Lactadherin Deficiency Leads to Apoptotic Cell Accumulation and Accelerated Atherosclerosis in Mice

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Background—Atherosclerosis is an immunoinflammatory disease; however, the key factors responsible for the maintenance of immune regulation in a proinflammatory milieu are poorly understood.

Methods and Results—Here, we show that milk fat globule-EGF factor 8 (Mfge8, also known as lactadherin) is expressed in normal and atherosclerotic human arteries and is involved in phagocytic clearance of apoptotic cells by peritoneal macrophages. Disruption of bone marrow–derived Mfge8 in a murine model of atherosclerosis leads to substantial accumulation of apoptotic debris both systemically and within the developing lipid lesions. The accumulation of apoptotic material is associated with a reduction in interleukin-10 in the spleen but an increase in interferon-γ production in both the spleen and the atherosclerotic arteries. In addition, we report a dendritic cell-dependent alteration of natural regulatory T-cell function in the absence of Mfge8. These events are associated with a marked acceleration of atherosclerosis.

Conclusions—Lack of Mfge8 in bone marrow–derived cells enhances the accumulation of apoptotic cell corpses in atherosclerosis and alters the protective immune response, which leads to an acceleration of plaque development.

Key Words: atherosclerosis ■ apoptosis ■ immune system ■ inflammation ■ interleukins

A well-recognized characteristic of atherosclerotic plaques is the accumulation of cell debris during plaque progression toward the advanced stages. Cell debris mostly results from the accumulation of apoptotic lipid-laden macrophages, leading to the formation of an acellular lipid core. Membranes of apoptotic cells are particularly rich in proinflammatory oxidized phospholipids, which suggests that accumulation of apoptotic cells and debris within the plaque might increase the risk of plaque progression and complications.

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Phagocytic clearance of apoptotic cells by professional scavengers is the most important determinant of the steady state and the physiological consequences of apoptosis in vivo, suppressing proinflammatory signaling and activating antiinflammatory pathways. However, the mechanisms that control apoptotic cell accumulation in this disease and their direct impact on disease activity and progression are currently unknown. Milk fat globule-EGF factor 8 (Mfge8, also known as lactadherin) expression by macrophages promotes the phagocytosis of apoptotic thymocytes by forming a bridge between phosphatidylserine on apoptotic cells and integrin on phagocytes. Here, we tested our hypothesis that Mfge8 expression by macrophages controls the accumulation of apoptotic membrane fragments in atherosclerotic plaques and substantially limits lesion development.

Methods

Animals

The generation of Mfge8−/− mice has been described previously. Eight-week-old low-density lipoprotein (LDL) receptor–deficient (Ldlr−/−) C57BL/6 (10× backcross) mice (Charles River Laboratories, l'Abresle, France) were subjected to medullar aplasia by 9.5 Gy of lethal total body irradiation. The mice were repopulated with an intravenous injection of bone marrow cells isolated from femurs and
tibias of sex-matched C57BL/6 Mfge8−/− mice or wild-type littermates. After 4 weeks of recovery, mice were fed a proatherogenic diet that contained 15% fat, 1.25% cholesterol, and 0% cholate for 8, 15, or 19 weeks.

**Extent and Composition of Atherosclerotic Lesions**

 Plasma cholesterol was measured with a commercial cholesterol kit (bioMérieux, Marcy l’Etoile, France). Lesion size and composition were assessed as described previously.8,10 Active caspase-3 was detected with a specific polyclonal rabbit antibody (Cell Signaling Technology, Danvers, Mass). Mfge8 expression was detected with a specific anti-Mfge8 antibody.8 Terminal dUTP nick end-labeling (TUNEL; ApopDETEK kit, ENZO Diagnostics, DAKO, Trappes, France) was considered as positive when at least 2 of 6 sections per mouse showed TUNEL staining.

**Cell Recovery and Purification, Cell Culture, Proliferation, and Cytokine Assays**

Methods used for cell purification from spleen and peripheral lymph nodes, their culture, stimulation, and assessment of T-cell proliferation and cytokine production were described previously in detail.10

**Bromodeoxyuridine Labeling and Cell Cycling Analysis**

This experiment was performed as described previously before animals were euthanized on Ldlr−/− mice reconstituted with either Mfge8−/− or Mfge8+/+ bone marrow and put on a high-fat diet for 10 weeks.

**Flow Cytometry**

Spleen or lymph node cells were assayed for CD4, CD25, and Foxp3 expression as described previously.10 Additional stainings were performed, particularly CD69 (H1.2F3, BD Biosciences, Rungis, France).

**Real-Time Polymerase Chain Reaction Analysis**

Total RNA was recovered from spleen, thoracic aorta, or human carotid plaques. The real-time polymerase chain reaction for Mfge8, Del-1, Hprt, GAPDH, and 18S was performed as described previously.8,10

**Plasma Microparticles**

Microparticles of platelet-free plasma were positively labeled with fluorescein-conjugated annexin V. They were analyzed on an EPICS XL flow cytometer (Beckman Coulter, Roissy, France).12

**Apoptosis Studies**

Bone marrow–derived macrophages were incubated with or without copper-oxidized human LDL at 100 μg/mL for 48 hours.13 In additional experiments, murine red blood cells were used fresh or were artificially aged by Ca2+ oxidation.14 Fresh or oxidized red blood cells were incubated with murine macrophages, and apoptosis was assayed 24 hours later. Eight different wells were examined for each experimental condition.

**Phagocytosis Studies**

Mfge8−/− (n=6) and Mfge8+/+ (n=6) mice received an intraperitoneal injection of apoptotic (dexamethasone-treated) carboxylfluorescein succinimidyl ester (CFSE)-labeled thymocytes (20×10⁶) or saline 3 days after thioglycollate (7%, 2 mL) injection. Phagocytosis of CFSE-positive cells by CD11b-positive macrophages was quantified 1 hour later by flow cytometry.

**Human Atherosclerotic Tissue**

Tissue sections were prepared from human carotid or coronary atherosclerotic plaques, as described previously.15 Mfge8 expression was examined either by Western blot or immunohistochemistry, as described previously.8

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**Statistical Analysis**

Values are expressed as mean±SEM. Differences between values were examined with the Mann-Whitney test. Differences in the percentage of lesions showing TUNEL positivity were compared with the Fisher exact test. Probability values were considered significant at *P*<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Mfge8 Is Expressed in Normal and Atherosclerotic Human Arteries**

We first examined Mfge8 expression in normal and atherosclerotic human arteries.8 As expected,8 Western blots...
Accelerated Atherosclerosis and Formation of Large Acellular Cores With Apoptotic Debris in the Absence of Mfge8

We next examined the direct physiological role of Mfge8 expression in the development of atherosclerosis. In the initial disease stages, fatty streaks are mostly composed of infiltrating macrophages that ingest oxidized lipids.18,19 Thus, we examined the potential role of these phagocytes in the clearance of apoptotic cells within the developing lesion. We irradiated and reconstituted atherosclerosis-susceptible Ldlr−/− mice with either a wild-type or Mfge8−/− deficient bone marrow. After recovery, the chimeric mice were put on an atherogenic diet. This model may be preferable to that with apolipoprotein (Apo)E deficiency, because ApoE per se, but not Ldlr, modulates clearance of apoptotic bodies in vitro and in vivo, which results in a systemic proinflammatory state in ApoE-deficient mice.20

After 8 weeks of diet, female Ldlr−/− mice with Mfge8−/− bone marrow developed typical fatty streak lesions in the aortic sinus (Figure 2A), rich in lipid-laden macrophages, with marginal accumulation of smooth muscle cells and collagen (Table 1). These early lesions did not show large acellular cores and stained negative for TUNEL (no TUNEL positivity in 7 tested mice, 0%), which indicates the absence of detectable cell death (Figure 2B). In contrast, we detected a marked accumulation of TUNEL-positive cells in the lesions of mice reconstituted with Mfge8−/− bone marrow (4 of 6 tested mice with TUNEL-positive lesions, 66%; P=0.02 versus Mfge8+/− mice), which led to the formation of large acellular cores with TUNEL-positive debris (Figure 2B and 2D), a feature of the “necrotic” lipid core. Detection of TUNEL positivity was associated with the detection of active caspase-3 (Figure 2C), a feature of apoptosis. We also assessed the level of phosphatidylserine-bearing apoptotic microparticles on platelet-free

<table>
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<th>TABLE 1. Weight, Plasma Cholesterol Levels, Lesion Size, and Composition in Ldlr−/− Mice Reconstituted With Mfge8+/− or Mfge8−/− Bone Marrow and Fed a High-Fat Diet for 8 Weeks</th>
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<td>Weight, g</td>
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<td>Total cholesterol, g/L</td>
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<td>Percent macrophages (MOMA-2)</td>
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*P<0.01.
plasma and found a marked accumulation of this apoptotic and thrombogenic material in the peripheral blood of mice with Mfge8−/− bone marrow (Figure 2E). Interestingly, despite similar serum cholesterol levels (Table 1), the accumulation of apoptotic debris in mice with Mfge8−/− bone marrow was associated with a marked 70% increase in lesion size compared with mice reconstituted with Mfge8+/− bone marrow (P<0.005; Figure 2A; Table 1). We obtained similar results using male Ldlr−/− mice (lesion size 146±294 vs 101±749 μm² in Mfge8−/− and Mfge8+/− mice, respectively, n=4 per group, P<0.05).

We next examined mice euthanized after 15 weeks of high-fat diet. Compared with controls, mice reconstituted with Mfge8−/− bone marrow still showed increased accumulation of TUNEL-positive debris (5 of 7 Mfge8−/− mice with TUNEL positivity [71%] versus 1 of 10 Mfge8+/− mice [10%], P<0.05; Figure 3D) and showed larger acellular cores (Data Supplement Figure IV). In addition, the TUNEL-positive area was much larger in these lesions than in lesions of Mfge8+/− mice (Figure 3D). This was associated with a significant increase in lesion size (Figure 3A) and a progression toward a mature phenotype, as revealed by the increase in smooth muscle cell and collagen contents (Figure 3B and 3C; Table 2). We also examined mice euthanized after 19 weeks of high-fat diet and obtained very similar results (lesion size 575±24 885 μm² versus 372±26 374 μm² in Mfge8+/− [n=4] and Mfge8−/− mice [n=5], respectively, P=0.01).

Increased TUNEL detection in lesions of Mfge8−/− mice occurred in the absence of enhanced susceptibility of Mfge8−/− macrophages to apoptosis in vitro (Figure 4A and 4B) and was associated with defective in vivo clearance of
apoptotic thymocytes by Mfge8−/− macrophages (Figure 4C). Thus, the present results suggest that Mfge8-dependence of apoptotic cells critically controls lesion development. The present results may also explain, at least in part, the intriguing and unexpected substantial increase in atherosclerotic lesion formation in mice with αβ3 deficiency,21 a critical integrin required for Mfge8-mediated clearance of apoptotic cells via the RGD domain of Mfge8.7

We have already reported that Mfge8-deficient mice develop autoimmune disease with advanced age (≥40 weeks old),3 like those reported by Hanayama et al.22 However, the Ldlr−/− mice used in the present study were euthanized after only 8 to 19 weeks of Mfge8 deficiency. Thus, they were deficient in Mfge8 for much less than 40 weeks. We examined spleens, anti-sDNA, and anti-dsDNA antibodies in these mice. Mice euthanized after 8 or 15 weeks of high-fat diet did not show splenomegaly (93.7±4.8 versus 91.0±4.5 g in Mfge8−/− and Mfge8+/+ mice after 15 weeks of high-fat diet, respectively, P=0.7) and had no detectable anti-DNA levels. Only mice euthanized after 19 weeks of high-fat diet showed splenomegaly (121.8±7.3 versus 93.0±6.5 g in Mfge8−/− and Mfge8+/+ mice, respectively, P<0.05). Thus, the acceleration of atherosclerosis in this model preceded the appearance of overt autoimmunity.

**Reduced Interleukin-10 Production and Increased Interferon-γ Expression in the Absence of Mfge8**

Phagocytosis of apoptotic cells has immunosuppressive effects on phagocytes, inducing the production of antiinflammatory cytokines, particularly interleukin (IL)-10 and transforming growth factor-β.5,8,23 Inhibition of Mfge8-mediated apoptotic cell phagocytosis by macrophages reduces IL-10 production.24 We examined whether Mfge8 deficiency in bone marrow cells altered the immunoinflammatory response. Splenocytes from mice with Mfge8−/− bone marrow produced similar IL-12 p70 levels but showed a marked reduction of IL-10 production in response to lipopolysaccharide/interferon (IFN)-γ compared with controls (Figure 5A), which suggests a proinflammatory antigen-presenting cell phenotype. Accordingly, CD3-stimulated purified CD4+ T cells from mice reconstituted with Mfge8−/− bone marrow produced similar IFN-γ levels but showed a marked reduction of IL-10 production compared with controls (Figure 5B). Reduced T-cell–derived IL-10 levels were also observed in cells recovered from nontransplanted nonatherosclerotic mice (564.3±31.6 versus 719.0±7.6 pg/mL in Mfge8−/− and Mfge8+/+ lymphocytes, respectively, P<0.05). We did not detect significant transforming growth factor-β production. To assess in vivo cytokine expression, we performed quantitative reverse transcription–polymerase chain reaction on spleen mRNA, because spleen cytokine expression adequately reflects both the systemic and plaque-associated immune response25 and is less subject to sampling errors than plaque cytokine expression. Interestingly, we found a profound decrease in IL-10 mRNA expression (Figure 5C) but a significant increase in IFN-γ levels (Figure 5D) in the spleens of mice reconstituted with Mfge8−/− bone marrow compared with controls. We also
detected a 2-fold increase in IFN-γ mRNA levels in ascending atherosclerotic aortas of mice reconstituted with Mfge8−/− bone marrow compared with controls (Figure 6). These results suggest a switch of the immune response toward a Th1 proinflammatory phenotype in vivo in the absence of Mfge8 expression.

**Reduced Suppressive Activity of Regulatory T Cells in Mice With Mfge8 Deficiency**

Phagocytosis of apoptotic cells has been suggested to play a potential role in the induction of immune tolerance.26,27 Defective apoptotic cell phagocytosis in Mfge8−/− mice could have contributed to the proimmunoinflammatory phenotype of these animals by altering the natural regulatory T-cell function that controls immune homeostasis. We therefore assessed the proliferative potential of CD4+ cells. Using in vivo bromodeoxyuridine staining, we found similar levels of CD4+ proliferation but increased CD69 expression (Figure 7A) in Mfge8−/− compared with Mfge8+/+ chimeras, which suggests enhanced T-cell activation. Effector CD4+CD25− cells purified from chimeric Mfge8−/− mice showed reduced in vitro proliferation compared with chimeric Mfge8+/+ mice, after CD3 stimulation in the presence of CD11c+ dendritic cells (Figure 8A, CD25− alone). Such reduction in effector T-cell proliferation in vitro has been reported previously in mice with alteration in immune regulation and increased T-cell activation.28 The percentage of CD4+CD25+ cells, determined in spleens and lymph nodes, was similar between chimeric Mfge8−/− and Mfge8+/+ mice (Figure 7B). Protein and mRNA expression levels of Foxp3, a transcription factor specific for the regulatory T-cell lineage, were comparable between the 2 groups of mice (Figure 7C and 7D). Remarkably, however, CD4+CD25− cells purified from chimeric Mfge8−/− mice after 8 to 10 weeks of high-fat diet failed to inhibit the proliferation of effector CD4+CD25+ cells (Figure 8A and 8B). This alteration was observed in 4 of 5 different experiments with male or female Mfge8−/− mice but was never observed in 5 different experiments with male or female Mfge8+/+ mice (2 to 3 mice per group in each experiment). In addition, a significant alteration in suppressive T-cell function was observed in pure nonatherosclerotic Mfge8−/− mice compared with wild-type controls (percent inhibition of T-cell proliferation at 1:8 ratio, 0.01.

**Figure 5.** Lactadherin deficiency is associated with a switch of the immune response toward a proinflammatory phenotype. A, Significant reduction in IL-10 production by cultured splenocytes recovered from chimeric Mfge8−/− mice after stimulation with lipopolysaccharide/IFN-γ. B, Significant reduction in IL-10 production by purified CD4+ T cells recovered from chimeric Mfge8−/− mice and stimulated with anti-CD3 antibody. C and D, Results of quantitative reverse transcription-polymerase chain reaction showing significant reduction of IL-10 mRNA expression but upregulation of IFN-γ in the spleens of chimeric Mfge8−/− mice compared with controls. Values are mean±SEM; n=3 per group. **P<0.01.

**Figure 6.** Lactadherin deficiency is associated with increased IFN-γ mRNA expression in atherosclerotic lesions. A, Similar levels of IL-10 mRNA in ascending atherosclerotic arteries of chimeric Mfge8−/− and Mfge8+/+ mice. B, Significant increase in IFN-γ mRNA expression in atherosclerotic lesions of chimeric Mfge8−/− mice after stimulation with Mfge8−/− compared with controls. Values are mean±SEM relative to 18S; n=3 to 6 per group. *P<0.05.
21.7±2.7% versus 46.3±6.6% for Mfge8⁻/⁻ and Mfge8⁺/⁺ cells, respectively, P<0.05). Because alteration of the suppression potential of regulatory T cells in vitro was observed despite normal Foxp3 levels, we hypothesized that alteration in dendritic cell function might be the missing link. Thus, regulatory T cells or dendritic cells from either Mfge8⁻/⁻ or Mfge8⁺/⁺ chimeras were intercrossed in the coculture suppression experiment. As shown in Figure 8C, CD25⁺ regulatory T cells from Mfge8⁻/⁻ mice recovered their suppressive potential when incubated with wild-type dendritic cells. In addition, CD25⁺ regulatory T cells from Mfge8⁺/⁺ mice showed reduced suppressive potential when incubated with Mfge8⁻/⁻ dendritic cells (Figure 8D). These results strongly suggest a dendritic cell–dependent alteration in regulatory T-cell suppressive potential in the absence of Mfge8, which is an...
agreement with a recent report showing a central role for dendritic cells in regulatory T-cell function.29

Discussion
Recent studies have clearly shown that accumulation of apoptotic macrophages or debris coincides with sites of plaque rupture and thrombosis30 and greatly determines the thrombogenicity of the lipid core31; however, whether apoptotic cell accumulation within the lipid core was also associated with plaque progression remained unknown. Apoptotic cell death has been observed in advanced and often complicated human atherosclerotic plaques, but it is almost undetectable in the early lesions of atherosclerosis.1 This might be due either to a reduced susceptibility of lesion-associated cells to apoptosis in the first stages of lesion development or to efficient phagocytosis of apoptotic cells and their rapid clearance from the developing lesion.3 Hitherto, neither of these 2 hypotheses has been substantiated by direct experimental data. In the present study, we detected increased accumulation of TUNEL-positive cells and debris in Mfge8-deficient mice despite no signs of increased susceptibility of Mfge8-deficient macrophages to apoptosis. Thus, the present results clearly suggest that apoptosis occurs early during plaque development but is hardly detectable in the first stages of the process owing to efficient Mfge8-dependent phagocytosis by professional macrophages. The larger lesion size in Mfge8-deficient mice does not explain the higher frequency of apoptotic cell accumulation in these lesions. Despite similar lesion size, lesions of Mfge8-deficient mice at 8 weeks of high-fat diet accumulated much more apoptotic debris (66% of lesions showed large areas of TUNEL positivity) than lesions of Mfge8+/+ mice at 15 weeks of high-fat diet (only 10% of lesions showed faint TUNEL positivity; Figures 2 and 3; Tables 1 and 2). Thus, there is clearly an Mfge8-dependent effect on apoptotic cell accumulation within the lesions.

The second important finding is that accumulation of apoptotic debris within the lesion is associated with the formation of large acellular cores and a marked acceleration of atherosclerosis. A previous study has shown that Fas ligand deficiency on an ApoE−/− background resulted in substantial acceleration of lesion development in association with increased accumulation of apoptotic debris.32 However, other factors, including abnormal lymphocyte count and function, as well as overt autoimmunity in the absence of Fas ligand, may have contributed to this phenotype. Transglutaminase-2 deficiency in bone marrow cells, shown to impair apoptotic cell phagocytosis and macrophage ABCA1 expression in vitro, did not significantly alter necrotic core formation in Ldlr−/− mice and led to a small increase in lesion size,23 which suggests only a modest role in vivo compared with Mfge8. Other studies have examined the effects of modulation of macrophage apoptosis on lesion size. Inhibition of a proapoptotic signaling pathway in macrophages, as shown with p53-deficient34,35 or Bax-deficient36 bone marrow transplantation, led to reduced macrophage apoptosis or increased macrophage proliferation, resulting in acceleration of lesion development. Thus, naturally occurring macrophage apoptosis modulates lesion development, at least in part, through modulation of lesion cellularity. On the other hand, others have found that absence of a macrophage survival protein, AIM, led to an increase in macrophage susceptibility to apoptosis and to lesion reduction.37 However, AIM-deficient mice display major alterations in T-cell number and function,38 which could per se explain a reduction in lesion size.

The absence of Mfge8-dependent phagocytosis was associated with a decrease in antiinflammatory IL-10 production by immune cells and increased IFN-γ expression within the lesions. In addition, the present results suggest that Mfge8 expression in bone marrow–derived cells contributes to the maintenance of a normal regulatory immune response in the periphery, probably through the maintenance of tolerogenic antigen-presenting cells. We and others have shown that IL-10 production or normal regulatory T-cell function control both the development and the inflammatory phenotype of atherosclerotic lesions.10,39–41 We propose that continuous efficient phagocytosis of apoptotic debris is critical to the maintenance of an antiinflammatory milieu, counterregulating the proinflammatory response and limiting lesion progression. The present results may explain in part the substantial increase in atherosclerosis risk in young patients with systemic lupus erythematosus42,43, a disease characterized by defective phagocytosis and increased accumulation of apoptotic cells.44,45 Interestingly, the acceleration of atherosclerosis in Mfge8-deficient mice preceded the appearance of autoimmunity. This is particularly important in light of results in humans showing that accelerated atherosclerosis in lupus patients is independent of signs of autoimmunity.42,43

In conclusion, lack of Mfge8 expression in bone marrow–derived cells enhances apoptotic cell accumulation in atherosclerosis, impairs the regulatory immune response, and accelerates lesion development. The present results suggest that defective phagocytosis of apoptotic cells may be a major risk factor of accelerated atherosclerosis in young patients and a novel target for disease modulation.

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A well-recognized characteristic of atherosclerotic plaques is the accumulation of apoptotic debris during plaque progression toward the advanced stages; however, the mechanisms responsible for this accumulation and their potential effects on plaque progression remain poorly understood. We hypothesized that phagocytic clearance of apoptotic cells by milk fat globule-EGF factor 8 (Mfge8, also known as lactadherin) is required for the maintenance of an antiinflammatory milieu and controls the development of atherosclerosis. Here, we show that Mfge8 is expressed in normal and atherosclerotic mouse and human arteries. Disruption of bone marrow–derived Mfge8 in a murine model of atherosclerosis leads to substantial accumulation of apoptotic debris, both systemically and within the developing lipid lesions, and to alteration of the antiinflammatory and regulatory immune response. These events are associated with a marked acceleration of atherosclerosis. In conclusion, efficient removal of apoptotic debris maintains a protective immune response and limits plaque development. Our results may explain, at least in part, the substantial increase in atherosclerosis risk in young patients with systemic lupus erythematosus, a disease characterized by defective phagocytosis and increased accumulation of apoptotic cells.
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