Mononuclear cell adhesion to collagen ex vivo is related to pulse pressure in elderly subjects

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Abstract

Mononuclear cells and platelets are intimately involved in the pathogenesis and complications of cardiovascular disease. Platelet activation has been reported in hypertension, though the activation-state of monocytes has received less attention. In this study the adhesiveness of monocytes and platelets was assessed and any relationship between the adhesive properties of these cellular elements and plasma levels of soluble adhesion molecules and blood pressure parameters determined. Fifty six elderly volunteers, of whom 32 were classified hypertensive (daytime SBP ≥ 135 mmHg), underwent 24 h blood pressure monitoring, assessment of monocyte and platelet adhesion and measurement of the plasma soluble adhesion molecules ICAM-1, L-selectin, E-selectin and vWF. In the elderly hypertensive subjects, monocyte adhesion to collagen coated (P < 0.05) and tissue culture plastic microwells (P < 0.05) was significantly elevated compared to their normotensive counterparts. A significant correlation was found to exist between monocyte adhesion to collagen and daytime pulse pressure (r = 0.39, P < 0.01) and also between plasma levels of soluble E-selectin and clinic DBP (r = 0.40, P < 0.001). The increased monocyte adhesion witnessed in hypertensive subjects and with increasing pulse pressure may contribute to the increased risk of cardiovascular disease in hypertension. Whether this increased adhesiveness is a property of the monocytes, or reflects endothelial cell activation, remains to be determined. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Monocytes; Platelets; Adhesion; Pulse pressure; Hypertension

1. Introduction

Mononuclear cells and platelets are intimately involved in athero-thrombotic vascular disease [1] a condition whose prevalence increases with age and blood pressure. The relationship between platelet reactivity and blood pressure has previously been studied [2–4] and although reports have been conflicting, the consensus indicates that platelets are activated in subjects with hypertension [5,6]. In contrast the activation-state of mononuclear cells, which play a pivotal role in atherogenesis, has received less attention.

Adhesion of monocytes to the arterial wall, via specific cell surface adhesion molecules, prior to transendothelial migration is an important early event in the development of atherosclerotic lesions [7]. It has been suggested that various risk factors such as hypercholesterolaemia, hypertension, smoking and diabetes may lead to endothelial dysfunction and activation [8–10]. Upon activation one of the earliest events is the upregulation of the expression of the adhesion molecules of the selectin family, which results in the tethering and rolling of monocytes along the endothelium [11]. Some of these monocytes will disengage and re-enter the circulation, while others will become more firmly attached via integrin mediated adhesion [12]. Monocytes subsequently migrate across the endothelium under the influence of chemotactic factors. Once within the subendothelial space they differentiate into macrophages and take up modified low-density lipoproteins to become the characteristic lipid laden foam cell of an atherosclerotic lesion.
The aim of the present study was to assess the adhesiveness of monocytes and platelets ex vivo in elderly hypertensive and normotensive subjects free of clinical vascular disease and to determine whether the adhesive properties of these cellular elements were related to plasma levels of the soluble adhesion molecules L- or E-selectin, ICAM-1 and von Willebrand factor (vWF) or the blood pressure parameters.

2. Methods

2.1. Materials

RPMI 1640 was purchased from Gibco (Uxbridge, UK). All other reagents including apyrase and prosta-
cyclin were purchased from Sigma Chemical (Dorset, UK).

2.2. Subjects

Healthy elderly (60–80 years) volunteers were screened to exclude those with a history of symptomatic vascular disease, established hypertension, diabetes mellitus or subjects using medication including aspirin and vitamin supplements, and current smokers. This research has been carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and ethical approval was granted by the Local Ethics Committee prior to the start of the study. Informed consent was obtained from all subjects. Conventional blood pressure was recorded in triplicate on three occasions, taking the mean of three sitting blood pressures. Home 24-h blood pressure monitoring was undertaken with the SpaceLabs 90 207 ambulatory blood pressure device (Space Labs Inc, Redmond, Washington, USA) programmed to take readings every 15 min; daytime blood pressure was defined as the mean of readings from 07:00 to 22:00 h. During the final clinic visit blood samples were taken between 09:00 and 11:00 h for the measurement of plasma lipid levels, glucose, urea, electrolytes, soluble L- and E-selectin, soluble ICAM- and vWF and assessment of monocyte and platelet adhesion.

2.3. Mononuclear cell isolation

Whole blood anticoagulated with 1/10th volume of 0.013 mol/l trisodium citrate was centrifuged at 200 × g for 20 min and the upper platelet-rich layer removed without disturbing the ‘buffy-coat’. The blood was re-
stored to its original volume with phosphate buffered saline (PBS), and 5-ml aliquots overlaid onto Ficoll–Paque. Blood mononuclear cells were prepared as previously described [13]. Briefly, the tubes were centrifuged at 400 × g for 30 min; the mononuclear cell layer was recovered and washed twice in five volumes of PBS. Immediately prior to use, the cells were resus-
pended in serum-free RPMI 1640 medium at a concent-
tration of 6 × 10⁶/l. Prior to use, cell viability was assessed by trypan blue exclusion, and on all occasions viability exceeded 95%. The content of contaminating platelets was low, with a ratio of platelets:mononuclear cells being < 1.

2.4. Measurement of monocyte adhesion

Mononuclear cell (MNC) suspensions in serum-free RPMI 1640 were added to plastic and collagen coated (20 μg/ml, overnight at 4°C) 96 well microtitre plates. The plates were incubated at 37°C for 30 min, and non-adherent cells removed by washing twice with PBS. Monocyte specific adherence was determined by a modi-
fication of the method described by Bath et al. [14]. This method relies on conversion of a colourless sub-
strate tetramethyl benzidine (TMB) to a blue product by the action of myeloperoxidase, which is present in monocytes but not lymphocytes [14]. The cells con-
tained in each well were lysed in 100 μl hexade-
cyltrimethyl-ammonium bromide (5 mg/ml in PBS, pH 5.0) at 37°C for 60 min. A fresh solution of TMB [0.1 mg/ml in 0.05 mol/l phosphate citrate buffer (pH 6.0) containing 0.3 mg/ml sodium perborate] was added to each well and the plate incubated for 10 min at room temperature. The reaction was stopped by the addition of 2.5 mol/l sulphuric acid and the absorbance of each well was measured at 450 nm using an Anthos HTIII automatic microplate reader. A standard curve of cell number versus absorbance was constructed for each batch of MNC and absolute adhesion calculated by reference to this curve using Biolise software (Labtech International, East Sussex, UK).

2.5. Platelet isolation

Whole blood anticoagulated with 1/10th volume of 0.013 mol/l trisodium citrate was centrifuged at 200 × g for 20 min and the upper platelet-rich layer collected. Apyrase (final concentration 10 μg/ml) and prostacyclin (final concentration 0.33 μg/ml) were added to the platelet-rich plasma to prevent premature platelet activa-
tion, followed by centrifugation at 800 × g for 15 min. The platelet pellet that resulted was resuspended in Ca²⁺-free Tyrodes buffer (10 mmol/l HEPES, 145 mmol/l NaCl, 2.7 mmol/l KCl, 1.8 mmol/l MgCl₂, 5.55 mmol/l glucose, 5.95 mmol/l NaHCO₃ and 0.42 mmol/l NaHPO₄, pH 7.4) containing bovine serum albumin (2 mg/ml) and the count adjusted to 1 × 10¹¹/l in Ca²⁺-free tyrodes buffer. Platelets were allowed to equilibrate at ambient temperature for 1 h prior to use.
2.6. Measurement of platelet adhesion

Platelet adhesion was measured according to the method of Bellavite et al. [15], which quantitates the number of platelets by measuring the activity of acid phosphatase, a platelet enzyme whose activity is stable independently of stimulation and is not released [15]. One hundred μl of the washed platelet suspension was added to plastic and collagen coated (20 μg/ml, overnight at 4°C) wells of a 96 well microtitre plate and incubated at 37°C for 1 h. Nonadherent platelets were removed by washing the plates twice with PBS. Subsequently 150 μl of 0.1 mol/l citrate buffer, pH 5.4, containing 0.1% (v/v) Triton-X-100 and 5 mmol/l p-nitrophenol phosphate was rapidly added to each well and the plate incubated for 1 h at room temperature. The reaction was then terminated by the addition of 50 μl of 4 mol/l NaOH and the absorbance of each well was measured at 450 nm using an Anthos HTIII automatic microplate reader (Labtech International, East Sussex, UK). A standard curve of cell number versus absorbance was constructed for each batch of platelets and absolute adhesion calculated by reference to this curve using Biolise software (Labtech International, East Sussex, UK).

2.7. Measurement of plasma soluble adhesion molecules

Fasted blood samples were collected into lithium heparin tubes (1.5 international units/ml) and centrifuged at 1500 × g for 10 min at 4°C. The plasma obtained was stored in the dark at −70°C. Plasma levels of sE-selectin, sL-selectin and sICAM-1 were determined by the use of commercial monoclonal antibody–based enzyme immunoabsorbent assay (ELISA) kits (R&D Systems, Abingdon, Oxfordshire, UK). Plasma levels of vWF were also determined using a commercial ELISA (Shield Diagnostics, Dundee, UK). All analyses were performed in duplicate.

2.8. Statistical analysis

Results are expressed as mean ± S.E.M. Differences between two means were compared using unpaired Student’s t-test. To evaluate correlations between variables Pearson’s r correlation test was performed. All calculations were performed on MINITAB issue 10 (State College, PA, USA). P < 0.05 was considered statistically significant.

3. Results

3.1. Baseline characteristics

Fifty six subjects (29 male) mean age 69 ± 5 years (range 64–80 years) underwent 24-h blood pressure monitoring, assessment of monocyte and platelet adhesion and measurement of plasma soluble ICAM-1, E-selectin, L-selectin and vWF. All subjects had blood glucose, urea and electrolytes within normal ranges. Selected clinical and biochemical characteristics of the hypertensive and normotensive subjects are summarised in Table 1. No significant differences were observed

<table>
<thead>
<tr>
<th>Hypertensive</th>
<th>Normotensive</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>70 ± 1</td>
<td>67 ± 1</td>
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<tr>
<td>M:F ratio</td>
<td>18:14</td>
<td>11:13</td>
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<tr>
<td>Mean total plasma cholesterol (mmol/l)</td>
<td>6.41 ± 0.27</td>
<td>6.08 ± 0.18</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27.35 ± 0.87</td>
<td>25.84 ± 0.67</td>
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</table>

Clinic blood pressure measurements

<table>
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<tr>
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<th>Normotensive</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinic SBP (mmHg)</td>
<td>153 ± 3</td>
<td>133 ± 6</td>
</tr>
<tr>
<td>Clinic DBP (mmHg)</td>
<td>90 ± 1.5</td>
<td>81 ± 3.5</td>
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<tr>
<td>Clinic pulse pressure (mmHg)</td>
<td>64 ± 2</td>
<td>51 ± 3</td>
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</tbody>
</table>

24-h blood pressure monitoring

<table>
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<th>P value</th>
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</thead>
<tbody>
<tr>
<td>24-h SBP (mmHg)</td>
<td>140 ± 1</td>
<td>118 ± 5</td>
</tr>
<tr>
<td>24-h DBP (mmHg)</td>
<td>79 ± 1</td>
<td>71 ± 3</td>
</tr>
<tr>
<td>Daytime SBP (mmHg)</td>
<td>147 ± 1</td>
<td>122 ± 5</td>
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<tr>
<td>Daytime DBP (mmHg)</td>
<td>84 ± 1</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>Night-time SBP (mmHg)</td>
<td>131 ± 2</td>
<td>111 ± 5</td>
</tr>
<tr>
<td>Night-time DBP (mmHg)</td>
<td>71 ± 1</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>24-h pulse pressure (mmHg)</td>
<td>62 ± 1</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>Daytime pulse pressure (mmHg)</td>
<td>63 ± 1</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>Night-time pulse pressure (mmHg)</td>
<td>60 ± 2</td>
<td>48 ± 2</td>
</tr>
</tbody>
</table>

* Values are expressed as mean ± S.E.M.
Table 2
Correlation of monocyte adhesion to collagen coated microwells with pulse pressure measured on ambulatory monitoring and clinic readings

<table>
<thead>
<tr>
<th></th>
<th>24-h PP</th>
<th>Day PP</th>
<th>Night PP</th>
<th>Clinic PP</th>
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</thead>
<tbody>
<tr>
<td>r</td>
<td>0.34</td>
<td>0.38</td>
<td>0.25</td>
<td>0.29</td>
</tr>
<tr>
<td>P</td>
<td>0.012</td>
<td>0.005</td>
<td>0.076</td>
<td>0.051</td>
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</table>

Fig. 1. Correlation between monocyte adhesion to collagen (%) and daytime pulse pressure (PP day) (mmHg); $r = 0.3796$, $P = 0.0051$.

between the two groups, except for systemic blood pressure (Table 1).

3.2. Monocyte adhesion

Univariate correlation coefficients of monocyte adhesion to collagen coated microwells with 24 h, daytime, night time and clinic pulse pressure are shown in Table 2. Monocyte adhesion to collagen coated microwells correlated significantly with daytime pulse pressure ($r = 0.39$, $P = 0.005$) (Fig. 1); on multiple linear regression analysis this relationship was independent of age, vitamin E, total, low density and high density cholesterol ($R^2 = 8\%$, $P = 0.016$). In contrast monocyte adhesion to tissue culture plastic microwells was not significantly correlated with daytime or clinic pulse pressure ($r = 0.21$, $P = 0.13$, $r = 0.24$, $P = 0.11$, respectively). Linear correlations of monocyte adhesion with blood pressure were not significant. However, subjects ($n = 32$) with daytime SBP $\geq 135$ mmHg, a level above which subjects can be defined as hypertensive [16,17] compared to those with lower SBP, had greater monocyte adhesion to collagen coated ($73 \pm 3.5$ vs. $61 \pm 3.7\%$, $P = 0.04$) and tissue culture plastic microwells ($79 \pm 2.1$ vs. $70 \pm 3.5\%$, $P = 0.04$) (Fig. 2).

3.3. Platelet adhesion

Platelet adhesion to collagen coated or tissue culture microwells was not significantly correlated with any of the ambulatory blood pressure, clinic blood pressure or pulse pressure measurements. Basal platelet adherence to collagen coated ($41.71 \pm 1.33$ vs. $46.30 \pm 2.66\%$, $P > 0.05$) or tissue culture plastic microwells ($34.10 \pm 1.82$ vs. $39.14 \pm 2.88\%$, $P > 0.05$) was not significantly different in hypertensive (daytime SBP $\geq 135$ mmHg) compared to normotensive subjects.

3.4. Soluble adhesion molecules

Circulating levels of soluble ICAM-1 ($389.49 \pm 20.21$ vs. $327.65 \pm 10.47$ ng/ml, $P < 0.01$) (Fig. 3) and soluble

![Fig. 3. Levels of soluble ICAM-1 in hypertensive (HT) and normotensive (NT) subjects. Data are expressed as means, with S.E.M. shown by vertical error bars. ** $P < 0.01$.](image1)

![Fig. 4. Levels of soluble E-selectin in hypertensive (HT) and normotensive (NT) subjects. Data are expressed as means, with S.E.M. shown by vertical error bars. * $P < 0.05$.](image2)
E-selectin (69.82 ± 7.14 vs. 47.96 ± 4.78 ng/ml, \( P < 0.05 \)) (Fig. 4) were significantly raised in the hypertensive (daytime SBP ≥ 135 mmHg) compared to normotensive subjects, while basal levels of vWF and soluble L-selectin were not significantly different between these two groups. A significant correlation existed between plasma levels of soluble E-selectin and clinic DBP (\( r = 0.396, \ P < 0.001 \); Fig. 5). The regression equation used to predict clinic DBP (\( r^2 = 0.157, \ P < 0.001 \)) was

\[
\text{E-selectin (ng/ml)} = 79.6 + 0.131 \text{ clinic DBP (mmHg)}
\]

No significant correlation existed between any of the parameters measured and soluble ICAM-1, soluble L-selectin and vWF.

4. Discussion

In the present study we found that mononuclear cell adhesion to collagen ex vivo was significantly greater in elderly hypertensive subjects compared to normotensive individuals. Whether this increased monocyte adhesion can be attributed to properties of the monocyte itself or is merely a reflection of increased activation of the endothelium remains to be conclusively determined. Increased monocyte adhesion in hypertension has been demonstrated in both in vitro studies [18,19] and in animal models [20,21]. It may be attributed to increased endothelial cell adhesion molecule expression including VCAM-1 and ICAM-1 [21,22]. Elevated monocyte chemotactic protein-1 (MCP-1) expression has also been demonstrated in the aortae of hypertensive rats [23] and in endothelial cells following cyclic strain [24]. Angiotensin II can also increase monocyte adhesion independent of endothelial adhesion molecule expression [19].

Pulse pressure, the difference between systolic and diastolic blood pressure, is strongly correlated with increased risk for cardiovascular disease [25,26]. With increasing age, hypertension is characterised by predominant elevation of systolic rather than diastolic pressure leading to a wide pulse pressure. A wide pulse pressure may reflect the increased peripheral vascular resistance, whereby large arteries become increasingly stiff and less compliant [25,27]. We have found that there is a significant correlation between monocyte adhesion to collagen under static conditions, and pulse pressure. Although this association was weak, the increased adhesiveness of monocytes with increasing pulse pressure may be another way in which pulse pressure contributes to the increased risk of cardiovascular disease. It also probably indicates that monocytes from the hypertensive subjects express higher levels of adhesion molecules. Although circulating monocytes are unlikely to come into contact with collagen in the absence of endothelial damage, our data has possible implications for atherosclerotic lesion expansion through enhanced monocyte binding to disrupted atherosclerotic plaques, or regions of denuded endothelium, for example following balloon angioplasty.

Co-existing coronary risk factors may alter MNC adhesiveness and confound for effects of hypertension. For example, monocytes isolated from patients with hypercholesterolaemia adhered to cultured human umbilical vein cells to a greater extent than those from subjects with normal cholesterol levels [28–30]. In the present study an attempt was made to control for possible confounding variables. Hypertensive and normotensive subjects were screened to exclude those with clinical evidence of vascular disease or diabetes mellitus, and those who smoked. Despite entering other possible confounding variables, including blood lipids, into the regression equation, blood pressure remained significantly correlated with monocyte adhesion to collagen coated microwells.

In contrast to the results for mononuclear cell adhesion, no significant association was found between platelet adhesion and either blood pressure or pulse pressure. Increased platelet activation has been reported in hypertensive subjects [5,6]. Despite this and the observation of Andrioli et al. [2] that hypertensives exhibited increased platelet adhesion compared to healthy normotensive controls, we failed to observe any significant differences in basal platelet adhesion between our hypertensive and normotensive subjects or any correlations with blood pressure.

In our study significantly higher levels of soluble ICAM-1 and E-selectin were observed in hypertensive compared to normotensive subjects, this is in accordance with Gearing et al. [31] and Blann et al. [32], who have also observed that hypertensive subjects exhibit increased plasma levels of soluble ICAM-1 and E-selectin respectively. A significant correlation was found between soluble E-selectin and clinic DBP, simi-
lar to that also observed by Blann et al. [32], who suggested that the increased levels of soluble E-selectin might indicate endothelial activation. A failure to demonstrate an increase in the levels of vWF the established marker of endothelial cell damage in hypertension, in our study and that of Blann et al. [32], may suggest that the endothelium is activated as opposed to damaged in hypertension, or that proposed activation resulting in elevated E-selectin is brought about by a different mechanism to that which results in vWF release.

Soluble adhesion molecules in plasma probably result from proteolytic cleavage from the cell surface. The mechanism by which levels of soluble adhesion molecules are increased is unknown, but their levels are increased in conditions in which expression on the cell membrane has been shown to be increased [33,34]. Therefore, it is possible that a raised blood pressure causes endothelial cell activation in vivo. This in turn results in increased adhesion molecule expression, which has been demonstrated in vivo in an animal model [21] and ex vivo upon cytokine stimulation [18,22].

It has been suggested that soluble adhesion molecules may influence leucocyte adhesion [35,36]; however, in this study we failed to observe any correlation between any of the soluble adhesion molecules and monocyte adhesion. If increased levels of soluble ICAM-1 and E-selectin truly represent increased expression of these molecules on the endothelium, this may result in increased monocyte adhesion in vivo. Adhesion and transmigration of monocytes do not necessarily correspond and once adhered to the endothelium, monocytes may become detached [12]. Although the detachment mechanism is unclear, perhaps this transient adhesion may result in activation of monocytes, which is reflected by increased monocyte adhesion ex vivo.

In summary, hypertension and especially increasing pulse pressure in elderly hypertensive subjects appears to have little effect on platelet adhesion but is associated with marked mononuclear cell adhesion, an early step in the formation and extension of atherosclerotic lesions. This association of pulse pressure with mononuclear cell activation may contribute to the progression of atherosclerotic lesions and increased atherothrombotic disease seen in older hypertensives.

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References


