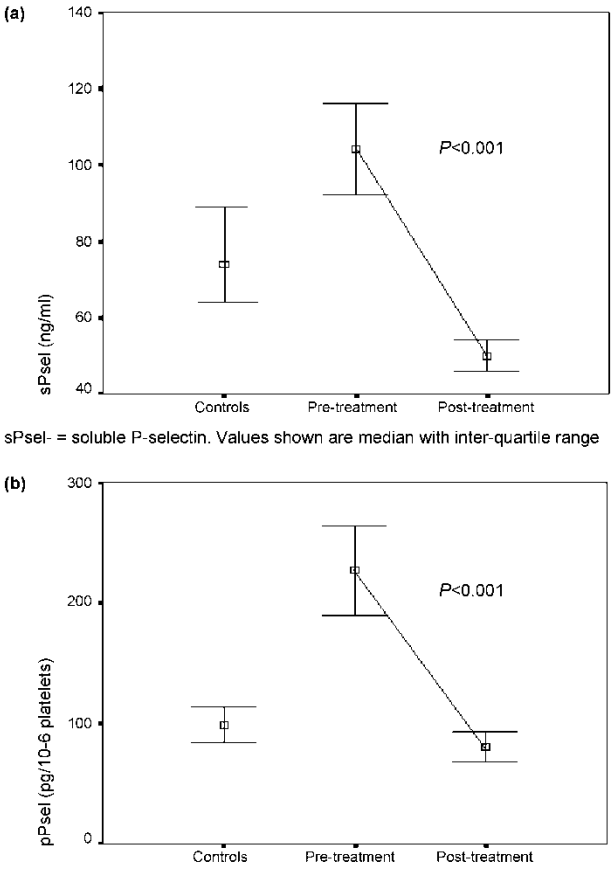

Figure 1. Effect of treatment on P-selectin in the plasma and platelets **(a)** Effect on plasma P-selectin **(b)** Effect on platelet P-selectin.

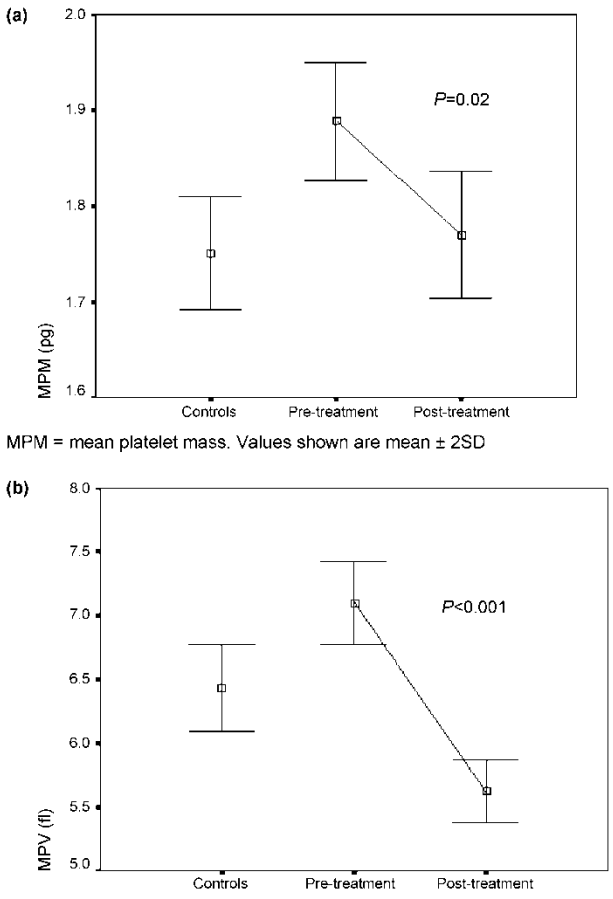

Figure 2. Effect of treatment on platelet morphology **(a)** Effect on platelet mass **(b)** Effect on platelet volume.

native mechanisms by which blood pressure reduction influences platelet function.

The biochemical mechanism explaining the beneficial effects of amlodipine is in interfering with calcium, an important role in the regulation of platelet function (5, 6). Platelet cytosolic calcium is involved in several aspects of platelet activation such as shape change, secretion and aggregation. Cytosolic calcium increases during platelet activation both as a result of release from intracellular stores, like the dense tubular system, and *via* influx from the extracellular space (25, 26). However, in contrast to smooth muscle cells or cardiac myocytes, platelets lack voltage-dependent calcium channels of the L-type; the influx of Ca^{2+} during platelet activation occurs through receptor operated calcium channels and through channels activated by the GpIIb/IIIa receptors (27, 28). Thus, the effects of calcium channel blockers on platelet function *in vivo* may involve indirect mechanisms (e.g., *via* shear stress, neuro-hormonal activity) or other mechanisms other than L-type calcium channel inhibition *per se*. Another method by which calcium channel blockers may reduce platelet activation could be by their effects on the endothelium (28, 29). By improving endothelial function, these agents increase the amount of endogenous nitric oxide, a potent anti-platelet agent. Also, calcium channel blockers themselves may also increase nitric oxide by mechanisms independent of improved endothelial function (30).

A limitation of our study is the absence of a control arm where blood pressure was reduced by another antihypertensive agent. However, our aim in this study was principally to study the effect of blood pressure lowering on platelet indices, rather than the specific effect of calcium channel blockers *per se*. As such our data contribute to a growing mass of data on the benefit of this class of agents on platelets (31–33).

In conclusion, treatment of hypertension using amlodipine-based anti-hypertensive therapy results in a reversal of the platelet morphology abnormalities and indices of platelet activation. This may contribute to a reduction in thrombosis-related complications seen in those whose blood pressure lowering is effective.

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