Atherosclerosis is a complex process involving inflammation. S100A8 and S100A9, the Ca\(^{2+}\)-binding neutrophil cytosolic proteins, are associated with innate immunity and regulate processes leading to leukocyte adhesion and transmigration. In neutrophils and monocytes the S100A8-S100A9 complex regulates phosphorylation, NADPH-oxidase activity, and fatty acid transport. The proteins have anti-microbial properties, and S100A8 may play a role in oxidant defense in inflammation. Murine S100A8 is regulated by inflammatory mediators and recruits macrophages with a pro-atherogenic phenotype. S100A9 but not S100A8 was found in macrophages in ApoE\(^{-/-}\) murine atherosclerotic lesions, whereas both proteins are expressed in human giant cell arteritis. Here we demonstrate S100A8 and S100A9 protein and mRNA in macrophages, foam cells, and neovessels in human atheroma. Monomorphic and complexed forms were detected in plaque extracts. S100A9 was strongly expressed in calcifying areas and the surrounding extracellular matrix. Vascular matrix vesicles contain high levels of Ca\(^{2+}\)-binding proteins and phospholipids that regulate calcification. Matrix vesicles characterized by electron microscopy, x-ray microanalysis, nucleoside triphosphate pyrophosphohydrolase assay and cholesterol/phospholipid analysis contained predominantly S100A9. We propose that S100A9 associated with lipid structures in matrix vesicles may influence phospholipid-Ca\(^{2+}\) binding properties to promote dystrophic calcification. S100A8 and S100A9 were more sensitive to hypochlorite oxidation than albumin or low density lipoprotein and immunoaffinity confirmed S100A8-S100A9 complexes; some were resistant to reduction, suggesting that hypochlorite may contribute to protein cross-linking. S100A8 and S100A9 in atherosclerotic plaque and calcifying matrix vesicles may significantly influence reduct- and Ca\(^{2+}\)-dependent processes during atherogenesis and its chronic complications, particularly dystrophic calcification.

Atherosclerosis is a progressive chronic disease for which the prevailing “response-to-injury” hypothesis emphasizes endothelial dysfunction and accommodates the various risk factors, underscoring the importance of the immune system and chronic inflammation in its initiation, progression, and complications. Advanced atherosclerosis is often associated with dystrophic calcification, and there is a relationship between calcification and the severity of coronary disease. Dystrophic calcification results in less compliant and more rupture-prone vessels, precipitating ischemic and thrombotic complications. Initiation of calcification occurs within matrix vesicles (MVs)\(^4\), extracellular microstructures originating from budding of plasma membranes (2). Calcifying MVs contain high levels of Ca\(^{2+}\)-binding acidic phospholipids, particularly phosphatidylserine (PS) (2) and membrane phosphatases, which hydrolyze a variety of naturally occurring organic phosphoesters (3, 4) ultimately forming nascent calcium phosphate mineral. The membrane-invested internal microenvironment protects the precrystalline mineral nucleus in a pre-crystalline state, before conversion to hydroxyapatite (5). The presence of bone morphogenic proteins and non-collagenous bone matrix proteins including osteopontin, osteonectin, and matrix Gla protein (MGP) in atherosclerotic lesions indicates that vascular calcification is actively regulated (6, 7), although mechanisms are unclear.

Inflammation-associated “calgranulins,” S100A8 (MRP-8) and S100A9 (MRP-14), members of the S100 family of Ca\(^{2+}\)-binding proteins (8), are highly expressed in numerous inflammatory conditions, including giant cell arteritis (9), cystic fibrosis, rheumatoid arthritis, dermatoses, some malignancies, and autoimmune diseases (10, 11). They are major cytosolic proteins in neutrophils and monocytes and found in subsets of macrophages at chronic inflammatory sites, but not in tissue macrophages (10, 11). Monocytes infiltrating early atherosclerotic lesions, and macrophages within mature plaque of ApoE\(^{-/-}\) mice were reported as S100A9\(^{-/-}\)-S100A8\(^{-/-}\) (12). Intracellular S100A8 and S100A9 essentially regulate phagocyte migration through calcium and mitogen-activated protein kinase (MAPK) transduction pathways affecting the microtubular system (13). Murine (m) S100A8 is a potent leukocyte chemoattractant (14); mS100A8-elicited macrophages exhibit a “pro-atherogenic phenotype,” expressing high levels of CD11b/CD18, Fc and scavenger receptors that accumulate acetylated low density lipoprotein (LDL) in vitro and in vivo, with cholesterol-ester profiles similar to those in human plaque and thus may contribute to potential foam cell (FC) development (15). Extracellularly, the S100A8-S100A9 complex (known as calprotectin) exerts antimicrobial activity (10), stimulates IL-8 production by airway epithelial cells (16), and transports arachidonic acid to endothelial cell (EC) targets affecting pathological responses in inflammation and atherosclerosis (10).

S100A8 is secreted by appropriately activated macrophages (10) and is exquisitely sensitive to oxidation, particularly by peroxide and hypo-

\(^4\) The abbreviations used are: MVs, matrix vesicles; PS, phosphatidylserine; MGP, matrix Gla protein; mS100A8, murine S100A8; LDL, low-density lipoprotein; FC, foam cell; EC, endothelial cells; MPO, myeloperoxidase; r, recombinant; OxS100A8, HOCl-oxidized S100A8; DC, dendritic cell; PBS, phosphate-buffered saline; NEM, N-ethylmaleimide; TBS, Tris-buffered saline; TEM, transmission electron microscopy; SEM, scanning electron microscopy; NTPPPH, nucleoside triphosphate pyrophosphohydrolase; ECM, extracellular matrix; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay.
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chlorite (HOCl/OCI') (17), and a role in antioxidant defense is proposed (18). HOCl from activated leukocytes generates inter-/intramolecular covalent cysteine-lysine sulfamidic bonds in S100A8 (17). HOCl is generated by myeloperoxidase (MPO), which co-localizes with macrophages (19) in human plaque where it oxidizes LDL to a proatherogenic form (20) and may also regulate proteases contributing to plaque rupture (21). Other S100s, such as S100A2 (22), are also implicated in cell responses to oxidative stress and S100B and S100A1 may link Ca²⁺- and redox-mediated signaling pathways, possibly by Ca²⁺-modulated S100 cysteine thiol-glutathione covalent interactions (23, 24). Intracellularly, the S100A8-S100A9 complex forms a scaffold for NADPH-oxidase components (10) and regulates casein kinase activity (25).

This is the first report of calgranulins in macrophages and FC in human atheroma and in areas of vascular calcification. S100A8 and S100A9 mRNA and protein were also expressed by microvessels in areas of neovascularization. Monomeric and complexed forms of S100A9 were predominant in MVs isolated from human carotid artery and aorta suggesting a role in regulation of dystrophic calcification. We show high amounts of S100A8 and S100A9 complexes in plaque extracts and demonstrate the exquisite sensitivity, particularly of S100A8, to oxidation by HOCl, strongly suggesting it as a ready target for oxidation in vivo.

**MATERIALS AND METHODS**

**Proteins and Antibodies**—Recombinant (r) S100A8 and rS100A9 were generated as described (26). The monoclonal antibody against HOCl-modified LDL (HOP-1) (20) was a gift from Dr. R. Stocker. Rabbit IgGs against S100A8, HOCl-oxidized S100A8 (OxS100A8) (17) or S100A9 were raised and purified as described (26). Preimmune IgGs were unreactive in Western blotting/immunohistochemistry. S100 proteins share regions of high sequence homology and cross-reactivities of antibodies were tested by Western blotting. Anti-S100A1/B (Dako Carpintaria, CA) recognized S100A1 and S100B (Sigma) but not the other S100 proteins; anti-S100A8, -OxS100A8, and -S100A9 were immunospecific. Anti-OxS100A8 reacted more strongly with OxS100A8 than S100A8 by ELISA (27).

**Carotid and Aortic Specimens**—Aortic and carotid artery specimens containing atherosclerotic lesions and apparently normal mural areas, collected according to the Declaration of Helsinki, were obtained from 39 patients undergoing endarterectomy and 14 (aged 32–86 years) undergoing aortic reconstruction, respectively, at St Vincent’s and Concord General Repatriation Hospitals, Sydney.

**Immunohistochemical Analysis**—For immunohistochemistry, 11 carotid and 5 aortic specimens were used. Tissue processing and single and double immunostaining were performed as reported (28). Optimal antibody concentrations were determined by calibrating staining intensities in various tissues. Anti-S100A1/S100B, -S100A9, or -S100A8 IgGs were used at 6.4, 1, and 2 µg/ml, respectively. Anti-CD3 (T-cell marker; Sigma), anti-CD68 (macrophage marker), anti-von-Willebrand factor (EC marker), and fascin (dendritic cell (DC) marker, all from Dako) were used at 1:50 dilution and anti-a-smooth muscle actin (Dako) at 1:400 v/v. Secondary antibodies were biotinylated goat anti-rabbit and rabbit anti-caprine (Vector Laboratories, Burlingame, CA) IgGs. For negative controls, primary antibodies were omitted, or sections treated with pre-/nonimmune rabbit IgG.

Control sections were negative. Immunostaining in sections stained with Alizarin red S, which identifies atherosclerotic calcification, was difficult, so calcified deposits were visualized by counterstaining with Mayer’s hematoxylin.

**In Situ Hybridization**—Tissue sections were washed (15 min) in phosphate-buffered saline (PBS, 25 mM PO₄⁻/H11001, 250 mM CI⁻, pH 7.5) containing Triton X-100 (0.3%), treated with 0.2 M HCl (15 min), permeabilized with proteinase K (25 µg/ml, MERCK; Darmstadt, Germany) in 0.1 M Tris-HCl/50 mM EDTA, pH 8.0 (37 °C, 30 min), washed with 0.2% glycine followed by PBS, and post-fixed with 4% paraformaldehyde in PBS (5 min). Photobiotin-labeled cDNA as whole plasminoids (vector and insert; 100 µl/slide) were used for hybridization. Stringency washes and detection were performed according to a sensitive method (29), with biotinylated alkaline phosphatase and streptavidin (both from Sigma) at 1:200 and 1:100 v/v, respectively, during four sequential 10-min applications for detection. S100A8 (360 bp) and S100A9 (413 bp) full-length cDNAs were synthesized from human neutrophil RNA and subcloned at BamHI-EcoRI and EcoRI-HindIII sites of pGEM-3Z vector (Promega). Integration was confirmed using restriction enzyme digestion. Vector alone, or unrelated plasmid vectors were used as negative controls; hybridizations with these were negative. Detection of GAPDH mRNA was the internal positive control.

**Arterial Tissue Extraction**—Specimens from 11 endarterectomy patients were pulverized under liquid nitrogen. Samples of apparently normal carotid artery (taken from a 74-year-old male who died of chronic obstructive pulmonary disease; 25-h post-mortem delay) were similarly processed and because of the low protein content, pooled extracts from two different mural sections analyzed. Samples (~500 mg) in PBS/10 mM EGTA (1.5 ml, pH 7.5) containing protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) were sonicated for 5 min, rotated at 4 °C for 2 h then centrifuged (1,600 × g, 20 min, 4 °C). Supernatants were preclarified with caprine anti-rabbit IgG (~1 µg/ml, 1 h at 4 °C) and protein A-Sepharose (~25 µl/500 µl, 15 min, 4 °C) or agarose-conjugated caprine anti-rabbit IgG (Sigma), centrifuged and ~25–30 µg of total protein (bicinchoninic acid assay, Sigma) used for analysis. Because of the propensity of S100 proteins to form disulphide (30) and sulfamidic cross-linking (17) under oxidative conditions, five additional endarterectomy specimens were prepared in PBS containing 1 mM sodium azide (inhibits MPO that generates HOCl) (31) and 10 mM N-ethylmaleimide (NEM; derivatizes free thiols) (18).

**Isolation and Characterization of Calcifying Vesicles**—MV’s from 12 carotid and 5 aortic specimens were isolated as described (32). All buffers contained protease inhibitors (Roche Applied Science), 10 mM EGTA, 10 mM NEM, 1 mM azide, and 1 mM benzamidine (Sigma). Specimens (~1 × 2 cm²-1 × 4 cm², ~0.5 g–1.5 g) were preserved in 1% methyl cellulose and 1.15 M sucrose for 1 h at 4 °C, subsequently frozen in liquid nitrogen and transferred to ~80 °C before extraction. The MV pellets were dissolved in 1 ml of Tris-buffered saline (25 mM Tris/250 mM Cl⁻; TBS) and preclarified as above prior to Western analysis.

The procedure for transmission electron microscopy (TEM) was adapted from a previous study (33). To quantitate the calcium and phosphorous content of MVs, suspensions (25 µl) were dried at 22 °C for 2 h and then mounted onto carbon disks coated with Balzers carbon fiber. Element content was assessed using a Cambridge S-360 scanning electron microscope (SEM) with Iridium software. The SEM was equipped with an EDS detector (1XRF Kevex with beryllium window) and 1–5 nA of beam current at 20 kV were used to acquire x-ray data. To confirm pyrophosphorylase activity, the nucleoside triphosphate pyrophosphorylase (NTPPPH) assay was performed on duplicate samples as described (34). Results are expressed as mmol of p-nitrophenol formed/min/mg MV protein. Protein concentrations were determined with the bicinchoninic acid assay.

Extraction of cholesterol and lipids from isolated MVs was performed as described (35). To determine the cholesterol content, membrane
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RESULTS

S100B, S100A8, and S100A9 Expression in Plaque—Arteries lacking features of atherosclerotic transformation did not contain cells expressing S100A8 (Fig. 1A) or S100A9 (not shown) in the intima. Nerve endings in the adventitia were S100A1+/S100B+ in normal and diseased tissue, and some cells distributed irregularly throughout all layers of the arterial wall, and particularly frequent around neovessels, were S100A1–/S100B– and fascin-positive, identifying them as DC (28) (not shown).

All vessel segments affected by early- and late-stage atherosclerosis contained large numbers of irregularly distributed, intensely stained S100A8+ FC and macrophages (Fig. 1, B and C), some of which were S100A8+–CD68+ although the majority around neovessels were CD68+–S100A8+. About 50% of neovascular EC were S100A8+ (Fig. 1B). In intimal areas containing CD68+ macrophages, many FC were S100A8+ (Fig. 1C). S100A9 was present in FC and non-FC, particularly CD68+ macrophages around neovessels, and in EC of all neovessels. The surrounding extracellular matrix (ECM) exhibited relatively intense S100A9 immunoreactivity (Fig. 1D). In situ hybridization indicated S100A9 (not shown) and S100A9 mRNA in FC, extravasating monocytes around neovessels and microvessels in areas of neovascularization (Fig. 1E). S100A8 or S100A9 were not detected in the luminal endothelium of arteries or the larger vasa vasorum (not shown).

Consecutive sections indicated no S100A8 or S100A9 expression in T lymphocytes or smooth muscle cells (not shown). Overlapping patterns of distribution of S100A8 and S100A9 were apparent in FC and non-FC (not shown), particularly CD68+ macrophages around neovessels.

In particular, anti-S100A9 immunoreactivity was associated with early (Fig. 2A) and large advanced calcified deposits (Fig. 2, B–E) that were surrounded by S100A9+ cells, mainly macrophages (CD68+) and some FC, some of which were closely attached to calcified deposits (Fig. 2C). These deposits consistently contained S100A9 in the ECM (Fig. 2E). S100A9 associated with calcified deposits was evident in all 11 carotid and 9 aortic specimens examined. Serial sections of the same specimens stained with anti-S100A9 showed markedly less reactivity; some S100A8 was associated with calcified deposits in 4 carotid arteries and 2 aortas (not shown).

Characterization of MV Isolates—TEM confirmed the heterogeneous structural appearance of MV isolates (Fig. 3, A–C). Size varied from 40–1,200 nm; some had electron-lucent content (Fig. 3A), whereas others were filled with material of middle electron density or contained an electron dense core (Fig. 3B). Some MVs were clustered (Fig. 3C), and
the membrane was absent in the contact zones suggesting that MVs coalesced. NTPPPH activity in MV isolates from 8 carotid specimens (mean: 315/11006 173 nmol/min/mg protein) was similar to levels reported for human vascular MVs (32). The lipid composition of two MV isolates was analyzed. Total sterol was 762.8/11006 23.3 nmol/mg protein and total phospholipid 745.5/11006 141 nmol/mg protein (TABLE ONE). Cholesterol esters comprised 14% of total sterols. HPLC analysis indicated that cholesteryl dodecanoate was the major ester (79%); 9.5% was cholesteryl oleate, and 2.3% was cholesteryl arachidonate.

The mineral composition of carotid MV isolates (Fig. 3D), assessed by energy-dispersive x-ray microanalysis and SEM, indicated a Ca-to-P ratio of 0.66:1. Lower levels of sodium, sulfur, and chlorine were also present. In three isolates, the ratio ranged from 0.47–0.76:1 with a mean value of 0.66 ± 0.17:1.

**Western Blotting of MV Extracts**—Strong anti-S100A9 immunoreactivity in 2 carotid MV extracts separated under reducing/non-reducing conditions is shown (Fig. 4A) and is representative of 12 of 12 atherosclerotic carotid and 5 aortic MV isolates. Components migrating with a mass equivalent to S100A9 monomer (~14 kDa) were prominent, and DTT-resistant components with masses of homodimers (S100A92, ~28 kDa), trimers (S100A93, ~42 kDa), and possibly S100A8-S100A9 heterodimer (~24 kDa) were evident. The component migrating at ~56 kDa could represent S100A91 and was reduced in both samples suggesting disulfide-bonded complexes. The minor components (~65 kDa)
observed following reduction may represent higher complexes derived from proteins that did not enter the gel.

In contrast to S100A9, S100A8 monomer levels in MV were less intense, and high interspecimen variability was obvious (Fig. 4B), which was not seen with anti-S100A8/immuno reactive components in whole carotid extracts (Fig 5). M1 and M2 contained higher amounts of monomer (10 kDa) relative to the low levels of S100A8 homodimer (~20 kDa, S100A8-S100A8) and S100A8-S100A9 heterodimer; 6 of 10 isolates had similar patterns. MV isolates from 4 carotid samples lacked S100A12 (lane 2, Fig. 4C), or S100A1/S100B (lane 5, Fig. 4C), other S100 proteins expressed in atherosclerotic lesions (28), suggesting a role, particularly for S100A9 in dystrophic calcification.

S100A8 and S100A9 in Arterial Extracts—Preliminary characterization of the structural properties of S100s in plaque indicated components with masses equivalent to S100A8 (10 kDa) and S100A8 homodimers, in 15 of 16 specimens (S1–S16; four of the most representative are presented in Fig. 5). Other prominent complexes migrated at 24, 30, 34, 40, and 48 kDa and may represent S100A8-S100A9, S100A8-S100A9, S100A8-S100A9, and S100A8-S100A9. Some extracts with high amounts of monomer generally had lower levels of the complexes (24 and 34 kDa) and some lacked monomer, indicating interspecimen variability (not shown). Anti-OxS100A8, which reacted more strongly with HOCl-oxidized S100A8 (compare Figs. 5, G and F), detected similar components (Fig. 5, C and D) but was more reactive with the complexes than anti-S100A8 (Fig. 5, A and B). Although reduced samples contained higher oligomers, reduction did not increase levels of monomeric S100A8, suggesting DTT-resistant oxidative modifications such as dityrosine and sulfaminamide cross-links. The complexes migrating at ~60 kDa following reduction may be derived from larger oligomers that failed to enter the gel, or from complexes that were less reactive with the antibody.

Components equivalent to S100A9 (14 kDa) and S100A9, (28 kDa) were prominent in 15 of 16 specimens (Fig. 6, A and B, 3 of the most representative are shown); those at 24, 40, and 58 kDa have masses equivalent to S100A8-S100A9, S100A8-S100A9, and S100A9-S100A9. The DTT-resistant S100A9 complexes (24, 28, and 40 kDa; Fig. 6A) also suggest cross-linking. Carotid extracts prepared in buffers either lacking (not shown, 11 specimens) or containing (5 specimens) the MPO inhibitor, sodium azide, and NEM, which prevents fortuitous disulfide interactions, contained anti-S100A8 and anti-S100A9-immunoreactive complexes with the same mobility, confirming that post-translational modifications occur in vivo and not during extraction.

Apparently normal regions of artery did not contain cells expressing S100s (Fig. 1A), and extracts contained no monomeric S100A8 (Fig. 5E), and insignificant levels of monomeric S100A9 (Fig. 6C) were detected by anti-OxS100A8 and anti-S100A9, respectively. Low reactivity with components migrating at ~40 and ~60 kDa was observed with both antibodies; reduction decreased levels of the 60-kDa complex. Blood neutrophils express high amount of the S100s and represent another potential contaminating source. Although neutrophils lysates contained both protein monomers, no complexes were observed (Figs. 5E and 6C).

S100A8-S100A9 cross-linked complexes were eluted from anti-S100A9-Sepharose (Fig. 6, D and E). DTT-resistant S100A8-S100A9 heterodimer (24 kDa), S100A9 (28 kDa), and the S100A9-S100A9 complex (40 kDa) were detected using both antibodies. The failure to elute larger complexes may indicate that aggregates either fail to bind, or cannot be eluted from the column. Elution of S100A8 (10 kDa) also indicated the likelihood of non-covalent association that commonly occurs between S100A8 and S100A9 (10).

HOP-1 was used to confirm HOCl-modified proteins in atherosclerotic plaque (20). Reactivity was weak and variable between specimens and the relative masses of HOP-1-positive components (32, 52, 68, 89 kDa, not shown) distinct from those of S100A8 and S100A9 complexes. Some samples were HOP-1-negative but contained S100A8-S100A9 or S100A8-S100A9 heterodimers (not shown). HOP-1 did not react with S100A8 or S100A8 oxidized with HOCl, but recognized BSA aggregates generated by 100-fold molar excess HOCl in 2 h (OxBSA; Fig. 7A, lane 9). BSA oxidized with lower amounts of HOCl was unreactive (not shown). In contrast to BSA, as little as 0.5–1-fold molar excess HOCl rapidly (10 min) oxidized S100A8, forming DTT-resistant homodimers of apparent mass 20 kDa; S100A8, formed with 5-fold excess and larger complexes with ~20-fold excess HOCl (Fig. 7B). Minor components (16, 24, 35 kDa) may represent HOCl-induced fragmentation products (Fig. 7A) (39). Anti-OxS100A8 reacted more strongly with the large
complexes than did anti-S100A8 (Fig. 7A, lanes 3 and 7); neither antibody recognized OxBSA (Fig. 7A, lanes 1 and 5). S100A9 dimer formation required 5-fold excess HOCl and larger DTT-resistant complexes were generated with 5–20-fold excess (Fig. 7C). S100A8 and S100A9 DTT-resistant homodimers also form because of spontaneous oxidation upon storage (compare S100A8 standard in Fig. 5B with Fig. 7B; S100A9 in lane 1, Fig. 7C with S100A9 standard in lane 4, Fig. 6A and in Fig. 6D, lane 2). S100A8–S100A9 complexes generated by HOCl were described by us (18).

**DISCUSSION**

S100 proteins are emerging as important mediators of diverse processes in chronic inflammation. S100A1/B is found in DC associated with T lymphocytes, and may represent antigen-presenting cells in plaque (28). S100A9- and S100A12-positive monocytes reported in the ascending aortae of ApoE−/− mice (12, 40) were suggested to regulate monocyte transmigration and endothelial activation even though there are no sequences related to human S100A12 within the rat and mouse genomes (8). Key cytokines involved in initiation and maturation of atheromatous plaque up-regulate S100 genes in numerous cell types. IL-1 induces strong S100A8 and S100A9 expression in murine microvascular EC (41) and in elicited murine macrophages (42). Here we demonstrate high expression of S100A8 and S100A9 in human plaque but not normal intima (Figs. 1A, 5E, and 6C), with distribution distinct from DC expressing S100A1/B (28). S100A8 and S100A9 were expressed in macrophages and FC although some CD68+ cells were...
negative (Fig. 1C), suggesting differential activation of macrophage populations, similar to the heterogeneity of MPO-expressing macrophages seen in plaque (19). Expression of S100A8 contradicts findings in murine plaque where only S100A9 was found, and questions the proposal that the absence of S100A8 would favor atherogenesis because of its ability to negate S100A9-enhanced leukocyte adhesion (12). Importantly, intracellular S100A8 and S100A9 essentially regulate phagocyte migration by integrating the calcium and MAPK transduction signals, thereby controlling reorganization of the phagocyte microtubular system (13) and monocytes expressing membrane-bound S100A8/S100A9 secrete more TNF and IL-1 than their surface negative counterparts (43) thus potentially favoring atherogenesis.

All neovessels, but not larger vessels, were strongly S100A9/H11001/S100A8/H11001 (Fig. 1). Expression of S100A8/S100A9 by EC is controversial. Others suggest that adherent myeloid cells release the proteins and S100A9 may bind to venules via heparan sulfate glycosaminoglycans (44) and that this process triggers EC activation (45). Although this may also be the case, we showed up-regulation of the two genes in three microvascular endothelial cell lines by IL-1/TNF (41). Here we demonstrate S100A8 and S100A9 mRNA (Fig. 1E) in neovessels, strongly supporting our proposal that they are expressed by appropriately activated microvessels, but not larger vessels, and may regulate leukocyte migration in the microcirculation (41). S100A9 was more prominent than S100A8 on the surrounding ECM (Fig. 1D) and in areas of early and advanced calcification (Fig. 2), possibly because of its strong heparin affinity.

S100A8 and S100A9 may be involved in bone remodeling. Here we show S100A9+ cells with ill-defined contours apposed to calcified deposits in intima (Fig. 2); little S100A8, but high levels of S100A9 monomer and complexes were found in enzymatically active MV extracts (Fig. 4), suggesting S100A9 involvement in mural dystrophic calcification. In contrast, MV extracts lacked S100A12, which is also expressed.

5 H. Zreiqat, C. R. Howle, B. Freeman, S. Gronthos, and C. L. Geczy, manuscript in preparation.
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in macrophages and FC in plaque, and S100B, which is exclusively found in DC within atheromatous lesions (46). There is emerging evidence for a role for S100 proteins in calcification/bone remodeling. Calbindin-D9k, is also present in MVs and enhances mineralization and interstitial Ca\(^{2+}\)-absorption (47). S100A6 stimulates osteoblast proliferation, expression of alkaline phosphatase and promotes osteoblastogenesis in response to simvastatin, which stimulates bone formation (48), whereas, S100A4 may inhibit mineralization, possibly by modulating osteoblast differentiation (49). Other high affinity Ca\(^{2+}\)-binding proteins, including osteoprotegerin, MGp, and fettuin protect against dystrophic calcification (50).

The Ca:P ratio in MV isolates (Fig. 3D) was 0.66±1.0, which is less than the Ca:P ratio of 1.66:1, reported for vascular hydroxyapatite (51). However, MV isolates were structurally heterogeneous (Fig. 3), and high concentrations of phosphate indicate incomplete mineralization, which occurs when phosphate esters localize to the MV membrane before hydroxyapatite forms (52). The MVs also contain high amounts of phospholipids, thereby elevating total P, levels (53) and local concentrations of P increase by enzymes such as the ATPases and NTPPPH (47). Osteopontin and MGP inhibit vascular calcification within atherosclerotic lesions (54, 55) and phosphorylated osteopontin (56), and its phosphopeptides inhibit hydroxyapatite formation (57). S100A8 and S100A9 can inhibit casein kinase activity (25) and S100A9 is phosphorylated at Thr\(^{113}\) in activated monocytes and neutrophils (58, 59). Although the kinases responsible for S100A9 phosphorylation are unclear, phosphorylated isoforms of S100A9 translocate to the plasma membrane and cytoskeleton and exhibit increased Ca\(^{2+}\) binding compared with the non-phosphorylated isoforms, suggesting that phosphorylation modulates Ca\(^{2+}\) binding and membrane translocation (60).

Lipid analysis of human vascular MV isolates has not been reported. These contained approximately equimolar amounts of phospholipids and sterols of which ca. 2.3% comprised cholesteryl arachidonate (TABLE ONE). This contrasts with chicken MVs, which contain mostly phospholipids with smaller amounts of free cholesterol, fatty acids, and triacylglycerols (2). During MV calcification, extensive phospholipid degradation occurs and levels of fatty acids increase. However, PS, which complexes to nascent mineral within the MV and is only extractable after demineralization, is not only protected from degradation but is apparently synthesized (61). The S100A8-S100A9 heterocomplex binds reversibly to unsaturated fatty acids in vitro, and it is the major arachidonic acid-binding protein in human neutrophils and may contribute to endothelial cell fatty acid uptake (10). S100A8 and S100A9 translocate into cholesterol- and sphingolipid-rich lipid rafts, other lipid structures and cell membranes in a detergent-resistant and Ca\(^{2+}\)- and tubulin-dependent manner (62). Annexins are the main group of proteins in MVs; annexins A2, A5, and A6 facilitate MV Ca\(^{2+}\) influx by forming ion channels (47). Annexin A5 binds PS in a Ca\(^{2+}\)-dependent manner within the nucleoletal core of growth plate MVs stimulating crystal growth (2) and annexin A2 may facilitate mineralization in osteoblastic lipid rafts (63). Several S100 proteins interact with annexins (64, 65) and considering the co-localization within MVs and common Ca\(^{2+}\) and lipid-binding properties of annexins and S100A8/S100A9, their interaction is worthy of investigation.

Macrophages in human atheroma produce MPO (19) and MPO-mediated reactions oxidize apoB-100 in preference to mural LDL lipids (66). The stronger reactivity of anti-OxS100A8, compared with anti-S100A8, with components in plaque extracts (Fig. 5), prepared with or without the MPO inhibitor, sodium azide, indicates that S100A8 may undergo oxidative modifications in vitro. The S100A8 gene is up-regulated by oxidative stress (67) and is strongly implicated in antioxidant defense because of its ability to scavenge oxidants such as peroxide and HOCl (17, 18). Peroxide generates disulfide-bonded dimers (18) whereas complex inter- and intramolecular interactions and novel covalent sulfamidate cross-links between Cys\(^{-\text{D}}\) and ε-amino groups on lysine residues in mS100A8 are generated by reagent or neutrophil-derived HOCl generated by MPO (17). Oxidative modifications in murine S100A8 regulates its chemicotactic activity in vitro and in vivo (17, 18). S100A8 and S100A9 can be similarly cross-linked (18). The DTT-resistant ~24-kDa component with a mass equivalent to the heterodimer, and complexes equivalent to S100A9, and S100A9, in MV extracts (Fig. 4A) are unlikely to be non-covalent Ca\(^{2+}\)-dependent complexes (10) as EGTA was used for extraction. Apparently normal artery (Figs. 1A, 5E, and 6C) contained negligible S100s whereas diseased carotid extracts contained complexes with sizes equivalent to S100A8\(_2\), S100A9\(_2\), S100A8-S100A9, S100A8\(_3\), S100A8\(_2\)-S100A9, S100A8\(_2\)-S100A9\(_2\), S100A8\(_2\)-S100A9\(_3\), and S100A8\(_3\)-S100A9\(_2\) (Figs. 5 and 6); the majority were resistant to strong reduction (100 mM DTT). Importantly, DTT-resistant complexes, typical of those containing sulfamidate bonds generated by HOCl oxidation in vitro (17, 18), were confirmed by anti-S100A9 immunoaffinity (Fig. 6, C and D), suggesting Cys-Lys cross-linking in vivo. Other S100 proteins including S100A2, S100A1, and S100B are also proposed to regulate redox mechanisms (22–24).

HOCl-oxidized proteins, particularly LDL, are found in plaque extracts (20). HOCl generated DTT-resistant S100A8 dimers and trimers in vitro within 10 min, with as little as equimolar, to 5-fold excess (Fig. 7B). Intrachain sulfamidate bonds predominate with low molar levels of HOCl but these cannot be identified by SDS-PAGE (17). S100A9 was somewhat less susceptible, requiring 5–20-fold excess HOCl (Fig. 7C). The rapid oxidation of these proteins with equimolar levels of HOCl contrasts markedly with those required to oxidize LDL or BSA (~1,800, or ~1:100, respectively for 2 h (20); Fig. 7A) in vitro. OxS100A8 was not recognized by HOP-1, suggesting that levels of HOCl-modified proteins in plaque may be greater than earlier estimates (20). In addition to HOCl cross-linking LDL via chloramines, which can subsequently react with lysine or histidine residues (66), a previously unsuspected oxidation pathway via cysteine-lysine interactions was also proposed, with likely relevance in the pathogenesis of atherosclerosis (68). Our evidence strongly supports this possibility, particularly as MPO (69) and S100A9 (Figs. 1D and 2E) bind glycosaminoglycans on EC and the ECM, and S100A8 preferentially forms non-covalent complexes with S100A9 (10), so the close proximity of these proteins may initially reduce oxidative damage. S100A8 and S100A9 in atherosclerotic plaque and calcifying MVs may significantly influence redox- and Ca\(^{2+}\)-dependent processes during the pathogenesis of atherosclerosis and its chronic complications particularly dystrophic calcification.

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