Membrane Vesicles Nucleate Mineralo-organic Nanoparticles and Induce Carbonate Apatite Precipitation in Human Body Fluids*

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**Significance:** Membrane vesicles (MVs) released from various cells are associated with human diseases.

**Results:** MVs isolated from human serum induce the formation of mineralo-organic nanoparticles in culture.

**Conclusion:** MVs represent a nucleating factor that promotes the formation of mineralo-organic nanoparticles and the precipitation of mineral deposits in body fluids.

**Significance:** The ectopic precipitation of carbonate apatite deposits in body fluids and tissues may be initiated in part by MVs.

Recent studies indicate that membrane vesicles (MVs) secreted by various cells are associated with human diseases, including arthritis, atherosclerosis, cancer, and chronic kidney disease. The possibility that MVs may induce the formation of mineralo-organic nanoparticles (NPs) and ectopic calcification has not been investigated so far. Here, we isolated MVs ranging in size between 20 and 400 nm from human serum and FBS using ultracentrifugation and sucrose gradient centrifugation. The MV preparations consisted of phospholipid-bound vesicles containing the serum proteins albumin, fetuin-A, and apolipoprotein A1; the mineralization-associated enzyme alkaline phosphatase; and the exosome proteins TNFR1 and CD63. Notably, we observed that MVs induced mineral precipitation following inoculation and incubation in cell culture medium. The mineral precipitates consisted of round, mineralo-organic NPs containing carbonate hydroxyapatite, similar to previous descriptions of the so-called nanobacteria. Annexin V-immunogold staining revealed that the calcium-binding lipid phosphatidylserine (PS) was exposed on the external surface of serum MVs. Treatment of MVs with an anti-PS antibody significantly decreased their mineral seeding activity, suggesting that PS may provide nucleating sites for calcium phosphate deposition on the vesicles. These results indicate that MVs may represent nucleating agents that induce the formation of mineral NPs in body fluids. Given that mineralo-organic NPs represent precursors of calcification in vivo, our results suggest that MVs may initiate ectopic calcification in the human body.

Recent studies indicate that various NPs present in the environment constantly interact with the human body and may be associated with disease conditions. For instance, the inhalation of asbestos nanofibers derived from fireproof materials is associated with the development of lung cancer and fibrotic lung damage known as asbestosis (1). Similarly, titanium dioxide NPs, which are widely used as whitening agents in commercial products, induce acute toxicity to various organs in laboratory animals (2). Given that NPs show unexpected size-dependent properties and increased access to various body compartments, the potential toxicity of such nanomaterials represents an important safety concern and is now a major research focus in the fields of nanomedicine and nanotoxicology (3).

Mineralized NPs called NB were initially described as the smallest living microorganisms on earth (4) and as the possible causative agent of various human diseases, including Alzheimer disease, atherosclerosis, cancer, chronic kidney disease, kidney stones, and prostatitis (5–7). However, recent studies performed by us (8–17) and others (18–22) have shown that the so-called NB actually represent non-living mineralo-organic NPs that possess biomimetic properties, including the ability to increase in size and number in culture. Although the hypothesis that NB represent living microorganisms has been discarded, several studies have indicated that mineral NPs similar to NB do form in the growing bones and teeth of vertebrates (23–25). Mineralo-organic NPs have also been observed in undesired, ectopic calcification associated with various human and animal conditions (26–30). As such, these mineral NPs may represent calcification precursors found in both physiological and pathological mineralization processes.

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3 The abbreviations used are: NP, nanoparticle; ALP, alkaline phosphatase; apoA-I, apolipoprotein AI; CPP, calciprotein particle; DIR, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide; EDX, energy-dispersive x-ray; HAP, hydroxyapatite; HS, human serum; MV, membrane vesicle; NB, nanobacteria; PS, phosphatidylserine; TEM, transmission electron microscopy; VSMC, vascular smooth muscle cell.
Membrane Vesicles Nucleate Mineralo-organic Nanoparticles

Human body fluids have been shown to contain membrane vesicles (MVs) in the form of small lipid-bound vesicles released from various cells (31). These vesicles have been broadly categorized as exosomes, microvesicles, and apoptotic bodies (32). Exosomes represent 30–100-nm vesicles released from cellular endosomes and are thought to be involved in cell-to-cell signaling (32–34). Microvesicles are a more heterogeneous group of 100–1,000 nm vesicles released from the plasma membrane of various cells; they have been implicated in intercellular communication, homeostasis, and cellular waste disposal (32, 35). Apoptotic bodies represent particles ranging from 50 nm to 5 μm, which are released by apoptotic cells and removed through phagocytosis by macrophages (32, 34). Given that the level of MVs is elevated in the body fluids of humans afflicted with various ailments, some authors have proposed that these vesicles may play a role in disease conditions that include arthritis, atherosclerosis, cancer, and chronic kidney disease (31, 36, 37). On the other hand, it remains unclear whether MVs represent a consequence of the disease process or whether they play an active role in pathogenesis.

Matrix vesicles represent a different type of extracellular vesicles implicated in physiological and pathological calcification processes in vertebrates (38–40). Matrix vesicles are 20–200-nm vesicles released from mineralizing cells, such as osteoblasts and odontoblasts, which induce mineralization in bones and teeth, respectively. During ectopic vascular calcification, these vesicles are also released by vascular smooth muscle cells (VSMCs), which develop into osteoblast-like cells and induce calcium phosphate precipitation when exposed to excess phosphate, inflammation, or hyperlipidemia (38–40). Matrix vesicles are thought to induce mineralization by concentrating calcium and phosphate ions in various ways, including via specific ion transporters; in addition, these vesicles contain enzymes, such as alkaline phosphatase (ALP), which degrades calcification inhibitors (e.g. pyrophosphate) and releases phosphate from various organic molecules. Although MVs similar to matrix vesicles and apoptotic bodies have been repeatedly described in calcified tissues (30, 41), the possibility that such vesicles may induce the formation of mineralo-organic NPs in body fluids has not been investigated.

Phosphatidylserine (PS), a phospholipid usually confined to the inner lipid layer of the cell membrane (42), has been found on the surface of various populations of MVs (32, 40). When present on platelet-derived microvesicles, PS induces blood coagulation, a phenomenon associated with an increased risk of thrombosis (42, 43). On the surface of apoptotic bodies, PS is thought to represent a signal that induces phagocytosis and clearance of the vesicles by macrophages (42). Notably, PS possesses calcium-binding properties and may provide a nucleating site for calcium phosphate formation on both matrix vesicles (38–40) and apoptotic bodies (44). Whether PS found on the surface of MVs may induce the mineralization of MVs in body fluids remains to be examined.

Several studies have been conducted to identify the factors that induce formation of the so-called NB and mineralo-organic NPs. Cisar et al. (18) observed that the cell membrane lipid phosphatidylinositol produces mineral NPs similar to NB after inoculation in DMEM and incubation in cell culture conditions. Raoult et al. (22) speculated that fetuin-A, a systemic calcification inhibitor (45, 46) associated with NB (9, 22), may initially inhibit NB formation but eventually act as a nucleator of NB formation following a conformational change similar to prion conversion. Our own experiments have shown that serum proteins like albumin and fetuin-A fail to induce NB formation under the conditions tested, although these proteins may form seeds for the formation of mineralo-organic NPs once the concentrations of calcium and phosphate ions exceed saturation (11). These results suggest that other molecules or structures, possibly in the form of lipid membranes, may represent factors that induce the formation of mineralo-organic NPs similar to the so-called NB in body fluids.

In the present study, we examined the possibility that mineral NP formation may be induced by MVs present in body fluids. We isolated a population of MVs from human serum (HS) and FBS and characterized the morphological and biochemical composition of these particles. Our results show that the isolated serum MVs induce the formation of mineralo-organic NPs when inoculated and incubated in cell culture medium, suggesting that MVs may serve as a nucleating agent of mineral NPs in culture and as a factor that induces ectopic calcification in human body fluids.

MATERIALS AND METHODS
Isolation of Membrane Vesicles—Blood was collected from healthy human volunteers using a conventional venipuncture method. Written informed consents were obtained from the volunteers, and the use of human samples was approved by the Institutional Review Board of Chang Gung Memorial Hospital (Linkou, Taiwan). Whole blood was collected into Vacutainer tubes without anticoagulant (BD Biosciences). After centrifugation at 1,500 × g for 15 min at room temperature, the supernatant corresponding to HS was collected and stored at −20 °C. HS and commercial FBS (Biological Industries) were filtered through 0.2-μm pore membranes prior to use.

MVs were isolated as before (47, 48), with minor modifications. Briefly, 10 ml of HS and FBS was centrifuged at 800 × g for 15 min at 4°C to spin down and remove large cell debris. The resulting supernatant was centrifuged for 30 min at 10,000 × g. The supernatant obtained this way was centrifuged for 60 min at 15,000 × g (SW27 rotor, Beckman Instruments). Material present in the supernatant was pelleted by ultracentrifugation at 200,000 × g for 2 h at 4°C (SW41 rotor, Beckman Instruments). The pellet was suspended in 1 ml of HEPES buffer (20 mM HEPES, 140 mM NaCl, pH 7.4) and is referred in the present study as “membrane vesicles” (MVs). In some experiments, sodium azide was added at 0.02% to prevent microbial contamination.

Dynamic Light Scattering—One ml of resuspended MVs was transferred to disposable plastic cuvettes (Kartell) and mixed by gentle inversion prior to reading using a Delta Nano Submicron Particle Analyzer (Beckman Coulter). Measurements were performed at room temperature at an incident angle of 165°. Although the relative particle unit used in the y axis of Fig. 1, A and B, represents arbitrary values, this unit correlates in a linear manner with the observed particle amount under the conditions used.
**Optical and Electron Microscopies**—Aliquots of MVs resuspended in HEPES buffer were deposited on glass slides and observed without fixation or staining with a BX-51 optical microscope (Olympus) equipped with a ×100 oil immersion objective with iris (UPlanFLN, Olympus) and a dark field condenser (Cerbe Distribution). Specimens were observed at a magnification of ×1,000, and images were acquired with a Spot Flex color camera (Diagnostic Instruments).

For negative stain TEM, MV preparations were deposited onto Formvar-carbon-coated grids and negatively stained with 0.5% aqueous uranyl acetate, followed by drying overnight at room temperature. In some experiments, MVs were treated with 0.1% (v/v) Triton X-100 prior to processing for negative staining. Specimens were examined under an EMU-3C (RCA) or JEM-100B (JEOL) transmission electron microscope.

Precipitates obtained following incubation of DMEM containing MVs (with or without added serum) were pelleted by centrifugation at 12,000 × g for 15 min and washed twice with HEPES buffer prior to resuspension in the same buffer. A small aliquot was deposited onto carbon-coated grids before drying overnight. In this case, the particles were observed without fixation or staining by TEM. Electron diffraction patterns were obtained with the JEM-100B transmission electron microscope.

Fluorescence Spectroscopy—MVs were quantified as before (49, 50) by measuring the concentration of total proteins using a commercial Bradford protein assay (Bio-Rad). To detect lipid markers, MVs were treated with 0.1% (v/v) Triton X-100 prior to processing for negative staining. Specimens were examined under an EMU-3C (RCA) or JEM-100B (JEOL) transmission electron microscope.

Western Blotting—SDS-PAGE and Western blot analysis were performed as before (9). A fraction of MVs corresponding to 60 μg of MV proteins, 60 μg of proteins from mineralo-organic NPs seeded by MVs, or 60 μg of proteins from either HeLa cells or whole serum was dissolved in 5 × “loading buffer” (0.313 M Tris-HCl, pH 6.8, 10% SDS, 0.05% bromophenol blue, 50% glycerol, 12.5% β-mercaptoethanol) to a final concentration of 1×, prior to heating at 95°C for 5 min. After separation under denaturing and reducing conditions on 10% SDS-PAGE using a minigel system (Hoefer). HeLa cells were purchased from the ATCC and cultured in minimum essential medium containing 10% FBS and 100 units/ml of both penicillin and streptomycin. The primary antibodies used were goat polyclonal anti-tissue nonspecific ALP (sc-15065, Santa Cruz Biotechnology), mouse monoclonal anti-LAMP2 (lysosome-associated membrane protein 2) (sc-18822), goat polyclonal anti-human TNFR1 (tumor necrosis factor receptor 1) (sc-31349), rabbit polyclonal anti-annexin V (sc-8300), goat polyclonal anti-CD63 (cluster of differentiation 63) (sc-31214), and rabbit polyclonal antibodies prepared in-house as described below. The secondary antibodies used were horseradish peroxidase-conjugated anti-goat, anti-mouse, anti-sheep, or anti-rabbit antibodies (Santa Cruz Biotechnology). Primary and secondary antibodies were diluted based on the instructions provided by the manufacturer. The polyclonal antibodies generated in house were used at a dilution of 1:1,000. The blots were revealed using enhanced chemiluminescence (Amersham Biosciences) and autoradiographic films (Molecular Technologies). Membranes were stripped by using the ReBlot Western blot recycling kit (Chemicon).

**Production of Polyclonal Antibodies**—New Zealand White rabbits were obtained at ~12 weeks of age. Human serum albumin, human serum fetuin-A, and human apolipoprotein AI (apoA-I) as well as bovine serum fetuin-A and bovine serum albumin were purchased from Sigma. The purified proteins (500 μg) were dissolved in 1 ml of DMEM and mixed with 1 ml of Freund’s complete adjuvant (Sigma) using two syringes connected by a 3-way stopcock (Nipro). Each protein-adjuvant mixture was administered intradermally on the back of the animals. Three weeks after the first immunization, one booster dose (200 μg of protein in a 1:1 mixture of DMEM and incom-
complete Freund's adjuvant; Sigma) was administered every month for a total of four times. Pre- and postimmunization blood was collected from the ear vein. For apoA-I, 28–30 kDa gel bands corresponding to ~200 μg of protein from SDS-PAGE bands of whole serum were used for the immunizations as described above.

Seeding of Mineralo-organic NPs by MVs—MV preparations obtained by either ultracentrifugation or sucrose gradient centrifugation were added into DMEM with or without 10% serum to obtain a final volume of 1 ml. The 24-well plates were incubated under cell culture conditions in a humidified cell culture incubator at 37 °C with 5% CO₂. Photography of the plates and A₆₅₀ readings were performed as before (9). Day 0 pictures and turbidity measurements were taken after reagent mixing. Mineralo-organic NPs obtained after incubation were pelleted by centrifugation at 12,000 × g for 30 min and washed twice with HEPES buffer prior to resuspension in the same buffer for further morphological and spectroscopy analyses.

**Calcium Deposition Assay**—The ability of MVs to calcify was assessed using a non-radioactive calcium phosphate deposition assay (49). Briefly, phospholipid-rich MV fractions corresponding to 30 μg of total MV proteins were added into DMEM and incubated in cell culture conditions. The incubation was terminated at each time point by centrifugation at 12,000 × g for 30 min to obtain a co-precipitate of MVs and mineralo-organic NPs. The mineral precipitate was solubilized in 0.6M HCl for 24 h. The calcium content of the HCl supernatant was then determined colorimetrically by using the O-cresolphthalein complexone method (BioVision). Briefly, 2 μl of acidified supernatant was incubated for 1 min with 150 μl of calcium working reagent (chromogenic + base reagent mix) prior to measurement with a microplate spectrophotometer. Absorbance was read against a blank at 575 nm within 10 min of reagent mixing.

To evaluate the contribution of PS to mineral seeding, we mixed MVs (2 mg of total proteins) with 0.2 μg of either anti-PS or anti-CD63 antibody in a final volume of 1 ml of HEPES buffer and incubated the resulting solution for 1 h at room temperature with gentle mixing. MVs were pelleted and washed following ultracentrifugation at 200,000 × g for 2 h at 4 °C. MVs were resuspended and incubated in 1 ml of DMEM in cell culture conditions. After 1 week of incubation, mineral pellets were retrieved and washed using centrifugation at 12,000 × g for 15 min. The calcium deposition assay was used to measure the level of mineral precipitate formed.

**MV Co-Precipitation Assay**—A MV fraction corresponding to 30 μg of total MV proteins was added into DMEM prior to the addition of 0.1–3 mM CaCl₂ and NaH₂PO₄ each. After incubation in cell culture conditions for 24 h, the precipitate was harvested by centrifugation at 12,000 × g for 30 min. After washing in HEPES buffer, the precipitate was resuspended in 50 μl of HEPES buffer and subjected to SDS-PAGE and Western blotting with the indicated antibodies. Samples that were not incubated were used in parallel for comparison.

**Spectroscopy Analyses**—Energy-dispersive x-ray spectroscopy (EDX) was performed as described previously (10). Briefly, material obtained after incubation of DMEM containing MVs (with or without serum) was pelleted by centrifugation at 12,000 × g for 30 min and washed twice with HEPES buffer prior to resuspension in water. Mineralo-organic NPs were dried overnight, and EDX spectra were acquired with an SEM S-3000N scanning electron microscope (Hitachi Science Systems) equipped with an EMAX Energy EX-400 EDX device (Horiba).

Fourier transform infrared spectroscopy (FTIR) was performed as before (11). Briefly, the spectra were acquired using a Nicolet 5700 FTIR spectrometer (Thermo Fisher Scientific) equipped with a deuterated triglycine sulfate detector. Mineralo-organic NPs were prepared by adding 1 mM CaCl₂ and NaH₂PO₄ each in 5% FBS, followed by incubation for 1 week in cell culture conditions (Fig. 4D, FBS-NPs). The particles were collected by centrifugation at 12,000 × g for 15 min and washed twice with HEPES buffer prior to drying overnight at room temperature. Commercial CaCO₃ (Mallinckrodt Baker), Ca₃(PO₄)₂ tribasic (Kanto Chemical) and HAP (buffered aqueous suspension, 25% solid; Sigma) were used for comparison.

**Statistical Analysis**—All experiments were performed in triplicate. The values shown represent means ± S.E. Comparisons between control and experimental groups were performed using Student's t test.

**RESULTS**

Isolation of Lipid-containing MVs from Serum—Mineralo-organic NPs initially described as NB have been shown to form when serum or other body fluids are inoculated into a cell culture medium and incubated in culture conditions for several days (4, 5). In contrast, incubation of the cell culture medium alone failed to produce mineral NPs, suggesting that nucleating agents exist in serum and body fluids. To verify whether MVs may represent a nucleating agent that induces the formation of mineralo-organic NPs in body fluids, we first isolated MVs from serum using an established protocol (see “Materials and Methods”). We hypothesized that MVs may induce mineral nucleation on their surface and thus become the core of mineralo-organic NPs. Given that mineralo-organic NPs and the so-called NB have sizes ranging from 50 to 500 nm (5, 9), we focused our attention on the smallest MVs present in body fluids and used a protocol involving ultracentrifugation to obtain vesicles with diameters below 100 nm.

After ultracentrifugation of serum, we obtained a pellet containing small, round particles of variable sizes when observed under optical, dark field microscopy (Fig. 1, A and B, insets). Based on dynamic light scattering analysis, the particles derived from HS showed a peak size of 37 ± 14 nm, whereas the particles obtained from FBS peaked at 28 ± 3 nm (Fig. 1, A and B). The sizes of the particles obtained were relatively heterogeneous; polydispersity index values of 0.29 and 0.22 were obtained for HS-MVs and FBS-MVs, respectively, indicating that the sizes of HS particles were more heterogeneous than for FBS particles.

Under negative stain TEM, MV preparations consisted of round particles with sizes ranging from 20 to 400 nm (Fig. 1C, Control). In order to determine whether these particles were delineated by a lipid membrane, we treated the MV preparations with Triton X-100, a non-ionic detergent with lipid-dispersing properties (48, 51). This treatment destroyed the
integrity of serum-derived particles, leaving only membrane remnants (Fig. 1C, Triton X-100). To confirm these observations, we used the lipophilic tracer DiR, which is weakly fluorescent in an aqueous environment but becomes highly fluorescent when intercalated within a lipid membrane. Accordingly, DiR did not fluoresce in HEPES buffer used as a negative control but produced fluorescence when incubated with human acute monocytic leukemia THP-1 cells used as a positive control (Fig. 1D). DiR treatment also produced fluorescence in the presence of MVs obtained from either HS or FBS (Fig. 1D), confirming that the isolated MVs were lipid-bound vesicles.

**Biochemical Characterization of Serum-derived MVs**—Next, we fractionated the MV preparations by sucrose gradient centrifugation and determined the biochemical composition of each 1-ml fraction. The concentration of phospholipids, cholesterol, triglycerides, and lipoproteins (i.e. LDL and HDL) in each fraction was determined using standardized biochemical assays. Our results showed that MV fractions contained relatively high concentrations of phospholipids, averaging from 30 to 93 μM for HS-derived vesicles (Fig. 2A) and from 4 to 15 μM for FBS-derived vesicles (Fig. 2B). Cholesterol was detected in low amounts in HS-derived MVs (Fig. 2A, fractions 4–6, peak at 2 μM), but none was found in FBS-MVs (Fig. 2B). Triglycerides, LDL, and HDL were not detected in the MV fractions tested (Fig. 2, A and B; data not shown), indicating that the isolated MV fractions did not contain lipoproteins.

We then performed Western blot analysis to examine the proteins associated with serum-derived MVs. We found that ALP, a glycosylated membrane-bound enzyme associated with biomineralization processes (52, 53), was associated with the phospholipid-rich MV fractions (Fig. 2, C and D). The molecular weight of the ALP found in HS-MVs (55 kDa) was smaller than the one found in FBS-MVs (72 kDa), possibly indicating the presence of different ALP isoenzymes in these samples. We also detected the presence of the exosome-associated proteins TNFR1 and CD63 in phospholipid-rich fractions (Fig. 2, C and D), whereas other exosome proteins like LAMP2 were not detected in our specimens (data not shown).

Albumin, fetuin-A, and apoA-I were also found in association with serum-derived MVs (Fig. 2, C and D). Although fetuin-A and apoA-I were associated with fractions of lighter densities compared with fractions containing exosome proteins, the three serum proteins overlapped to some extent with exosome-associated proteins. These serum proteins have been consistently associated with mineralo-organic NPs derived from body fluids, and they have been shown to act as both inhibitors and seeders of mineral particles in culture (9–11).

The structural and morphological data reported here along with the co-distribution of phospholipids and exosome proteins confirmed that the particles isolated from serum consisted of MVs and included exosomes. Given that the sizes of the isolated MVs (20–400 nm) exceeded the sizes reported previously for exosomes (30–100 nm) (32–34), we concluded that other vesicles, possibly microvesicles and apoptotic bodies, may also be present in our MV preparations.

**Serum-derived MVs Induce Mineral Precipitation in Culture**—To determine whether serum MVs may induce the formation of mineralo-organic NPs and mineral precipitation in body fluids,
we inoculated MVs into DMEM and incubated the resulting mixture in cell culture conditions for several days (Fig. 3A). Although the addition of MVs into DMEM produced no turbidity following inoculation at day 0, precipitation visible to the naked eye was observed in most wells containing MVs after 1 week of incubation (Fig. 3A). The precipitation increased in a dose-dependent manner with the amount of MVs added and was more abundant for FBS-MVs than for HS-MVs. The level of visible precipitation continued to gradually increase with time, as seen after 2 weeks of incubation (Fig. 3A). To confirm that serum MVs induce the formation of mineral precipitates in culture, we used another assay that monitors calcium deposition in a non-radioactive manner. We noticed that DMEM containing MVs showed a time-dependent increase in calcium deposition during incubation, whereas no calcification was observed in DMEM alone (Fig. 3B). As seen earlier (Fig. 3A), precipitation levels produced by FBS-MVs were slightly higher than those produced by HS-MVs (Fig. 3B).

To identify the biochemical component of MV fractions that induces mineral precipitation, we inoculated MV fractions obtained by sucrose gradient centrifugation into DMEM and measured the level of mineral precipitation following incubation in different cell culture conditions. After 2 months of incubation, the MV fractions containing high levels of phospholipids induced higher levels of mineral precipitation than MV fractions containing low amounts of phospholipids (Fig. 3C). Similar results were obtained for incubation periods of either 2 weeks or 1 month (data not shown). These results indicate that phospholipid-rich MV fractions induce mineral precipitation in culture.

In order to evaluate whether MVs induce mineral precipitation by binding to calcium and phosphate ions in cell culture medium, we inoculated MVs into DMEM and treated the resulting mixture with calcium and phosphate ions to induce mineral precipitation. We have shown previously (9–11) that the addition of calcium and phosphate ions in DMEM containing a body fluid produces mineral precipitates without the delay usually required for mineral NPs to spontaneously form in culture. Here, a constant amount of MVs was added into DMEM, followed by the addition of calcium and phosphate ions at 0.1–3 mM each. After overnight incubation in cell culture conditions, the precipitates were harvested by centrifugation and subjected to Western blot analysis to detect MV proteins (Fig. 3, D and E).

Whole HS used as a control showed the presence of the proteins ALP (low signal), TNFR1, and CD63 as well as albumin, fetuin-A, and apoA-I (Fig. 3D, lane 1). In the case of whole FBS, all of these proteins were detected (Fig. 3E, lane 1). As shown earlier in Fig. 2, C and D, HS-MVs and FBS-MVs harbored ALP, TNFR1, CD63, albumin, fetuin-A, and apoA-I (Fig. 3D, lane 2). When MVs were added into DMEM and centrifuged at low speed (12,000 × g), the vesicles could not be pelleted, as seen by the absence of MV-associated proteins in this control experiment (Fig. 3, D and E, lane 3). However, after inoculation of MVs into DMEM and incubation for 1 day in cell culture conditions prior to low speed centrifugation, several MV proteins were detected (Fig. 3, D and E, lane 4), suggesting that MVs induced the formation of mineral precipitate following
incubation and that this precipitate was heavy enough to be pelleted by the low speed centrifugation used. When HS-MVs were incubated into DMEM with exogenous calcium and phosphate ions were added at 0.1–3 mM each, MV-associated proteins were also detected, and most proteins (ALP, TNFR1, CD63, and apoA-I) produced signals that increased in a dose-
dependent manner with the concentration of ions added (Fig. 3D, lanes 5–7), indicating that MVs interacted with the exogenous ions to produce a mineral precipitate. In the case of FBS-MVs, protein signals were also detected for all MV proteins (Fig. 3E, lanes 5–7; note that ALP, TNFR1, CD63, and apoA-I produced signals only after the addition of ions at 3 mM).

These results suggest that the isolated serum MVs induce the formation of calcium-containing precipitates in culture. These MVs may induce the formation of mineral precipitates by binding to exogenous calcium and phosphate ions in cell culture conditions.

Morphological and Mineral Characterization of Mineralo-organic NPs Seeded by MVs—To verify the nature of the precipitation induced by MVs, we submitted the precipitates (obtained in Fig. 3A) to TEM analysis without fixation or staining. The precipitates produced following inoculation of MVs into DMEM consisted of small, round, electron-dense particles of sizes varying from 10 to 400 nm (Fig. 4A). The fact that these particles could be observed without staining suggested that they may be coated with an electron-dense material like minerals (compare the negatively stained, non-mineralized MVs shown in Fig. 1C with the unstained, mineralized MV-seeded particles in Fig. 4A). Mineralization of the particles was confirmed by electron diffraction analysis, which showed several concentric rings corresponding to a polycrystalline mineral (Fig. 4A, insets). Particles of similar size, morphology, and electron density were noted in the precipitates seeded by FBS-MVs (Fig. 4A). We confirmed the presence of MV proteins in the seeded mineral NPs by Western blots (Fig. 4A, right panels). Overall, the particles seeded by MVs were similar to the particles described earlier as NB (6, 54). No mineral particles could be pelleted by centrifugation of incubated DMEM (data not shown), consistent with the observation that DMEM alone does not produce mineral NPs under these conditions (4, 5).

We also inoculated MVs into DMEM containing either 5% HS or FBS prior to incubation for 2 weeks to compare the nature of the particles obtained with and without serum. The mixtures of MVs, DMEM, and serum produced mineral NPs of a crystalline nature following incubation (Fig. 4B). Accordingly, the electron diffraction patterns of these particles (Fig. 4B, insets) showed concentric rings that were more pronounced than that of the particles seeded without serum (Fig. 4A, insets). The particles obtained this way appeared larger in size compared with the particles prepared without added serum (Fig. 4B, 20–500 nm). All of the proteins found earlier in MVs (Fig. 2, C and D) were detected within the MV-seeded mineral NPs (Fig. 4B, right panels).

Notably, the mineral particles seeded by MVs and serum were highly similar not only to NB (6, 54) but also to the secondary calciprotein particles (CPPs) observed by Jahnen-Dechent and colleagues in specimens obtained from patients undergoing dialysis (55) or suffering from calcifying peritonitis (27). These observations suggest that, in addition to MVs, serum may provide additional compounds that induce crystallization of the mineral particles following prolonged incubation.

Next, we used EDX and FTIR spectroscopies to identify the mineral phase associated with MV-seeded particles. Previous studies have shown that the so-called NB (5, 56) and the mineralo-organic NPs observed in calcified human tissues (30, 57) consist of carbonate HAP, a mineral similar to the one found in bones and teeth of vertebrates (58, 59). EDX spectra of mineral particles seeded by MVs in DMEM showed major peaks of carbon (C), calcium (Ca), oxygen (O), sodium (Na), phosphorus (P), and sulfur (S) (Fig. 4C, HS-MVs + DMEM and FBS-MVs + DMEM), consistent with the presence of carbonate-calcium phosphate in MV-seeded NPs. Similarly, the particles seeded by MVs in DMEM containing serum showed similar peaks consistent with the formation of carbonate-calcium phosphate (Fig. 4C, HS-MVs + DMEM + HS and FBS-MVs + DMEM + FBS).

FTIR analysis revealed major peaks of phosphate at 566 cm⁻¹, 604 cm⁻¹, 960 cm⁻¹, and 1,033–1,100 cm⁻¹ in the particles seeded by MVs in DMEM with or without serum (Fig. 4D; compare with the spectra of commercial Ca₁₀(PO₄)₆ and HAP shown for reference; see also Refs. 60 and 61). In addition, peaks corresponding to carbonate at 875 and 1,430 cm⁻¹ were found in MV-seeded particles (Fig. 4D; compare with the signals obtained for CaCO₃; see also Refs. 62 and 63). For comparison, a sample of mineralo-organic NPs prepared by adding calcium and phosphate into DMEM containing 5% FBS showed similar phosphate and carbonate peaks (Fig. 4D, FBS-NPs). These FTIR spectra were consistent with the presence of carbonate HAP within the MV-seeded particles. Other peaks corresponding to H₂O and the amide bonds of serum proteins were also detected in the seeded particles (Fig. 4D; see also Refs. 58, 64, and 65). These spectroscopy analyses confirm that the MV-seeded particles contain carbonate HAP similar to the particles described earlier as NB (7) and to the mineralo-organic NPs found in calcified human tissues (30).

PS on the Surface of MVs Contributes to Mineral NP Seeding—PS appears to represent a nucleator of calcium phosphate in matrix vesicles (38, 66). Several MV populations, such as those released by platelets and apoptotic cells, harbor PS on their surface (67, 68), but it is unclear whether PS acts as a nucleator in this context. We first used Western blot analysis to examine whether the PS-binding protein annexin V is present in serum MV fractions. Annexin V was not detected in either HS-MVs or FBS-MVs (Fig. 5A; a HeLa cell lysate was used as positive control). Next, we examined whether PS is present on MVs by using immunogold labeling and annexin V as a probe. TEM images showed that annexin V binds the surface of MVs isolated from HS or FBS (Fig. 5B). Similarly, using fluorescence spectroscopy, we observed that annexin V that was prelabeled with the fluoroochrome FITC interacts with MVs of both human and bovine origins. As a control, the interaction between PS and annexin V, which requires free calcium (69), was abrogated by the addition of the calcium chelator EDTA (Fig. 5C). These results confirm that PS is present on the surface of MVs isolated from serum.

We investigated whether PS found on the surface of MVs contributes to the seeding of mineralo-organic NPs by the vesicles. We incubated MVs with an anti-PS antibody and measured the seeding potential of MVs following the washing steps. Blocking PS moieties with the antibody significantly decreased seeding of mineral NPs by MVs, whereas an anti-CD63 antibody used as a negative control showed no effect on seeding (Fig. 5D). These data indicate that PS contributes to the seeding activity of MVs.
DISCUSSION

In the present study, we show that serum MVs isolated by ultracentrifugation and sucrose gradient centrifugation induce the formation of mineral NPs similar to the so-called NB following incubation in cell culture conditions. These results indicate that MVs may represent the long sought nucleator of mineralo-organic NPs in body fluids. Moreover, these observations support our previous studies showing that NB-like structures represent non-living mineral NPs that possess biomimetic properties (9, 16).

The MVs isolated from serum harbor various proteins, including ALP, exosome markers (TNFR1 and CD63), and...
serum proteins (albumin, fetuin-A, and apoA-I; Fig. 6A). The size of the isolated MVs (30–180 nm) and the presence of exosome markers indicate that exosomes represent the major vesicles present in our preparations, consistent with previous results obtained with a similar fractionation protocol (32). Given that the sizes of the MVs isolated here (30–180 nm) are slightly larger than the sizes reported previously for exosomes (30–100 nm), it is possible that microvesicles and apoptotic bodies may represent minor constituents of our MV specimens. The minor differences in the composition of the MVs isolated here and in previous studies may be due to the nature of the starting material (e.g., serum versus plasma) as well as variation in sample preparation (70), among others.

ALP, a phosphatase enzyme found on the surface of matrix vesicles (34, 53, 71), is found in association with the serum MVs described here. Hunter et al. (72) have observed that blocking ALP activity with specific inhibitors considerably decreases the formation of mineral particles from tissue homogenate culture. These observations support the possibility that ALP may contribute to the seeding of mineralo-organic NPs in this context.

In the case of serum proteins, we proposed in a previous study (11) that albumin and fetuin-A may initially inhibit mineral particle formation but may eventually nucleate particle formation and deposition once the concentrations of calcium and phosphate exceed saturation levels. The results obtained in the present study appear to support this proposal, because albumin and fetuin-A were found in association with MVs, which in turn induced the formation of mineralo-organic NPs in culture. The presence of PS on the surface of MVs suggests a possible mechanism to account for the formation of mineralo-organic NPs induced by serum MVs (Fig. 6B). PS possesses calcium-binding properties (38–40), and its presence on the surface of MVs suggests that this phospholipid may serve as a binding site for calcium and phosphate ions. Binding of calcium and phosphate on the surface of MVs may induce mineral precipitation and lead to the formation of fully crystallized mineral particles with time. Such mineral NPs have been shown to evolve into mineralized biofilm-like structures, as seen previously for the so-called NB (5) and mineralo-organic NPs (9). Notably, several human diseases, including cancer, diabetes, malaria, sickle cell anemia, thalassemia, and uremia, have been associated with increased exposure of PS on MVs or cells (73), suggesting that PS may also contribute to mineral NP formation in these conditions.
We observed that FBS-MVs possess a higher mineral seeding ability compared with HS-MVs (Fig. 3, A and B). Fetal serum (i.e., FBS) may represent a better pro-calcifying milieu needed for bone and teeth formation compared with adult serum (HS). If this is the case, FBS-MVs may show functional differences compared with HS-MVs; accordingly, ALP appears to be present in slightly higher amounts in FBS-MVs compared with HS-MVs (Figs. 2 (C and D) and 4 (A and B)), an observation that may partially explain the difference in seeding ability of these vesicles.

Our results may have important implications for the understanding of ectopic calcification in humans. For one, MVs may induce the formation of mineral NPs in tissues and body fluids under certain conditions. Although the exact nature of these circumstances has not been examined in the present study, various conditions could possibly induce MV-mediated seeding of mineral NPs in vivo. For instance, a decrease in the level of calcification inhibition proteins, an increase of calcium and phosphate concentrations due to kidney failure, a disturbance of vitamin D physiological activity, or an increase in the production of MVs due to inflammation or hyperlipidemia may lead to MV-induced formation of mineral NPs. In addition, given that MVs are present in most body fluids, it appears plausible that these entities may induce the formation of mineralogic NPs in other areas of the body. Further studies are currently under way to assess the relevance of MV-induced ectopic calcification in vivo.

A prominent factor that may induce the formation of MVs and ectopic calcification in vivo is inflammation (40). Chronic inflammation can induce the differentiation of VSMCs into osteoblast-like cells that produce matrix vesicles, which in turn induce ectopic calcification. A recent study by Aikawa et al. (74) has shown that inflammation precedes ectopic calcification in a mouse apolipoprotein E-deficient model of atherosclerosis, suggesting that inflammation may lead to ectopic calcification. These authors concluded that macrophages infiltrating the sites of lipid accumulation secrete cytokines that induce the phenotypic differentiation of VSMCs into functional osteo-blast-like cells. Given that calcium phosphate displays pro-inflammatory properties (75–77), Shanahan (78) suggested that these processes may lead to a vicious cycle of inflammation and calcification, which would eventually propitiate atherosclero-

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**FIGURE 6. Schematic model illustrating the composition of MVs and the seeding of mineralo-organic NPs by MVs.** A, illustration representing an MV isolated from serum in the present study. Our results indicate that the isolated MVs consist of phospholipid-bound vesicles containing ALP, TNFR1, CD63, albumin, fetuin-A, and apoA-I. PS was found in the outer phospholipid layer of MVs. B, calcium ions present in cell culture medium and body fluids may bind to PS exposed on the surface of MVs and induce mineral precipitation. With time, mineralization of MVs may further increase and lead to the formation of fully mineralized mineralo-organic NPs similar to the so-called NB. Molecules present in serum and body fluids may promote the crystallization of the mineral particles and produce mineral precipitates similar to those observed previously in human calcified tissues.
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sis. We recently found that mineralo-organic NPs of calcium phosphate do not activate pro-inflammatory reactions in macrophages but that large particle aggregates led to caspase-1 activation and secretion of pro-inflammatory IL-1β (17). These results suggest that mineral NPs nucleated by MVs may initially be inert in the body but may eventually induce inflammation and participate in disease processes after reaching a certain size.

Apoptosis represents another factor that may control the formation of mineral NPs and ectopic calcification in the body. A previous study by Proudfoot et al. (44) showed that VSMCs calcify after about 28 days in culture and that apoptosis appears prior to mineral precipitation. Blocking apoptosis with a pan-caspase inhibitor reduced calcification, whereas induction of apoptosis with anti-Fas IgM increased calcification in culture. In the end, calcification was attributed to the release of apoptotic bodies (44) similar to the MVs studied here.

Obviously, differences may exist between the in vitro system studied here and what might occur in the human body. For instance, the observation that PS is exposed on the surface of MVs suggests that these MVs could be subject to constant phagocytosis and clearance by macrophages of the reticuloendothelial system in vivo. It remains to be seen under what conditions procacifying MVs similar to the ones characterized here may accumulate at amounts high enough in body fluids to induce the formation of mineralo-organic NPs and larger calcified deposits.

Jahnen-Dechent and colleagues (79, 80) have proposed that mineral NPs in the form of CPPs may be part of a larger physiological cycle that controls the use and clearance of mineral ions in the body. These authors have proposed that serum proteins like fetuin-A may serve as systemic calcification inhibitors and chaperone molecules that stabilize excess calcium and phosphate in the form of CPPs, leading to clearance of the particles by the reticuloendothelial system (81). Recently, these authors have shown that the scavenger receptor-A present on macrophages is implicated in the recognition and rapid clearance of CPPs from the blood of mice (82).

From another perspective, we noticed that the MVs isolated from serum (Fig. 1, A and B) share similarities with a group of intriguing particles described previously in the blood under various names, including “microzymas,” “protils,” “somatids,” “cancer bacteria,” “pleomorphic bacteria,” and “filterable bodies,” among other terms (83–86). Vodyanoy and colleagues (87) have reported that metal nanoclusters may form structures referred to as “proteins” when they bind to denatured proteins in various body fluids, such as blood. Based on these and similar observations (8–17), we believe that the putative microzymas, protits, somatids, and related entities may represent a combination of various particles that form spontaneously in the blood during incubation, which may include MVs, mineralo-organic NPs, proteins, and cellular remnants. Further studies are needed to verify this intriguing possibility.

In summary, our results indicate that human and bovine sera contain MVs that are able to induce the formation of mineralo-organic NPs when incubated in cell culture medium. These results suggest that the formation of mineral NPs and ectopic calcification occurring in the human body may be induced by MVs similar to the ones described here.

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Note Added in Proof—We have recently extended the findings on calcium apatite nanoparticles to other types of cations which also assemble nano- and micro-scale structures resembling biological topologies, which we have termed collectively as bions (Wu, C. Y., Young, L., Young, D., Martel, J., Young, J. D. (2013) Bions: A family of biomimetic mineralo-organic complexes derived from biological fluids. PLoS One 8, e75501).

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