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Characterization of blood borne microparticles as markers of premature coronary calcification in newly menopausal women

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Departments of 1Surgery and Physiology and Biomedical Engineering, 2Biochemistry and Molecular Biology and Hematology, 3Internal Medicine, Divisions of Hematology, 4Cardiovascular Diseases, and 5Radiology, Mayo Clinic College of Medicine, Rochester, Minnesota; 6Los Angeles Biomedical Research Institute at Harbor University of California at Los Angeles, Torrance, California; and the 7Kronos Longevity Research Institute, Phoenix, Arizona

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Jayachandran M, Litwiller RD, Owen WG, Heit JA, Behrenbeck T, Mulvagh SL, Araoz PA, Budoff MJ, Harman SM, Miller VM. Characterization of blood borne microparticles as markers of premature coronary calcification in newly menopausal women. Am J Physiol Heart Circ Physiol 295: H931–H938, 2008.—While the risk for symptomatic atherosclerotic disease increases after menopause, currently recognized risk factors do not identify ongoing disease processes in low-risk women. This study tested the hypothesis that circulating cell-derived microparticles may reflect disease processes in women defined as low risk by the Framingham risk score. The concentration and phenotype of circulating microparticles were evaluated in a cross-sectional study of apparently healthy menopausal women, screened for enrollment into the Kronos Early Estrogen Prevention Study. Microparticles were evaluated by flow cytometry, and coronary artery calcification (CAC) was scored using 64-slice computed tomography scanners. The procoagulant activity of isolated microparticles was determined with a sensitive fluorescent thrombin generation assay. Chronological age, body mass index, serum lipids, systolic blood pressure (Framingham risk score) and hematocrit, used to calculate the Framingham risk score. The concentration and phenotype of circulating microparticles have been described in apparently healthy menopausal women, screened for enrollment into the Kronos Early Estrogen Prevention Study. Microparticles were evaluated by flow cytometry, and coronary artery calcification (CAC) was scored using 64-slice computed tomography scanners. The procoagulant activity of isolated microparticles was determined with a sensitive fluorescent thrombin generation assay. Chronic age, body mass index, serum lipids, systolic blood pressure (Framingham risk score), and smoking status, used to calculate the Framingham risk score (FRS), do not predict risk in many women (14, 25, 28, 39, 40). Therefore, an alternative approach that takes advantage of these cellular interactions may provide a way to link vascular pathophysiology and thrombosis and to stratify risk early in disease processes.

Cardiovascular disease increases in women after menopause, but standard markers of risk such as hypertension, circulating lipids, and smoking status, used to calculate the Framingham risk score (FRS), do not predict risk in many women (14, 25, 26, 41). Furthermore, the concentrations of circulating proteins and peptides to assess risk provide little insight into intravascular processes leading to the formation of an arterial lesion, since biochemical markers turn over rapidly and their source usually cannot be identified (5, 38, 39). Alternatively, circulating platelets and leukocytes have defined lifetimes and bear marks of interactions with each other or the vascular wall (20, 28, 39, 40). Therefore, an alternative approach that takes advantage of these cellular interactions may provide a way to link vascular pathophysiology and thrombosis and to stratify risk early in disease processes.

METHODS

Subjects. This was a cross-sectional study of a subset (n = 33) of apparently healthy, newly menopausal women (between 6 mo and 3 yr from their last menses; chronological age 42–58 yr) screened to...
participate \((n = 146)\) in the Kronos Early Estrogen Prevention Study (KEEPS; NCT000154180) at Mayo Clinic (Rochester, MN) \((18)\). KEEPS is a randomized, double-blind trial designed to test the hypothesis that menopausal hormone therapy started early in menopause will reduce the progress of atherosclerotic disease as defined by the progression of carotid intimal-medial thickness and coronary calcification. Because one exclusion criterion for the study is a coronary artery calcification (CAC) score of \(>50\) Agatston units (AU), all women undergo a coronary calcium scan at screening. Of the 146 women screened at Mayo, 5 had CAC \(> 50\) AU (range, 93–315 AU), 18 women had CAC between 0 and 50 AU (range, 0.3–32 AU), and 123 women had a score of 0 AU. For this ancillary study, all women with a CAC score \(\leq 0\) were included, and 10 women were randomly selected from the 123 women with a CAC score \(= 0\). Among the 33 women included in this study, none was a current smoker, hypertensive, diabetic, or had a history of thrombotic disease. This study was approved by the parent KEEPS Ancillary Studies Committee and the Institutional Review Board at Mayo Clinic. All participants gave written informed consent. Because these tests were performed on individuals being screened for KEEPS, none was on active medication. However, some had endogenous estradiol levels that made them ineligible for randomization into KEEPS but eligible for this ancillary study.

Antibodies and other reagents. Recombinant annexin-V and mouse anti-human cell surface marker antibodies conjugated with fluorescein isothiocyanate or R-phycoerythrin and TruCOUNT beads were obtained from BD Biosciences (San Jose, CA). Amine-modified polystyrene fluorescent yellow-green latex beads (1 and 2 \(\mu m\)) were from Sigma-Aldrich (St. Louis, MO). Human factor Xa, factor Va, and prothrombin were obtained from Haematologic Technologies (Essex Junction, VT). All other reagents were analytical grade.

Coronary calcification. CAC images were obtained using a 64 detector computed tomography scanner (Siemens Sensation 64, Siemens Medical Solutions, Forchheim, Germany) with a scan configured to cover the heart. Scans were gated to the cardiac cycle and obtained in end inspiration using the following parameters: rotation time \(= 0.33\) s and collimation \(= 24 \times 1.2\), with images reconstructed at 3-mm-thick slices; pitch \(= 0.2\), peak kilovoltage \(= 120\); and field of view \(= 30\); milliamphere-second (mA-s) was varied according to width of the patient on the scout image as measured across the liver. Subjects who measured \(\leq 32\) cm had mA-s \(= 126\). Subjects who measured 32–38 cm had mA-s \(= 385\). Subjects who measured \(>38\) cm had mA-s \(= 780\). Images were reconstructed with a B35 kernel at 65% of the R-R interval.

The calcium score was calculated using commercially available semiautomated software (GE Smartscore, GE Healthcare, Milwaukee,

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**Fig. 1.** Representative scatter plot obtained by FACSCanto flow cytometry. A: control gates of buffer with fluorescein-conjugated antibodies and calibration (size and TruCount beads) beads in the absence of sample. B: gates derived from adding a sample containing microparticles to the buffer with fluorescein-conjugated antibodies and calibration beads. Note that the gate to define the area of interest (P1) was set above the background noise of the machine. C and D: representative quadrants (Q) derived from the microparticle gates shown in A and B, respectively. Counts are separated by antibody binding with Q3 representing microparticles. FL, fluorescence. E and F: representative scanning (E) and transmission (F) electron microscopy of the isolated microparticles. Arrow heads, membranes. G: microparticles imaged by CytoViva dual fluorescence and optical microscopy at \(\times 150\). H: fluorescent beads (1 \(\mu m\) in diameter) imaged by the CytoViva cell imaging system at \(\times 150\).
Table 1. Characteristics of women participating in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Negative</th>
<th>Low (0 &lt; 50)</th>
<th>High (≥50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>10</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Menopause, mon</td>
<td>53.1 ± 0.3</td>
<td>53.1 ± 0.4</td>
<td>54.8 ± 1.2</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>7.2 ± 3.3</td>
<td>75.6 ± 2.7</td>
<td>78.7 ± 7.3</td>
</tr>
<tr>
<td>Basal Mean coronary calcium, Agatston units</td>
<td>0.0 ± 0.1</td>
<td>8.4 ± 2.3</td>
<td>151.2 ± 41.3</td>
</tr>
<tr>
<td>Mean coronary calcium (vol)</td>
<td>0.0 ± 0.1</td>
<td>9.0 ± 1.8</td>
<td>133.3 ± 30.8</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>208.5 ± 11.3</td>
<td>213.2 ± 9.5</td>
<td>213.0 ± 12.6</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>125.8 ± 10.8</td>
<td>133.8 ± 6.9</td>
<td>120.6 ± 10.8</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>62.8 ± 3.6</td>
<td>57.3 ± 4.4</td>
<td>59.0 ± 8.0</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>74.2 ± 11.9</td>
<td>114.3 ± 3.6</td>
<td>84.8 ± 38.3</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>91.0 ± 1.7</td>
<td>93.8 ± 2.3</td>
<td>102.4 ± 4.4</td>
</tr>
<tr>
<td>Follie-stimulating hormone, mU/l</td>
<td>83.4 ± 11.2</td>
<td>89.9 ± 9.5</td>
<td>74.2 ± 14.0</td>
</tr>
<tr>
<td>17β-estradiol, pg/ml</td>
<td>24.4 ± 2.5</td>
<td>26.3 ± 5.0</td>
<td>49.6 ± 15.6</td>
</tr>
<tr>
<td>Thyroid-stimulating hormone, mU/ml</td>
<td>2.2 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>4.5 ± 1.3*†</td>
</tr>
<tr>
<td>High-sensitive C-reactive protein, mg/l</td>
<td>2.0 ± 0.7</td>
<td>2.0 ± 0.4</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Framingham risk score, %</td>
<td>1.1 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>1.8 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of women. CAC, coronary arterial calcium. Framingham risk score was calculated using the National Cholesterol Education Program, Adult Treatment Panel III. *Statistically significant difference from women with Agatston scores of 0 < 50. †Statistically significant difference from women with Agatston scores of 0.

WI) according to the Agatston method (1). Calcified plaque within each epicardial vessel was detected by four contiguous pixels and a peak density >130 Hounsfield units (HU). The product of the area and the respective density factor (1 = 130 to 199 HU; 2 = 200 to 299 HU; 3 = 300 to 399 HU; and 4 if ≥400) constituted the vessel-specific coronary artery calcium score. The calcium scores across each individual epicardial vessel were summed to determine the total coronary artery calcium score.

Blood sample collection. Venous blood samples were collected for measurements of lipids, hormones, and high-sensitivity C-reactive protein (hs-CRP), chemistries, and platelet functions. The anticoagulant used for each assay was dictated by the requirement of that assay. For microparticle analyses, blood (4 ml) was collected through a 19-gauge butterfly needle into a tube containing 0.5 ml of 0.11 M Hanks’ solution with 0.05% glucose and buffered by 10.220.33.5 on October 15, 2017 http://ajpheart.physiology.org/ Downloaded from

H933 MICROPARTICLES AND CORONARY CALCIFICATION

Table 3. Platelet characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Negative</th>
<th>Low (0 &lt; 50)</th>
<th>High (≥50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (×10^9/l)</td>
<td>226 ± 18</td>
<td>245 ± 15</td>
<td>250 ± 7</td>
</tr>
<tr>
<td>Platelet P-selectin expression (% positive)</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Basal Fibrinogen receptor expression (% positive)</td>
<td>91.0 ± 1.1*</td>
<td>92.0 ± 0.6*</td>
<td>91.0 ± 2.1*</td>
</tr>
<tr>
<td>TRAP activated</td>
<td>1.4 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Basal TRAP activated</td>
<td>16.0 ± 2.9*</td>
<td>18.0 ± 2.8*</td>
<td>11.0 ± 2.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of women. TRAP, thrombin receptor agonist peptide. *P < 0.05, significant increase over basal expression.

Blood chemistry. Total cholesterol, low (LDL) and high (HDL) density cholesterol, triglycerides, blood glucose, follice-stimulating hormone, 17β-estradiol, thyroid-stimulating hormone, hs-CRP, sodium, potassium, chloride, bicarbonate, creatinine, phosphorus, total protein, albumin, bilirubin, alkaline phosphatase, aspartate, alanine transaminases, and urea were measured by Kronos Science Laboratories (Phoenix, AZ) and the Mayo Clinic Clinical Laboratories (Rochester, MN). Total white blood cells, differential leucocytes, hemoglobin, and hematocrit were also determined by Mayo Clinic Hematopathology Laboratories. Platelet counts were measured with a Coulter counter T660.

Isolation of blood microparticles. Blood samples were centrifuged at 3,000 g for 15 min within 30 min of phlebotomy. The plasma was frozen at −40°C. Frozen samples were thawed in a 37°C water bath for 5 min, vortexed, and then centrifuged at 3,000 g for 15 min. In preliminary experiments, it was determined that the recovery of microparticles was the same from fresh compared with plasma that had undergone a single-freeze thaw cycle. After centrifugation, samples were evaluated with the Coulter counter for the presence of other cells. This step validated that platelet counts of ≤1 and other cells were absent from the sample. Thus it is improbable that microparticles could be generated from cells remaining in the plasma during the subsequent isolation process. After the validation step, the plasma sample (0.5 ml) was then centrifuged at 20,000 g for 30 min, which recovers >90% of microparticles (data not shown). Supernatants were removed, and the remaining pellets (microparticles) were reconstituted with 0.5 ml Hanks’ solution with 0.05% glucose and buffered (pH 7.4) with 20 mM HEPES. Tubes containing reconstituted microparticles were vortexed and centrifuged again at 20,000 g for 30 min. After centrifugation, the pellets containing microparticles were reconstituted again (0.5 ml) and vortexed for 1 to 2 min before analysis. All buffers were filtered twice through a 0.2-μm membrane filter before use.

Microscopic observation of isolated microparticles. For scanning electron microscopy, a small drop of isolated microparticles was added to a parlodian carbon-coated grid and allowed to sit for 10 min. A small drop of 1% phosphotungstic acid (pH 7.2) was added to the grid, and, after, air drying was examined by scanning electron microscopy. For transmission electron microscopy, isolated microparticles were fixed and embedded in quetol resin by standard methods (31). Ultrathin (70–90 nm) sections were cut with a diamond knife, stained with lead citrate, and examined with a transmission electron microscope (FEI Technai-12 TEM, Hillsboro, Oregon). Freshly isolated microparticles were also observed using dual mode fluorescence and optical microscopy (CytoViva, Auburn, AL).

Identification of isolated microparticles. Flow cytometry (FACSCant, BD Biosciences) was used to define microparticles by size and positive fluorescence using marker-specific antibodies. The gates to define size
were set using an internal standard of 1- and 2-
from an individual woman.

Fig. 2. Analytical variability in total numbers of microparticles between 2
aliquots from the same sample incubated with four different
antibody, the total numbers of microparticles were measured in
of the total numbers of microparticles were affected by a specific
no annexin-V-positive events.

particles in phosphate-buffered saline without calcium, which yielded
positive (or negative) microparticles in each quadrant.

absolute number of annexin-V-positive or other cell specific marker-
test volume (see Fig. 1). The same calculation applies to determine the
numbers of the microparticles were calculated from the number of
V-PE, which gave congruent counts (data not shown). Absolute
validate specificity, the annexin-V assay was repeated with annexin-
tidylserine was measured with the use of annexin-V-FL. Stained
flow cytometry.

the TruCOUNT beads were added immediately before analysis by

limit set above the background noise level of the machine (Fig. 1).
A calibrated quantity of TruCOUNT beads of 4.2 µm diameter were
added to samples to enable the calculation of absolute particle counts
(Fig. 1). All buffers and antibodies were filtered twice through a
0.2-µm filter to eliminate chemical particles and reduce instrument
noise. Isolated microparticles (50 µl) were incubated with 4 µl of
specified antibodies conjugated to fluorescein (FL) and phycoerythrin
(PE) for 30 min. After incubation, the microparticles were fixed with
400 µl of 1% paraformaldehyde for 15 min. After fixation, 50 µl of the
TruCOUNT beads were added immediately before analysis by flow cytometry.

Annexin-V binding as a measure of microparticle surface phosphatidylserine was measured with the use of annexin-V-FL. Stained
microparticles were separated by quadrant (Q1–Q4, cf. Fig. 1). To validate specificity, the annexin-V assay was repeated with annexin-
V-PE, which gave congruent counts (data not shown). Absolute
numbers of the microparticles were calculated from the number of
events in the region containing microparticles divided by the number
of events in the calibration bead region times the number of beads per
test volume (see Fig. 1). The same calculation applies to determine the
absolute number of annexin-V-positive or other cell specific marker-
positive (or negative) microparticles in each quadrant.

Nonspecific annexin-V labeling was evaluated by preparing micro-
particles in phosphate-buffered saline without calcium, which yielded
no annexin-V-positive events.

To determine intra-assay variability and whether measurements of
the total numbers of microparticles were affected by a specific
antibody, the total numbers of microparticles were measured in
aliquots from the same sample incubated with four different antibodies.

Source (cells of origin) of microparticles. Platelet-derived micro-
particles expressing phosphatidylserine were identified with mouse
anti-human integrin-β1 (CD61) and glycoprotein IX (CD42a-PE). Granulocyte-, monocyte-, and endothelium-derived microparticles
and phosphatidylserine expression were identified with, respectively,
mouse anti-human CD11b-PE, CD14-PE, CD62-E-PE antibodies, and
annexin-V-FL. Microparticles expressing the cellular adhesion mole-
cules intercellular adhesion molecule-1 (ICAM-1) and vascular cell
adhesion molecule-1 (VCAM-1) were identified by ICAM-1-PE and
VCAM-1-PE antibodies, respectively.

Procoagulant activity of microparticles. A prothrombinase activity
assay was used to measure the phospholipid procoagulant activity of
the microparticles. For this assay, defined numbers (10,000) of mi-
croparticles from each individual were incubated in Tyrode buffer
containing (final concentrations in 100 µl) 5 nM Factor Xa and 10 nM
Factor Va at 37°C for 3 min. Then, 2 µM prothrombin and 50 µM
Asp-(Val-Pro-Arg)-6-amino-1-naphthalene-sulfonamide (final concen-
trations in 120 µl) were added, and changes in fluorescence
(thrombin activity) were measured immediately with a Thermo
Electron FluoroScan fluorometer (excitation = 355 nm; emission = 445 nm).

Statistics. Data are presented as means ± SE. One-way analysis of
variance followed by Bonferroni’s multiple comparison test was used
to determine statistical differences in numbers of annexin-V-positive
microparticles among groups of women with negative, low and high
CAC scores. Spearman’s rank correlation coefficient was used to
determine the correlation of endothelium-derived microparticles (Q1 +
Q2) with CAC scores. Bartlett’s test was used to identify analytical
variability in thrombin generating capacity of microparticles among
groups. A repeated measures analysis of variance was performed on
the natural log of the thrombin generating capacity. Statistically
significance was accepted at P < 0.05.

RESULTS

Characteristics of the study population. Women were grouped
according to their CAC scores as negative (no calcification), low (0 < 50; range, 0.3–32 AU) or high (>50, range, 93–315 AU). There were no significant differences among groups in

Fig. 3. Representative quadrants derived from flow cytometric scatter plots
(top) demonstrating annexin-V-negative (Q3) and -positive (Q4) microparticles. Bottom; cumulative data for numbers of total (annexin-V negative, Q3,
white bars, plus annexin-V positive, Q4, black bars) microparticles from
women with negative (0 Agatston units, n = 10 women) and positive coronary
calcification scores (low, <50 Agatston units, n = 18 women; and high, >50
Agatston units, n = 5 women). Data are presented as means ± SE. *P < 0.05,
statistical significance from those with zero calcium and low calcium scores.
FL1, green fluorescence; FL2, red fluorescence.
age, months past menopause, body weight, body mass index (BMI), lipid profile, follicle stimulating hormone, 17β estradiol, and CRP concentrations (Table 1). The concentrations of thyroid-stimulating hormone were significantly higher in women with high calcium scores compared with the other groups (Table 1). Serum chemistries (data not shown), total or differential leukocytes, hemoglobin, hematocrits, and platelet counts all were within normal range and did not differ among the groups (Tables 2 and 3).

Validation of microparticle isolation and assay. Basal platelets were <5% P-selectin and fibrinogen receptor positive and showed expected positivity after the stimulation with thrombin receptor agonist peptide (Table 3). The number of events measured by flow cytometry from filtered buffers and antibodies ranged between 100 and 250 events for a 3-min run (Fig. 1, A and C). More than 25,000 events were counted in the same buffer containing microparticles (Fig. 1, B and D). Isolated microparticles were heterogeneous in size and <1 μm in diameter (Fig. 1, G and H).

Total numbers of microparticles (Q1 + Q2+ Q3 + Q4) detected by flow cytometry varied among individuals (Fig. 2). However, the correlation between numbers of microparticles prepared in duplicated samples from single individuals and then analyzed with two different antibodies approached unity (Fig. 2). The coefficient of variation calculated from single samples measured with four different antibodies was 0.095.

Microparticles and CAC. The total numbers of microparticles increased with the CAC score (Fig. 3), and women with the highest calcium scores had significantly more microparticles positive for annexin-V than women in the other two groups. Annexin-V positive microparticles in women with high CAC scores were identified as being of platelet origin by positive binding to two different platelet-specific antibodies (Fig. 4), anti-CD61 (integrin-β3) and anti-CD42a (glycoprotein IX). Endothelium-derived (CD62-E positive) microparticles also were more abundant in women with high calcium scores (Fig. 5). In the 23 women with positive CAC scores, endothelium-derived microparticles were correlated to the Agatston score (Spearman’s rank coefficient = 0.57, P = 0.005). Leukocyte (CD11b positive)- and monocyte (CD14 positive)-derived microparticles and those positive for ICAM-1 and VCAM-1 did not differ among groups (Table 4).

There was large and significant variability in in vitro thrombin generation by microparticles containing large numbers of annexin-V-positive microparticles, i.e., those from women with high CAC (Fig. 6). Thrombin-generating capacity (compared by repeated-measures test of the log transformation of individual data) was statistically greater in the high CAC group compared with negative and low CAC groups (P < 0.03).

Microparticles and FRS. FRSSs (10 yr) calculated using the National Cholesterol Education Program (16a) ranged ≤1–3% and so placed these women in a low risk group. The mean FRS did not differ among groups based on CAC scores (Table 1). Within this range of FRS, the total numbers of microparticles, those positive for annexin-V and percentages of platelet and endothelium-derived microparticles, were significantly higher in women with FRS of 2 to 3 compared with those with scores of ≤1. Granulocyte- and monocyte-derived microparticles and those positive for ICAM-1 and VCAM-1 did not correlate with FRS (Table 5).
Agatston units, screened for KEEPS and positive for CAC is half that reported risk for cardiovascular disease. The percentage of women other blood parameters would be considered otherwise at low plasma lipids, the body mass index, CRP, triglycerides, or who are within three years of menopause, and on the basis of Table 4.

Leukocyte marker (CD11b and CD14)-, ICAM-1-, and VCAM-1-positive microparticles from women in whom CAC DISCUSSION

The present study documents CAC in about 16% of women who are within three years of menopause, and on the basis of plasma lipids, the body mass index, CRP, triglycerides, or other blood parameters would be considered otherwise at low risk for cardiovascular disease. The percentage of women screened for KEEPS and positive for CAC is half that reported in women of comparable chronological age from the estrogen-only arm of the Women’s Health Initiative. However, those women had undergone hysterectomies 10–20 yr before the CAC measurements, and thus were between 5–10 years beyond menopause (30).

An important novel finding of the present study is that both the total numbers of microparticles and those defined with a procoagulant marker (annexin-V binding to phosphatidylserine) were highest in women with CAC scores of >50 AU. We verified that the flow cytometric measure of annexin-V binding recapitulated procoagulant activity. Furthermore, procoagulant microparticles were both of platelet and endothelial origin. Endothelium-derived microparticles identified by E-selectin (CD62-E) (35) and a marker of possible endothelial dysfunction (21) were about tenfold higher in plasma of women with CAC. While this marker may not account for all endothelium-derived microparticles, it reflects those released during perturbation of the endothelium by inflammatory processes (13), perhaps related to the calcification process, rather than those released because of endothelial cell apoptosis (24).

Microparticles of endothelial origin have been identified also by CD31+/CD41- and CD31+/CD42- staining (2, 3, 11, 19, 21, 24, 37). However, those methods identify endothelial microparticles by elimination and are not specific, since CD31 may be present on microparticles derived from leukocytes, and do not differentiate microparticles derived from apoptotic compared with activated cells (36, 44). Therefore, in the present study, microparticles expressing CD62-E unambiguously re-

Fig. 6. Prothrombinase assay of thrombin generation by microparticles (10,000) derived from women with negative (0 Agatston units, n = 10 women; *) and positive coronary calcification scores (low, 0 < 50 Agatston units, n = 18 women; □, and high, >50 Agatston units; n = 5 women; ●). Control for buffer with factors but no microparticles (○; n = 2) is shown. Data are presented as means ± SE. *P < 0.03, significant variability by Bartlett’s test and thrombin-generating capacity by log transformation in the high compared with low and zero CAC groups.

Table 4. Leukocyte marker (CD11b and CD14)-, ICAM-1-, and VCAM-1-positive microparticles from women in whom CAC scores are known

<table>
<thead>
<tr>
<th>CAC Scores, Agatston units</th>
<th>CD11b</th>
<th>CD11b + annexin-V</th>
<th>CD14</th>
<th>CD14 + annexin-V</th>
<th>ICAM-1</th>
<th>ICAM-1 + annexin-V</th>
<th>VCAM-1</th>
<th>VCAM-1 + annexin-V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>10</td>
<td>1.6±0.8</td>
<td>7.3±3.2</td>
<td>0.1±0.0</td>
<td>0.8±0.2</td>
<td>3.9±1.5</td>
<td>11.9±5.5</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Low (0 &lt; 50)</td>
<td>18</td>
<td>1.2±0.5</td>
<td>3.3±1.1</td>
<td>0.0±0.0</td>
<td>0.7±0.2</td>
<td>4.1±0.9</td>
<td>14.9±3.6</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>High (&gt; 50)</td>
<td>5</td>
<td>1.7±1.0</td>
<td>7.9±3.5</td>
<td>0.0±0.0</td>
<td>1.2±0.6</td>
<td>3.6±1.1</td>
<td>21.1±6.8</td>
<td>0.1±0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE (n, number of women) of the percentage of total microparticles/µL plasma that expressed the marker.
of women with early calcification and in whom follow-up screening or additional testing might be warranted. The association of coronary calcification with elevation in thyroid-stimulating hormone warrants further evaluation in larger groups of women. The procoagulant nature of microparticles derived from women with the highest levels of calcification may identify women with increased propensity for thrombosis at sites of vascular lesions and may increase the specificity for risk stratification in women, especially those who are not smokers (see Table 5).

This study has some limitations. The number of individuals screened was 146, and as expected in this age group of women, the incidence of CAC was low. However, despite this low incidence, statistical significance prevailed. Clearly, these measurements need to be extended to larger and broader populations, including middle-aged men without elevated lipids and in older populations in whom coronary calcification is expected.

GRANTS

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