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Pleiotropic Roles of S100A12 in Coronary Atherosclerotic Plaque Formation and Rupture

Jesse Goyette,* Wei Xing Yan,† Eric Yamen‡ Yuen Ming Chung,* Su Yin Lim,* Kenneth Hsu,* Farid Rahimi,* Nick Di Girolamo,* Changjie Song,† Wendy Jessup,‡ Maaike Kockx,‡ Yuri V. Bobryshev,* S. Ben Freedman,† and Carolyn L. Geczy2* Macrophages, cytokines, and matrix metalloproteinases (MMP) play important roles in atherogenesis. The Ca2+-binding protein S100A12 regulates monocyte migration and may contribute to atherosclerosis by inducing proinflammatory cytokines in macrophages. We found significantly higher S100A12 levels in sera from patients with coronary artery disease than controls and levels correlated positively with C-reactive protein. S100A12 was released into the coronary circulation from ruptured plaque in acute coronary syndrome, and after mechanical disruption by percutaneous coronary intervention in stable coronary artery disease. In contrast to earlier studies, S100A12 did not stimulate proinflammatory cytokine production by human monocytes or macrophages. Similarly, no induction of MMP genes was found in macrophages stimulated with S100A12. Because S100A12 binds Zn2+, we studied some functional aspects that could modulate atherogenesis. S100A12 formed a hexamer in the presence of Zn2+; a novel Ab was generated that specifically recognized this complex. By chelating Zn2+, S100A12 significantly inhibited MMP-2, MMP-9, and MMP-3, and the Zn2+-induced S100A12 complex colocalized with these in foam cells in human atheroma. S100A12 may represent a new marker of this disease and may protect advanced atherosclerotic lesions from rupture by inhibiting excessive MMP-2 and MMP-9 activities by sequestering Zn2+. The Journal of Immunology, 2009, 183: 593–603.
is important given that overexpression of MMP-9 alone was insufficient to induce plaque rupture in apoE<sup>−/−</sup> mice, although overexpression of the autoactivating form destabilizes plaques (30). Because the calgranulins are abundantly expressed in atherosclerotic plaque by activated macrophages and FCs (15, 31), they could potentially regulate MMP activity and contribute to plaque stabilization. S100A12 also binds Zn<sup>2+</sup>, but its potential to inhibit MMPs has not been reported.

Here we investigated the potential functions of S100A12 in atherogenesis by assessing its ability to induce proinflammatory cytokines, and in plaque rupture by regulation of MMP gene expression and function. S100A12 was released from sites of plaque rupture and levels were elevated in serum from patients with CAD and correlated with C-reactive protein (CRP) levels. In contrast to rupture and levels were elevated in serum from patients with CAD or minor coronary disease (diameter stenosis, >50%) and correlated with C-reactive protein (CRP) levels. In contrast to rupture and levels were elevated in serum from patients with CAD or minor coronary disease (diameter stenosis, >50%). S100A12 was released from sites of plaque rupture and its potential to inhibit MMPs has not been reported.

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### Materials and Methods

**Recombinant S100A12 and Abs**

Recombinant S100A12 was produced as described (7). Rabbit IgG against S100A12 was as reported (7, 31). Anti-S100A12 hexamer (anti-hexS100A12) Abs were produced as reported (7); the recombinant was 50 μg cross-linked Zn<sup>2+</sup>-bound oligomeric S100A12 (see Chemical cross-linking and Western blotting). Preimmune IgG was unreactive in immunohistochemistry. Protein A-purified IgG was affinity purified on a Carbosynth/Lagarose support (Pierce Chemical) to which 1 mg of Zn<sup>2+</sup>-bound hexameric S100A12 was coupled. The mAb against HOCl-modified low-density lipoproteins was a gift from Dr. R. Stocker (Sydney University, Sydney, Australia; Refs. 31–33).

**Patients and peripheral blood sampling**

Sera were collected from 67 men: 38 patients (66 ± 11 years) with symptomatic angiographically documented coronary artery disease (CAD), divided into 23 stable angina pectoris (SA), 15 hospitalized with ACS, 5 unstable angina, and 10 non-ST elevation myocardial infarction (nonSTEMI); and 29 normal subjects (age 63 ± 7 years) attending preadmission clinic for minor surgery. Procedures in this and the coronary sinus study were approved by the institutional Human Ethics Committee, and written informed consent was obtained.

**Patient selection for coronary sinus and aortic blood sampling**

Patients (n = 34; 14 SA, 9 ACS, 11 control) were recruited from the cardiac catheterization laboratory before planned coronary angiography for investigation of chest pain and/or a positive stress test, or for evaluation following admission for ACS. Control subjects had normal coronary arteries or minor coronary disease (diameter stenosis, <50%). SA and ACS patients had ≥70% coronary stenosis. Patients had transcoronary gradients measured by drawing paired blood samples from the aorta and coronary sinus after coronary angiography. A subgroup of 6 SA patients with left anterior descending coronary artery stenosis had additional paired samples drawn 12–60 (mean ± SD, 39 ± 19) min after percutaneous coronary intervention (PCI). An additional six subjects were recruited before study commencement to validate sampling technique.

Patients with SA had typical exertional angina, a positive stress test, or both, and no change in symptoms in the 2 wk before recruitment. The ACS group included patients admitted with either Braunwald class IIb unstable angina (troponin T <0.01 ng/ml on serial testing, n = 4) or non-STEMI (troponin T >0.03 ng/ml on serial testing, n = 5). Patients were excluded if they reported active inflammatory, infective or neoplastic conditions, current use of glucocorticoids or nonsteroidal anti-inflammatory medications other than low-dose aspirin, or if STEMI had occurred during the index admission. All procedures were approved by the institutional Human Ethics Committee, and written informed consent obtained in all cases.

Patients were prepared for coronary angiography via the femoral artery using the Judkins approach. Heparin, 2000 U, was administered to all patients. After angiography, the coronary sinus (CS) was cannulated via the femoral vein using a 5F Simmons diagnostic angiography catheter, and the position was confirmed with contrast injection. For each patient, blood was drawn at least four times into collection tubes (Vacutainer; BD Biosciences) containing clot activator (for serum) and citrate (for plasma). The first 5 ml of each blood collection were discarded, as was blood that did not aspirate freely. For the measurement of transcoronary gradients, at the completion of the angiography blood was drawn from the aortic root (Ao) followed immediately by the CS. A subgroup of patients with SA who underwent PCI (balloon angioplasty with stent implantation) of the left coronary artery also had paired Ao-CS samples taken 12–60 (mean ± SD, 39 ± 19) min after balloon inflation. An additional six subjects were recruited at the beginning of the study to validate the sampling technique. This group had samples drawn from a femoral vein sheath before administration of heparin and contrast agent, followed by blood drawn from the inferior vena cava using a Simmons diagnostic catheter after administration of heparin and contrast; the CS was not cannulated in this group. After blood collection, citrate tubes were placed on ice; serum tubes were left at room temperature to clot. All samples were then centrifuged within 90 min of collection at 3000 rpm for 25 min; then serum and plasma aliquots were frozen at −80°C. Angiograms were analyzed for extent and severity of CAD by a cardiologist blinded to the assay results, with a validated scoring system (34); numbers of major coronary vessels with ≥50% stenosis were recorded. Patient data are summarized in Table I.

### Table I. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 11)</th>
<th>SA (n = 14)</th>
<th>ACS (n = 9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>52.6 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.9 ± 11.3</td>
<td>61.6 ± 13.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Male</td>
<td>6 (55)%</td>
<td>11 (79)</td>
<td>5 (56)</td>
<td>0.39</td>
</tr>
<tr>
<td>Hypertension</td>
<td>5 (45)</td>
<td>12 (86)</td>
<td>8 (67)</td>
<td>0.08</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>5 (45)</td>
<td>12 (86)</td>
<td>7 (78)</td>
<td>0.08</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (18)</td>
<td>4 (29)</td>
<td>3 (33)</td>
<td>0.66</td>
</tr>
<tr>
<td>History of smoking</td>
<td>5 (45)</td>
<td>8 (57)</td>
<td>5 (56)</td>
<td>0.70</td>
</tr>
<tr>
<td>BMI</td>
<td>30.2 ± 4.5</td>
<td>29.6 ± 4.2</td>
<td>28.4 ± 5.6</td>
<td>0.70</td>
</tr>
<tr>
<td>Aspirin</td>
<td>8 (73)</td>
<td>13 (93)</td>
<td>9 (100)</td>
<td>0.29</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>4 (36)</td>
<td>11 (79)</td>
<td>9 (100)</td>
<td>&lt;0.05 vs controls</td>
</tr>
<tr>
<td>Statin</td>
<td>7 (63)</td>
<td>10 (71)</td>
<td>8 (89)</td>
<td>1.0</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>6 (55)</td>
<td>9 (64)</td>
<td>4 (44)</td>
<td>0.70</td>
</tr>
<tr>
<td>Gensini score</td>
<td>1.0 ± 1.7</td>
<td>25.6 ± 8.7</td>
<td>24.3 ± 18.5</td>
<td>&lt;0.001 vs controls</td>
</tr>
<tr>
<td>Number of diseased coronary vessels</td>
<td>0 ± 0</td>
<td>1.86 ± 0.77</td>
<td>1.89 ± 0.60</td>
<td>&lt;0.001 vs controls</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SD.

<sup>b</sup> Numbers in parentheses, percent.

BMI, body mass index; Statin, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor; ACE, angiotensin converting enzyme. Diseased coronary vessel is defined as having at least one stenosis ≥50% luminal diameter reduction.
Serum soluble RAGE (sRAGE) was measured by ELISA (R&D Systems), and plasma tryptase was determined by fluorimmunoassay (UniCAP).

**S100A12 and CRP quantification**

Briefly, serum CRP was measured by nephelometry; the data used were generated as part of another study (35). Venous, aortic, and CS sera were assayed for S100A12 by double-sandwich ELISA using recombinant S100A12 as standard (7).

**Heparin affinity chromatography**

An Econopac heparin carboxylic (Bio-Rad) was loaded with 20 μg of S100A12 in HEPES buffer ± 15 μM Zn²⁺ and eluted with a gradient of 0–2 M NaCl in 2.5-ml fractions. Aliquots (500 μl) were transferred onto a polyvinylidene difluoride membrane using a dot-blotting apparatus (Bio-Rad) and immunoblotted with anti-S100A12 IgG.

**Arterial specimens**

Carotid artery specimens containing atherosclerotic lesions and apparently normal mural areas, collected according to the Declaration of Helsinki, were from 16 nondiabetic patients (8 male, 8 female, ages 32–68 years) undergoing endarterectomy for carotid stenosis. Processing and immunostaining were as reported (7).

**Immunohistochemistry**

Carotid artery specimens from patients undergoing endarterectomy were processed, and immunostaining was as reported (7). Briefly, 2-μm serial sections were heat-treated in citrate buffer (pH 6.0) for Ag retrieval. Anti-S100A12 IgG (10 μg/ml), anti-hex-S100A12 IgG (10 μg/ml), mouse anti-human RAGE (mAbA11; Chemicon; 1/400 v/v), mouse anti-MMP-2 (42-5D11, Calbiochem; 1:200 v/v), mouse anti-MMP-9 (IM37L; Calbiochem; 1/200 v/v) and mouse anti-CD68 monoclonal IgG (KP1; Dako; 1/50 v/v) were used. Negative controls were primary Ab from nonimmune rabbit IgG or mouse IgG isotype control. Secondary Abs were biotinylated goat anti-rabbit or anti-mouse IgG (Vector Laboratories and Chemicon), visualized using the alkaline phosphatase red (Vector Laboratories) system or the HRP-3,3'-diaminobenzidine (Dako) system followed by Mayer’s hematoxylin counterstaining.

**Immunoblotting**

Diseased carotid specimens from eight endarterectomy patients were processed as described (31), and 40–50 μg total protein separated by SDS-PAGE and Western blotting was performed. Human monocytic (apoE3/E3 phenotype) from mononuclear cell concentrates from healthy donors were differentiated into macrophages for 8 days (36). Cells were untreated or enriched with cholesterol by adding 50 μg/ml acetylated low-density lipoprotein (in RPMI 1640 containing 10% lipoprotein-deficient serum) for 24 h and then harvested, washed, and lysed; equal protein amounts (75 μg) were analyzed by Western blotting.

**Human monocyte-derived macrophages, PBMCs, and THP-1 differentiation and stimulation**

Human PBMCs isolated using Ficoll-paque plus (GE Healthcare) were cultured in RPMI 1640 with 2% bovine calf serum (BCS) and stimulated with multiple batches of recombinant S100A12 (1 μM) or LPS (10 ng/ml; Sigma-Aldrich) for 4 h before extracting total RNA, or supernatants were collected after 24 h for protein assay.

Human monocytes from mononuclear cells were differentiated into macrophages in 12-well plates (Greiner Bio-One) for 3 days in RPMI with 10% autologous heat-inactivated (56°C, 30 min) human serum and 25 ng/ml GM-CSF (R&D Systems) or 50 ng/ml M-CSF (R&D Systems) and then for 4 days in RPMI with 10% autologous serum (36). After 7 days human monocyte-derived macrophages (HMDM) were stimulated with S100A12 (1 μM) and/or TNF-α (25 ng/ml; R&D Systems) for 4 and 24 h before extracting total RNA, or supernatants collected after 24 h for protein assays.

**The THP-1 human monocyteid cell line (American Type Culture Collection; TIB-202), was maintained in RPMI 1640 supplemented with 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 10% BCS. Cells (5 × 10⁶/ml) were differentiated to macrophages (37) by incubating for 72 h with 10⁻³ M 1,25-dihydroxycholecalciferol (vitamin D₃; Sigma-Aldrich); THP-1 macrophages (10⁶/ml) washed and resuspended in RPMI 1640 with 1% BCS were dispensed into 96-well flat-bottom plates (Greiner Bio-One) and stimulated with S100A12 (1 μM) or LPS (10 ng/ml). Media were sterilized by filtration through Zetapore membranes (0.2 μm pore size; Cuno) to remove contaminating LPS.

Media, S100A12, and reagents were routinely monitored (Limulus amebocyte lysate assay) and discarded if LPS was >10 pg/ml. IL-8 in supernatants harvested 24 h poststimulation was measured using a Duoset IL-8 ELISA kit (R&D Systems) according to the manufacturer’s instructions. Zymography was performed on supernatants as described (38).

**Real-time PCR**

RNA was isolated and real-time PCR performed as described (39). β-Actin was used as endogenous control. Primer sequences were: IL-8, 5'-CAC GATGACCTGTCATCACTA-3' and 5'-GTGCTGCTATCATGTGCTT-3'; IL-6, 5'-AACCTGACCTTCCAAAGATGG-3' and 5'-TCTGG CTTGTTTCTCTACT-3'; IL-8, 5'-GCTCCTGTGTTTGTTCGAC-3' and 5'-TGACTGACATCTAATGAATGAC-3'; TNF-α, 5'-ATGACGACTGAAGCATTG-3' and 5'-GAGGCGTGATTAG AGAGAGTC-3'; β-actin, 5'-AGTTGACGGTTCGACATCCCGT-3' and 5'-GCCAGAGCAATCTCTCCCT-3'; MMP-2, 5'-ATAACCCTGGA TGCCTGCTGT-3' and 5'-AGGACCCTTGAAGAATGAC-3'; MMP-3, 5'-ATGCGAAAAGGATACACACAGGA-3' and 5'-TGTGAGTGTAG GTAAGTGGG-3'; MMP-9, 5'-GGGACGCACGACTGCTAC-3' and 5'-TCGTGCTGCGAATTGGG-3'; MMP-13, 5'-ACTGAGAG GCTTCGCAAAGATG-3' and 5'-TGTATACGTCGAAGTTTGCCATGC-3'.

**MMP assays**

Recombinant MMP-2 or MMP-9 (100 ng/ml; Calbiochem) were activated with 2 mM 4-aminophenylmercuric acetate (APMA; Sigma-Aldrich) for 8 h at 25°C, diluted in assay buffer (50 mM Tris-HCl, 10 mM CaCl₂, 0.1% Brij 35, pH 7.5) to 5 ng/ml, and assayed at 25°C ± S100A12, Zn²⁺ or 1.10-phenanthroline, using a fluorogenic EnzChek Gelatinase assay kit (Invitrogen). Recombinant MMP-3 (5 μg/ml; R&D Systems) was activated for 30 min at 37°C with 50 pg of chymotrypsin; then PMSF (Sigma-Aldrich) was added to 2 mM, the enzyme was diluted in assay buffer to 100 ng/ml, and MMP-3 activity was assayed using a thiopeptide substrate (Bio- mol International) according to the manufacturer’s instructions. HMDM culture supernatant diluted 1/6.25 (v/v) was activated with APMA as for recombinant MMPs, diluted to 1/100 (v/v) with assay buffer, and assayed using the EnzChek assay. GraphPad Prism (V3–Windows GraphPad) was used to fit nonlinear regression curves of dose responses using the Hill equation (40).

**Chemical cross-linking and Western blotting**

Lyophilized S100A8, S100A9, or S100A12 was reconstituted (100 μg/ml) in 20 mM HEPES, 150 mM NaCl, pH 7.5 (HEPES buffer), with 1 mM CaCl₂, 15 μM CuCl₂, or 15 μM ZnCl₂, and divalent ions were left to bind for 30 min at room temperature. Proteins were then cross-linked with 5 mM amine-reactive cross-linker bis(sulfosuccinimidyl)suberate (BS³⁺; Pierce) for 30 min at room temperature in the dark. Cross-linking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Pierce Chemical) was performed as for BS³⁺ cross-linking except the buffer used for resuspension was 20 mM N-morpholinoethanesulfonic acid, 150 mM NaCl (pH 6.0), and 5 mM EDC was added after cation binding. Complexes were resolved on 10% SDS-PAGE gels and either silver stained (41) or analyzed by Western blotting with anti-S100A12, or anti-hex-S100A12.

**Statistical analysis**

Data are expressed as means ± SEM. Comparisons between groups were assessed by Student’s t test or the Mann-Whitney U test and between multiple groups by one-way ANOVA for parametric data (p < 0.05 considered significant). Categorical variables were compared using Fisher’s exact test. Paired A-OCS concentrations were compared using the paired t test. Correlations were analyzed using Pearson’s test, and nonparametric data were logarithmically transformed or correlated using Spearman’s test.

**Results**

**S100A12 is elevated in peripheral venous sera from patients with CAD and is released from sites of spontaneous or mechanical plaque rupture**

Mori et al. (2) recently showed a correlation between intimal medial thickness and plasma S100A12 levels in patients with end-stage renal disease on hemodialysis. We first wanted to investigate a possible correlation between atherosclerosis and circulating S100A12 protein in a wider cohort. Fig. 1A shows that S100A12...
levels in sera from patients with CAD (775.0 ± 87.6 ng/ml) were significantly higher \((p = 0.0002)\) than levels in sera from normal subjects (282.1 ± 22.9 ng/ml), although no significant difference was observed between the SA and ACS groups. In the total 45 subjects (19 SA, 13 ACS and 13 normal), we found a positive correlation between serum S100A12 and CRP levels (Fig. 1B; Spearman \(r = 0.36, p = 0.016\)).

To investigate the relative contributions of plaque-associated and circulating leukocytes to S100A12 levels in coronary disease, we measured serum S100A12 in the Ao and CS in patients with and without angiographically demonstrable CAD. In our validation cohort, serum S100A12 and plasma tryptase levels did not change after administration of heparin and contrast medium \((p = 0.61\) and 0.56, respectively), and there were no differences between measurements taken from the diagnostic catheter compared with the femoral sheath.

There were no significant differences in demographics or cardiovascular risk factors between subject groups (Table I). Subjects with demonstrable coronary disease were more likely to be taking clopidogrel than controls; use of other medications did not differ between groups. SA and ACS groups both had more severe coronary disease than controls, as shown by higher Gensini scores, but the extent of coronary atherosclerosis was similar in SA and ACS groups. Similar to the cohort studied with only peripheral venous sampling, in this cohort, aortic S100A12 concentration was higher in both SA and ACS than in controls, whereas levels in SA and ACS were similar (data not shown). In ACS and patients with SA following PCI, S100A12 levels in the CS were consistently and significantly higher than those in the Ao; this pattern was not seen in control or SA groups (Fig. 1C). In the PCI group, the increase in CS S100A12 was seen within 1 h of plaque disruption. Myocardial necrosis was not a prerequisite for the increased S100A12 gradient, given that none of the PCI patients had a postprocedure troponin rise and, within the ACS group, all subjects demonstrated a transcoronary increase in S100A12, irrespective of evidence of myocardial necrosis before blood sampling. In keeping with the association between S100A12 and CAD, there was a weak positive correlation between aortic S100A12 levels and the number of stenosed coronary arteries (Spearman \(r = 0.35; p = 0.04\) but...
not with Gensini score ($p = 0.42$). Systemic S100A12 showed no relation to gender ($p = 0.62$), presence of diabetes ($p = 0.69$), or other risk factors including history of smoking or hyperlipidemia or family history of CAD.

Because S100A12 activates mast cells (9), we measured serum tryptase levels. Aortic tryptase was similar in all subject groups (6.3 ± 5.0 μg/L in controls, 6.3 ± 3.7 μg/L in SA, 3.9 ± 1.9 μg/L in ACS; $p = 0.28$), and tryptase in CS blood was not increased in any patient group. Similarly, because the antagonist sRAGE may modify function (10), the relationship with sRAGE was assessed. Aortic sRAGE did not differ between groups (2263 ± 843 pg/ml in controls, 2561 ± 970 pg/ml in SA, 2145 ± 714 pg/ml in ACS; $p = 0.50$) and was similar in diabetics and nondiabetics (2186 ± 803 and 2422 ± 888 pg/ml, respectively; $p = 0.75$). No significant transcoronary sRAGE gradient was seen in any subject group. No relationship was found between sRAGE and S100A12 levels in the overall group.

Collectively these results confirm that S100A12 is released from sites of atherosclerotic plaque rupture.
S100A12 colocalizes with RAGE

To confirm previous results demonstrating colocalization of S100A12 with RAGE in atheroma (15), we performed on sections of human carotid artery. Strong anti-S100A12 immunoreactivity was evident in FCs in atherosclerotic plaque (Fig. 2A) and S100A12+ cells were prominent around neovessels (Fig. 2B). RAGE was absent in nonatherosclerotic areas (not shown) but variably and weakly expressed in atherosclerotic areas in 16 specimens. RAGE colocalized on some S100A12+ macrophage-like cells in three of six specimens (the 6 patients had no documented diabetic history; Fig. 2C). Anti-S100A12 immunoreactivity was evident in some CD68+ macrophages by double immunostaining (Fig. 2D). S100A12+ cells were located along the arterial lumen in early and advanced lesions, and S100A12 was weakly expressed by some microvascular ECs and smooth muscle cells (Fig. 2B).

Overall, there was high expression of S100A12 in all specimens (n = 16) containing advanced lesions. Arteries lacking features of atherosclerotic transformation did not contain cells expressing S100A12 in the intima (not shown).

Because we found extensive S100A12 staining on the extracellular matrix (ECM), we investigated its affinity for heparin. S100A12 bound heparin-Sepharose and eluted between 0.625 and 1 M NaCl (Fig. 2E), indicating relatively high-affinity binding. Zn2+ did not change the elution profile of S100A12.

Western blotting of carotid plaque extracts revealed varying levels of a component with the expected mass of S100A12 (10 kDa) and complexes (25, 37, 56, and 70 kDa), evident in six of eight samples (four shown; Fig. 2F), indicating interspecimen variability. Complexes resistant to DTT reduction. Because S100 proteins can undergo oxidative cross-linking by hypochlorite, HOP-1 was used to identify HOCl-modified proteins (31, 33); reactivity was weak and variable between specimens and HOP-1-reactive complexes (32, 52, 68, and 89 kDa; not shown) were of sizes distinct from anti-S100A12-reactive complexes.

S100A12 does not induce proinflammatory cytokines in human macrophages or PBMCs

S100A12 is proposed to induce proinflammatory genes in macrophages via ligation of RAGE. Because this function is based on studies with bovine S100A12 on murine macrophages (10), we wanted to confirm this in a homologous system. M-CSF promotes survival of foam cells, modulates cytokine production in mature macrophages and is implicated in the early stages of atherogenesis (42). GM-CSF is also abundant, although its role is ambiguous.
because exogenous administration or gene deletion of GM-CSF increased atherosclerotic lesion size in ApoE<sup>−/−</sup> mice (42). We therefore tested the effects of S100A12 on macrophages differentiated with GM-CSF or M-CSF. When HMDM from three donors were stimulated with S100A12, there was no induction of IL-1β (not shown) or TNF-α mRNA at 4 or 24 h (Fig. 3, A and B) poststimulation. Because TNF-α also contributes to atherogenesis (42), we tested whether S100A12 could potentiate this response, thereby enhancing the autocrine loop; no significant enhancement was seen (Fig. 3, A and B).

We next stimulated PBMCs with nine different batches of LPS-free S100A12 but found no induction of IL-1β or TNF-α (Fig. 3, C and D; six batches shown). Similarly, S100A12 did not induce IL-6, IL-8, or TNF-α in PBMCs (not shown) or stimulate secretion of IL-8 by undifferentiated or vitamin D<sub>3</sub>-differentiated THP-1 macrophages (Fig. 3E). In contrast, the expected responses to LPS were obtained. It is unlikely that this lack of activity was due to incorrect folding of the recombinant protein because several of the same batches were active in chemotaxis assays using THP-1 cells and provoked mast cell-dependent edema in mice (9).

**S100A12 does not regulate MMP expression in macrophages**

Because S100A8 or S100A8/A9 elevated MMP-3, -9, and -13 mRNA levels in murine bone-marrow-derived macrophages (16), we investigated whether S100A12 had a similar function. Because TNF-α induces MMP-9 in macrophages (43), we also tested whether S100A12 could potentiate this response. HMDMs, differentiated with GM-CSF or M-CSF, were stimulated with S100A12 and TNF-α ± S100A12 for 4 and 24 h and mRNA levels of MMP-2, -3, -9, and -13 measured by real time RT-PCR. Secreted MMP-2 and MMP-9 were assessed by gelatin zymography.

MMP-9 mRNA was the most abundant of the MMP mRNAs tested in the two macrophage populations and results are shown in Fig. 4A. S100A12, either alone or with TNF-α, had no significant effect on mRNA levels of MMP-9 (Fig. 4A), MMP-2, MMP-3, or MMP-13 (not shown). Similarly, MMP-2, -3, -9, and -13 and TIMP-1 and -2 mRNA were unchanged by S100A12 in vitamin D<sub>3</sub>-differentiated THP-1 cells (not shown). Quantification of zymograms indicated a modest but consistent increase (1.4-fold) in MMP-9 (Fig. 4, B and D), but not MMP-2 (Fig. 4C), in supernatants from GM-CSF-generated HMDM harvested 24 h poststimulation. Changes were much less pronounced in HMDM differentiated with M-CSF but followed the same trend. S100A12 did not potentiate MMP-9 levels induced by TNF-α. Although levels were lower than pro-MMP-9, activated MMP-9 was only elevated in supernatants from GM-CSF-HMDM stimulated by TNF-α, and S100A12 did not alter these (Fig. 4, C and E).

**S100A12 inhibits MMP-2 and MMP-9**

Because S100A12 binds Zn<sup>2+</sup> we investigated whether S100A12 could influence MMP activity. No effect was seen using standard gelatin zymography, possibly because S100A12 is not stabilized by disulfide bonds and SDS required for PAGE may disrupt its Zn<sup>2+</sup>-binding capacity. Therefore, a sensitive fluorogenic assay was used to determine how S100A12 affected the gelatinolytic activities of MMP-2 and MMP-9. Titration of S100A12 revealed that inhibition was nearly all-or-none, with a Hill slope of −9.5 for MMP-2 and -8.1 for MMP-9 (Fig. 5, A and B), indicating strong cooperative inhibition. A chromogenic assay showed that S100A12 also inhibited MMP-3 and that the Hill slope was steeper (−24.4; Fig. 5C). The steepness of the Hill slope is an indication of cooperativity between binding sites (40). In contrast to S100A12, the Zn<sup>2+</sup> chelator 1,10-phenanthroline yielded a more standard inhibition curve with a Hill slope of −2.2 (Fig. 5D).

The IC<sub>50</sub> of S100A12 was similar for MMP-2 (0.16 ± 0.0052 μM) and MMP-9 (0.18 ± 0.0049 μM) and higher for MMP-3 (0.40 ± 0.017 μM); maximal inhibition was somewhat greater for MMP-2 (94%) and MMP-3 (96%) compared with MMP-9 (81%). Heparin did not affect the IC<sub>50</sub> of S100A12 for MMP-9 (not shown). Zn<sup>2+</sup> reversed the inhibition of MMP-2 in a dose-dependent manner (Fig. 5E), indicating that S100A12 inhibited MMPs by chelating Zn<sup>2+</sup>.

Macrophages are the dominant source of gelatinases in atherosclerotic plaque (24). Zymography confirmed that the gelatinase activity in macrophage supernatants was predominantly due to MMP-9, given that its gelatinolytic component corresponded to the
mass of recombinant MMP-9 (Fig. 4, B and C), and the enzyme was predominantly latent (Fig. 4C). Gelatinase activity in APMA-activated supernatants was reduced by 85% when 1 μM S100A12 was included (Fig. 5F). The Zn²⁺ chelator 1,10-phenanthroline (1 mM) abolished the gelatinase activity, excluding the possibility that other proteinases were contributing to the overall activity (not shown).

**S100A12 forms a Zn²⁺-induced oligomeric complex**

The steepness of the Hill slope for inhibition of MMPs by S100A12 implies a highly cooperative process, possibly via allosteric interactions between subunits of a complex. S100A12 forms Ca²⁺-induced hexameric complexes (44); and as an initial approach to characterizing ion-induced changes in quaternary structure, chemical cross-linking was performed using BS³, an amine-reactive chemical cross-linker with an 11.4-Å spacer arm. Fig. 6A shows that, in the absence of Ca²⁺, cross-linked S100A12 was predominantly dimeric and Ca²⁺ (1 or 5 mM) generated low levels of tetramer (~40 kDa). In contrast, Zn²⁺ generated larger complexes of ~40, 50, and 60 kDa and inclusion of Ca²⁺ had no further effect. S100A12 also binds Cu²⁺ (45); cross-linking with BS³ in the presence of CuCl₂ indicated a relatively low propensity for 40- to 60-kDa complex formation compared with Zn²⁺ (Fig. 6A).

EDC is a zero-length cross-linker that creates a peptide bond between side chains with carboxyl and amino groups that are in close proximity. In contrast to BS³, EDC cross-linked multiple complexes up to the hexameric size in the presence of Ca²⁺, whereas only dimers and tetramers were detected when Zn²⁺ was included (Fig. 6B). When Zn²⁺ and Ca²⁺ were combined, the pattern of complexes was similar to Ca²⁺ alone (not shown). As expected, EDC was a less efficient cross-linker than BS³ as evidenced by the un-cross-linked monomer found in all samples. However, these results confirm that Ca²⁺ can induce formation of hexameric S100A12 as previously published (44, 46).

A polyclonal Ab that predominantly reacted with the tetrameric/hexameric forms of S100A12 was generated (anti-hexS100A12). Remarkably, this Ab did not cross-react with other quaternary structures of S100A12 or other calgranulins (Fig. 6C).

**Zn²⁺-dependent S100A12 complexes in lipid-loaded macrophages**

To determine whether Zn²⁺-dependent S100A12 complexes could form in macrophages, cell lysates were subjected to anti-S100A12 Western blotting. HMDMs or lipid-loaded HMDMs cultured in
We next attempted to identify Zn\(^{2+}\) and MMP-9. Anti-hexS100A12 showed similar reactivity with preimmune IgG showing the component with mass slightly larger than the 70-kDa component (Fig. 7B). Complexes detected in macrophage populations; weakly reactive complexes were also detected at 25 and 50 kDa in the lipid-loaded macrophage lysates (Fig. 7B). Blotting with preimmune IgG showed the component with mass slightly larger than the 70-kDa component (Fig. 7B, right). Complexes detected in macrophage and lipid-loaded macrophage lysates were of sizes similar to those detected in the carotid plaque extracts (Fig. 2F).

Zn\(^{2+}\)-dependent S100A12 complexes colocalize with MMP-2 and MMP-9

We next attempted to identify Zn\(^{2+}\)-dependent complexes in atherosclerotic plaque. Overlapping localization of S100A12, MMP-2, and MMP-9 was evident in serial sections of human atheroma (Fig. 8), mostly in CD68\(^+\) macrophages and FCs. Although staining was less intense, anti-hexS100A12 showed similar reactivity as S100A12, providing the first evidence for the formation of a Zn\(^{2+}\)-containing S100 complex in vivo (Fig. 8). In contrast to MMP-9, MMP-2 reactivity was weak, consistent with the zymographs of human macrophage supernatants, which indicated a size similar to those detected in hippocampal extracts (51); the nature of these complexes is unclear. The 60-kDa complex detected in macrophage lysates and plaque extracts is the size of hexameric S100A12; the 120-kDa complex could be a dimer of hexamers (Fig. 7). S100A12 has no cysteine residues, excluding disulfide bond formation, although sulfanamide cross-linking between lysine residues and reactive cysteine in other proteins such as S100A8 (52), dityrosine cross-linking, or transglutamination could generate reduction-resistant complexes as demonstrated for other S100 proteins (53).

Although S100A12 was expressed by macrophages in atheroma expressing RAGE, and elevated serum levels were associated with CAD, we could not substantiate the widely held assumption that it may contribute to atherogenesis by ligating RAGE to promote inflammation in the vessel wall. In contrast to the single report indicating that bovine S100A12 activated murine BV-2 microglial cells via RAGE (10), we were unable to induce IL-1\(\beta\) or TNF-\(\alpha\) in human macrophages, or proinflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), IL-6, IL-8, or MCP-1 in PBMCs or THP-1 monocyteoid cells differentiated to macrophages (Fig. 3). Experiments with RAGE\(^{-/-}\) mice indicate that RAGE ligation is unlikely to be involved in some cell-mediated immune responses (54), such as those proposed for development of atherosclerosis, and additional receptors for putative RAGE ligands are proposed. Importantly, we find S100A12-mediated activation of mast cells (9) and monocyte chemotaxis (11) are also RAGE-independent processes.

Human S100A8/A9 was suggested to inhibit MMPs by chelating Zn\(^{2+}\) (26). On the other hand, murine S100A8 induced MMP-3, -9, and -13 in murine bone marrow-derived macrophages (16). Here we show that S100A12 strongly suppressed MMP-2, MMP-9, and MMP-3 activities by chelating Zn\(^{2+}\) (Fig. 5) but did not influence MMP expression in macrophages (Fig. 4). The gradient of the Hill slopes (−9.5 for MMP-2, −8.1 for MMP-9, and −24.4 for MMP-3) suggests a cooperative mechanism of inhibition (40). A model in which hexameric S100A12 would have a much higher affinity for Zn\(^{2+}\) than the dimeric form, and in which hexamer formation is a highly cooperative process requiring a certain threshold concentration of S100A12, would fit the data. Although this hypothesis has interesting implications for other Zn\(^{2+}\)-dependent processes that may be regulated by S100A12, testing these was beyond the scope of this study.

*FIGURE 8.* Colocalization of S100A12, hexameric S100A12 (S100A12X), MMP-9, and MMP-2 in CD68\(^+\) cells in serial sections of human atheroma. Preimmune IgG was the control. ×1000.
Excessive activity of macrophage-derived MMP-2 and MMP-9 may weaken the fibrous cap and promote plaque rupture (18). In rodent models, overexpression of MMP inhibitors, including TIMP-1 or TIMP-2, protects from atherosclerosis and plaque rupture (55, 56). Because S100A12 was expressed by activated macrophages and FCs (Fig. 2), and the oligomeric Zn$^{2+}$-induced form colocalized with MMP-2 and MMP-9 in atheroma (Fig. 8), S100A12 may play in concert with TIMPs to stabilize plaques. Oligomeric S100A12 was also present in macrophages and FCs in lesions, where it colocalized with MMP-9, and in HMDM in vitro (Fig. 7), confirming generation of the Zn$^{2+}$-bound complex within cells.

S100A12 may play a pleiotropic role in atherogenesis. Our results indicate that S100A12 likely does not play a significant role in activating proinflammatory gene production in macrophages, although low concentrations are chemotactic for monocytes and mast cells (11) and may contribute to their accumulation. Higher levels may cause changes in the microcirculation by provoking mast cell activation (11) that potentiate atherogenesis (57). Conversely, activated macrophage- and FC-derived S100A12 may protect vulnerable plaque from rupture by inhibiting MMP activities.

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References


