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Leukocyte membrane bleb and pseudopod formation in hypertension

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Abstract

Leukocyte activation, including adhesion molecule expression, oxygen radical generation and, in animal studies, pseudopod formation, is a hallmark of hypertension. This study examined pseudopod and bleb formation and demonstrates that leukocytes from hypertensive individuals are more susceptible to produce membrane blebs than leukocytes from normotensive individuals. Bleb formation is likely indicative of apoptosis, thus this observation adds to previous observations of increased apoptosis in various tissues in hypertension.

Chronically elevated blood pressure (BP) is associated with a growing list of inflammatory markers at rest and with exaggerated inflammatory responses to acute stress.1,2 Animal models of hypertension show that in addition to other markers of leukocyte activation, pseudopod projections are upregulated alongside elevated BP. Pseudopods are temporary projections formed through F-actin polymerisation used by cells in phagocytosis and spreading and migration.3 Pseudopod formation may be important in BP control due to its contribution to vascular resistance in the microvasculature, especially in capillaries which demand single-file cell motion. Due to their relatively large size, leukocyte velocity is reduced in capillaries, and thus contributes to increase vascular viscosity and microvascular resistance.

In addition to pseudopod projections, leukocytes also project membrane blebs, typically in response to toxic stimuli, and their appearance precedes apoptosis.4 Blebs form when the cytoskeleton and membrane separate.5 Transient, spherical bleb membrane extensions are seen during mitosis or during spreading in eukaryotic cells, but much larger blebs which do not shrink are found during the early apoptotic process due to loss of membrane integrity. Physical and chemical stress have been shown to induce apoptotic blebbing, and recently

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neutrophils were observed to form large membrane blebs as a precursor to necrosis after exposure to cytotoxic factors.6

The current study investigated the effects of BP on the physical properties of circulating leukocytes at rest and in response to a moderate exercise challenge in otherwise healthy untreated individuals with a range of normal to elevated BP. We hypothesised that pseudopod formation would be greater in individuals with elevated BP, and that in response to exercise challenge pseudopod formation would be reduced in normal BP subjects but increased in subjects with elevated BP.

Twenty-two sedentary, healthy volunteers with normal or elevated BP (age 26–60 years, 9 female) gave written informed consent. The protocol was approved by the University of California, San Diego (UCSD) institutional review. All subjects underwent a history and physical examination by a physician and a normal electrocardiogram was confirmed. Subjects were grouped according to BP taken during a screening visit; Normal BP (N=9)<120/80 mmHg (mean±SD SBP=117.1±6.7mmHg, and DBP=70.2±5.3mmHg); Elevated BP (N=13)>120/80 mmHg (mean±SD SBP=142.5±8.2mmHg, and DBP=86.5±10.1mmHg). Subjects completed a VO_{2peak} treadmill test and, approximately 1 week after the peak exercise test, returned for a 20-min steady-state exercise task (65-70%VO_{2peak}). A blood sample was collected before (baseline) and during the last minute of exercise.

Blood was drawn into tubes containing Sodium Heparin, and immediately (within 30 seconds) 500μl was removed and added to 500μl 1% formaldehyde. After fixation for >10-min, red blood cells were lysed, and cells washed and collected by centrifugation. Cells were resuspended in 25μl PBS and stained with crystal violet for identification of nuclear morphology. Un-fixed, whole blood samples were incubated with PBS as the negative control and N-formyl-met-leu-phe (fMLP, Sigma) as the positive control (100ul for 5-min) and then fixed with formaldehyde and followed the same protocol. A light microscope was used to visualise the cells (100X) and photographs of 70 cells per sample were acquired using a 20X eyepiece camera (MiniVID, LW Scientific, Inc.).

Two blinded observers analyzed images of each cell for pseudopod and bleb formation. Membrane blebs were notable in a high proportion of samples. Cells were classified *positive for pseudopod formation* when projections were greater than 1μm in length. Cells were classified *positive for bleb formation* when projection was spherical and smooth and unstained by crystal violet (See Fig .1 insert). Cells observed were identified by morphology, >90% were neutrophils. Inter- and intra-observer variation in bleb and pseudopod classification was <4% and <2%, respectively. Positive controls showed 94-98% pseudopod formation, negative controls <4% pseudopod formation.

Data were analyzed by ANOVA and Pearson's correlations. Statistical significance was considered at $p<.05$, eta-squared (η^2) was determined as a measure of effect size. Apart from the expected differences in resting and exercise BP, individuals with normal BP were not different from those with elevated BP in age, BMI or VO_{2peak}, nor blood glucose or cholesterol. During 20-min exercise the two BP groups were not different for average HR,

VO_2, respiratory exchange ratio, or metabolic equivalents, indicating that all participants exercised at relatively same intensity as intended.

There was a trend toward greater pseudopod formation at post-exercise in cells from hypertensive participants (at pre-exercise Elevated BP=3.2±7%, Normal BP=2.3±5%, *p*=.75, at post-exercise Elevated BP=3.3±4%, Normal BP=0.7±1%, *p*=.07). Post-exercise pseudopod formation correlated with resting SBP (r=.429, *p*=.047) and resting DBP (r=.544, *p*=.009). At pre-exercise, subjects with elevated BP showed a significantly greater proportion of cells with bleb formation (Elevated BP=33.0±13%, Normal BP=13.5±8%, *p*=.001). At post-exercise, there was a trend toward greater bleb appearance in cells from elevated BP subjects (Elevated BP=35.2±20%, Normal BP=22.3±11%, *p*=.09). Figure 1 demonstrates the significant positive associations between proportion of cells with blebs at baseline with resting SBP (r=.623, *p*=.002) and resting DBP (r=.553, *p*=.008). Further, proportion of cells with blebs at post-exercise was also associated with both resting SBP (r=.471, *p*=.027), and resting DBP (r=.584, *p*=.004).

The current findings show that circulating leukocytes in individuals with elevated BP as well as normal BP exhibit low levels of pseudopod projection before and even after acute exercise (<4%). Activation of cells in the circulation rapidly leads to entrapment in capillaries as well as tethering and migration through the endothelium, and thus removal from the active peripheral circulation, which could lead to low levels of activation in the circulating pool of leukocytes drawn in whole blood. *In vivo* analysis in SHR rats has shown increased pseudopod formation in capillaries,3 but the circulating cells analysed in the current data from venous blood did not exhibit the same effect.

The fixation protocol using formaldehyde resulted in prominent bleb formation on leukocytes. The blebs observed were large in size, and given the peripheral circulation origin of the cells, unlikely to be formed in locomotion. The fixation protocol thus may have initiated apoptosis while fixing the cells, capturing the bleb formation. Alternatively, it may reflect a weaker binding of the membrane to the underlying cytoskeleton, potentially reflective of pre-existing cellular damage. Notably, cells from subjects with elevated BP were more susceptible to bleb formation after fixation than cells from subjects with normal BP. These findings are consistent with previous animal studies, where hypertension has been associated with enhanced apoptosis in several different cell types in the SHR,7 including the microvascular endothelium as well as in high-pressure regions of the vasculature. In human hypertension, circulating endothelial cells, have been noted8 (circulating endothelial cells have been found to be up to 90% apoptotic in other diseases), and apoptosis is increased in endothelial cells in atherosclerosis.9,10

Several limitations of this study need to be acknowledged. Firstly, our sample size was modest; however, the difference between groups was confirmed by the continuous data analysis, confirming the finding. Secondly, the methodology wasn't specifically designed to induce bleb formation. However, formaldehyde has previously been found to increase the intracellular concentration of Ca^{2+} and induce apoptosis,11 and Ca^{2+} homeostasis disturbance has been suggested as a major factor responsible for bleb formation.12

The current study demonstrates that leukocytes from hypertensive individuals are more susceptible to membrane bleb formation, indicative of apoptosis, than leukocytes from normotensive individuals. The association with blood pressure was continuous: if split to Normotensive (SBP<120mmHg), Pre-hypertensive (120mmHg<SBP>140mmHg) and Hypertensive (SBP>140mmHg), bleb formation showed graded increases of 13%, 27%, and 32%, indicating that future studies might explore the susceptibility of cells to bleb formation as an early marker of hypertension. Regarding pseudopod formation, future studies could examine *in vitro* cell activation stimulating naïve cells with plasma from subjects with elevated or normal BP, to avoid loss of activated leukocytes due to entrapment in the microcirculation.

Acknowledgments

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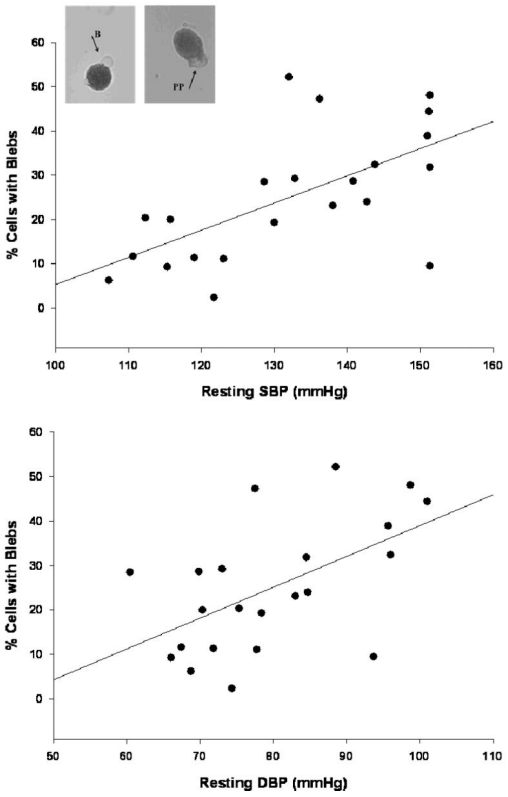

Figure 1.

Association between resting blood pressure (SBP upper panel, DBP lower panel) and % cells showing bleb formation at pre-exercise. *Insert; pseudopod(PP) and bleb(B) morphology.*

Summary Table

What is known about this topic

- Hypertension is associated with many different markers of elevated inflammation including pro-inflammatory cytokines, reactive oxygen species and markers of leukocyte activation.
- Leukocytes from spontaneously hypertensive rats (SHR) have been shown to exhibit increased pseudopod extension, which is hypothesized to contribute to microvascular resistance and thus elevated blood pressure.
- SHR models show increased apoptosis in various cell types.

What this study adds

- We demonstrate that leukocytes from hypertensive individuals are more susceptible to form membrane blebs, indicative of apoptosis, than leukocytes from normotensive individuals.
- Bleb formation was strongly associated with resting blood pressure.