Critical Role of Macrophages in Glucocorticoid Driven Vascular Calcification in a Mouse-Model of Atherosclerosis

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Objective—Macrophage-derived products are known to play a crucial role during atherogenesis and vascular calcification. Glucocorticoids (GC) are important modulators of immune cell functions, but their specific effects on macrophages behavior during plaque formation are not defined. The present study was therefore designed to investigate the effects of macrophage-specific deletion of the glucocorticoid receptor (GR$^{LyMCre}$) on atherogenesis and vascular calcification in a hyperlipidemic mouse-model.

Methods and Results—Bone marrow was isolated from GR$^{LyMCre}$ mice and wild-type controls (GR$^{lox}$) and subsequently transplanted into lethally irradiated LDL-receptor–deficient mice. Animals were fed a Western-type diet for 15 or 24 weeks, and atherosclerotic lesions within the aortic sinus were evaluated. At both time points, no significant difference in serum lipid and corticosterone concentrations, atherosclerotic lesion size and macrophage-content within the lesions could be observed. However, GR$^{LyMCre}$ mice showed less calcification as well as a significant reduction of RANKL, BMP2, and Msx2 expression within the vasculature. In vitro studies using conditioned media from macrophages which had been stimulated with dexamethasone demonstrated a dose-dependent increase in calcium deposition by vascular smooth muscle cells.

Conclusion—This study demonstrates that macrophage-specific glucocorticoid receptor inactivation reduces vascular calcification without affecting atherosclerotic lesion size in LDL receptor–deficient mice. (Arterioscler Thromb Vasc Biol. 2008;28:2158-2164.)

Key Words: atherosclerosis ■ inflammation ■ macrophages ■ glucocorticoid receptor ■ vascular calcification

The accumulation of macrophages within the vascular wall is part of the initiation in the development of atherosclerosis and vascular calcification. Macrophages are the main source of a variety of cytokines and growth factors, which may contribute to smooth muscle cell migration and proliferation via matrix degrading metalloproteinases and expression of coagulation and calcification stimulating proteins. Thus, macrophages could play an important role in the proliferative, inflammatory, and calcifying processes in atherosclerosis.

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Glucocorticoids (GCs) possess a wide variety of anti-inflammatory and antiproliferative effects in vivo and in vitro, thereby possibly modulating atherosclerosis and vascular calcification. GCs suppress the release of tumor necrosis factor alpha, (TNF-α)$^1$ inhibit the proliferation and migration of smooth muscle cells, (2$^1$ inhibit interleukin (IL) synthesis and the expression of inducible nitric oxide synthase, and reduce tissue factor activity in macrophages.$^3$ Experimental studies using animal models of hyperlipidemia-induced atherosclerosis have demonstrated a beneficial role of GC in cholesterol-induced atherosclerosis as well as in postangioplasty restenosis models.$^6$/7 However, next to their ability to decrease atherosclerosis and restenosis, GCs are also known to exert the risk of atherogenesis, depending on the experimental or clinical conditions.$^8$

Although exogenous GCs are known to promote osteoporosis,$^9$ physiological concentrations of GCs can exert an anabolic effect on bone formation.$^{10}$ Moreover, dexamethasone is commonly used in basic science studies to promote chondro-/osteoblast differentiation of mesenchymal stem cells and osteoprogenitor cells.$^{11}$ Several in vitro studies also suggest that GCs can promote the acquisition of a chondro-/osteoblast-like phenotype by smooth muscle cells and pericytes.$^{12}$ Even if the in vivo effect of GC on vascular cells is
still elusive, these studies suggest that GC can directly induce the osteogenic differentiation of cells resident/recruited in the vascular wall during pathological processes such as atherosclerosis. However, the relevance of physiological levels of GCs on the development of vascular calcification is not known. Several macrophage-derived factors have been shown to promote phenotypic transition of vascular cells toward an osteogenic phenotype. Whether GCs can modulate the production of procalcifying factors by macrophages needs to be clarified.

Using the Cre-loxP system, the following study investigates the effects of a macrophage-specific GC receptor deletion on the initiation and progression of atherosclerotic lesions as well as mechanisms of vascular lesion calcification in a hyperlipidemic mouse model of atherosclerosis.

Materials and Methods

Animals and Bone Marrow Transplantation

Eight-week-old female LDL-receptor−/− mice (LDLr−/−; Jackson Laboratory, Bar Harbor, Maine; n=20, C57BL/6 background) were lethally irradiated at a dose of 9 Gy and transplanted with bone marrow of mice with a macrophage-specific glucocorticoid receptor knock-out (GRlysMCre). These mice were generated as described earlier using the cre-loxP system and backcrossed on a C57BL/6 background for more than 8 generations. Twenty LDLr−/− mice were used as controls which were transplanted with wild-type bone marrow (GRflox). Four weeks after transplantation, mice were fed a Western-type diet (Altromin, Lage/Germany; Nr. 11320010: 0.15% cholesterol, 600,000 IE/kg vitamin D, 8840 mg/kg calcium, and 5080 mg/kg phosphate). Animals were kept within the animal care facility of the University of Heidelberg. The housing and care of animals and the procedures done in the study were performed in accordance with the guidelines composed by the Animal Care Committee of the University of Heidelberg and approved by the Regierungspräsidium Karlsruhe.

Animal Sacrifice and Preparation of Tissues

After 15 (n=20) and 24 weeks (n=20) on a Western-type diet, the mice were heavily sedated (Avertin, Aldrich), blood was collected from the inferior vena cava, and the animals were euthanized by exsanguination. The animals were perfused with 10 mL phosphate-buffered saline at physiological pressure, followed by a perfusion with 4% buffered formalin via the left ventricle. Before the perfusion with formalin, a ligation was tied around each aorta distal to the branch of the left subclavian artery. The aortas were collected beginning from the suture and extending to the bifurcation of the iliacal arteries, and the surrounding connective tissues were removed and immediately snap frozen in liquid nitrogen (LN.). The entire heart from each animal was dissected out and embedded in paraffin, and the aortic sinus was serially sectioned (5 μm). Every fifth section was stained with a modified Movat pentachrome stain.

Assessment of Chimerism

The reconstitution of the transplanted bone marrow was determined by polymerase chain reaction (PCR) on genomic DNA from bone marrow. Wild-type GRflox-1 gene and LDLr−/− was detected as described earlier.

Blood Parameters and Systolic Arterial Pressure Determination

Total serum cholesterol, HDL, LDL cholesterol, triglycerides, calcium, and phosphate were determined enzymatically in the serum of the mice. Corticosterone concentrations were determined via enzyme linked immuno assay. Systolic arterial pressure was obtained two times by tail-cuff method 8 weeks after bone marrow transplantation (n=5 per group). During this procedure, the animals were slightly sedated.

Evaluation of Lesion Size

Two investigators who were blinded to the study protocol determined the cross-sectional area of the lesions in each section by using computer-assisted morphometry (Image Pro, Media Cybernetics). The maximum plaque area per animal was compared between the two groups of mice (data expressed in μm²).

Evaluation of Calcium Deposition Inside the Atherosclerotic Lesions

Lesion calcification was established by using von Kossa staining and computer-assisted visualization. Calcified area inside the lesions was quantified and reported as μm².

Immunohistochemistry

Detection of monocytes/macrophages was performed by using monoclonal goat antimmune antibody (anti-Mac-2, Accurate Chemicals), detection of smooth muscle cells by using an antiswine smooth muscle actin antibody (Dianova), and evaluation of RANKL, OPG, aggrecan and typeII collagen by using polyclonal goat antimmune antibodies according to the manufacturers’ protocol (Santa Cruz, Biotechnology Inc). The content of positive staining within the lesions was determined by two investigators who were blinded to the study protocol using computer-assisted morphometry (Image Pro, Media Cybernetics).

Real-Time RT-PCR

Total RNA was extracted from frozen aortas using TriPure reagent (Roche Diagnostics GmbH) according to the manufacturer’s instructions. Reverse transcription was performed with 1 μg total RNA isolated from each separate aorta. To determine the levels of gene expression, real-time PCR quantification (TaqMan) was performed (ABI Prism 7700 Sequence Detection System; PE Biosystems). The primer sequences used in the study are listed in supplemental Table I (available online at http://atvb.ahajournals.org). The transcript for the constitutive gene product β-actin was used for data normalization.

RAW 264.7 Mouse Macrophages Stimulation With Dexamethasone and Conditioned Media Collection

RAW cells were plated at 1.5×10⁶ cells for 100 mm dish, grown in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Twenty-four hours after plating, cells were treated for 3 hours with dexamethasone (Sigma-Aldrich). After the treatment, the media was removed, cells were washed 3 times in PBS, and fresh media was added. The latter was collected after 12 hours, centrifuged, filtered (0.2 μm), and stored at −20 C° until used.

Aortic Bovine Smooth Muscle Cell Isolation and Treatment

Expplant-derived primary bovine aortic smooth muscle cells (BMSMCs) were used between passage 4 and 6. At confluence in 6-well plates, cells were treated with 2.0 mL of media (DMEM containing 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) supplemented with 0.5 mL of conditioned media from RAW cells treated with dexamethasone. The media was changed every third day, and the experiments stopped after 12 days. To promote calcium deposition, βglycerophosphate (10 mmol/L) was added to media.

Calcium Assay

Calcium deposition in the plates was quantified as previously described. Cells were decalcified using a 0.6 mol/L HCl solution. After collecting the supernatant the cells were washed with PBS and
solubilized in 0.1 N NaOH 0.1% SDS solution. The calcium content in the supernatant was quantified calorimetrically using the o-cresolphthalein complexone method (Chema Diagnostica) while the protein content was measured using a BCA protein assay kit (Pierce Protein). For each plate, calcium data were normalized to the protein content.

**Statistical Analysis**

All data were expressed as mean±SE. Significant differences between means in different serum profiles and blood pressure were determined with the 2-tailed Student’s *t* test. ANOVA followed by the 2-tailed Mann–Whitney *U* test. ANOVA followed by posthoc Fisher LSD test was used for the in vitro experiments. A probability value <0.05 was considered statistically significant.

**Results**

**Effect of Bone Marrow Transplantation**

PCR analysis of the bone marrow demonstrated a complete conversion of the origin LDLr<sup>−/−</sup> genotype to the donor’s wild-type, indicating that the bone marrow population had been reconstituted (data not shown). Furthermore, we were able to demonstrate the presence of Cre-recombinase in mice that received bone marrow of GR<sup>1yMCre</sup> mice (data not shown).

**Serum Determinations**

There were no significant differences in serum total cholesterol, total triglycerides, LDL, and HDL, between the recipients of GR<sup>1yMCre</sup> mice and GR<sup>lox</sup> bone marrow on a Western type diet (supplemental Table II). There was also no significant difference in serum corticosterone concentrations (172±33 ng/mL for controls versus 142±22 ng/mL for GR<sup>1yMCre</sup> mice, *P*=0.38). Furthermore we did observe any difference in serum phosphate (3.13±1.1 ng/mL for controls versus 3.69±2.2 ng/mL for GR<sup>1yMCre</sup> mice, *P*=0.79) and calcium levels (2.02±0.3 ng/mL for controls versus 2.2±0.3 ng/mL for GR<sup>1yMCre</sup> mice, *P*=0.21).

**Effect on Systolic Arterial Pressure**

We did not observe any significant difference in the systolic arterial pressure between mice transplanted with bone marrow from GR<sup>1yMCre</sup> mice in comparison to controls (112±4 mm Hg for controls versus 110±5 mm Hg for GR<sup>1yMCre</sup> mice, *P*=0.45).

**Maximum Lesion Area**

After 15 (lesion initiation) and 24 weeks (lesion progression) on the Western diet, the extent of atherosclerotic lesion development in the aortic sinus was evaluated. At the 15-week time point, we were not able to demonstrate a significant difference in lesion size (87.9±12.2×10<sup>3</sup> mm<sup>2</sup> for controls versus 89.9±12.1×10<sup>3</sup> mm<sup>2</sup> for GR<sup>1yMCre</sup> mice, *P*=0.89). After 24 weeks, we also did not observe any significant differences in atherosclerotic lesion size (137.1±13.2×10<sup>3</sup> mm<sup>2</sup> for controls versus 150.6±5.2×10<sup>3</sup> mm<sup>2</sup> for GR<sup>1yMCre</sup> mice, *P*=0.37).

**Positive Macrophage Staining Areas and Calcium Deposition Within the Atherosclerotic Lesions**

After 15 weeks on the Western diet, we were able to detect macrophage rich fatty streaks and enhanced foam cell formation evaluated by macrophage-specific immunostaining. In comparison after 24 weeks, macrophage content in atherosclerotic lesions was much less. However, the area of positive macrophage-staining did not differ significantly between GR<sup>lox</sup> and GR<sup>1yMCre</sup> mice recipients at both time points (30.2±5.7×10<sup>3</sup> mm<sup>2</sup> for GR<sup>lox</sup> mice versus 39.3±8.7×10<sup>3</sup> mm<sup>2</sup> for GR<sup>1yMCre</sup> mice at 15 weeks, *P*=0.23 and 14.6±2.3×10<sup>3</sup> mm<sup>2</sup> for GR<sup>lox</sup> mice versus 19.8±4.1×10<sup>3</sup> mm<sup>2</sup> for GR<sup>1yMCre</sup> mice at 24 weeks, *P*=0.25). Twenty-four weeks after bone marrow transplantation, we were able to observe advanced atherosclerotic lesions which demonstrated areas of calcification. Mice containing GR<sup>1yMCre</sup> macrophages showed less calcification within the atherosclerotic lesion determined by positive von Kossa Staining (59.4±0.2×10<sup>3</sup> mm<sup>2</sup> for GR<sup>lox</sup> mice versus 41.2±4.2×10<sup>3</sup> mm<sup>2</sup> for GR<sup>1yMCre</sup> mice, *P*<0.05; Figure 1A). The calcified area was distributed within the lateral regions/shoulder regions (Figure 1B and 1C) and the necrotic cores of the lesion (Figure 1D and 1E).
Immunocytochemical Expression of Receptor Activator of Nuclear Factor Kappa B Ligand (RANKL) and Osteoprotegerin Within the Atherosclerotic Lesions

Deletion of the GC Receptor in macrophages led to a significant reduced expression of receptor of RANKL within atherosclerotic lesions (17.2 ± 2.0 × 10³ µm² for GR^{flx} mice versus 11.0 ± 0.8 × 10³ µm² for GR^{LyMCre}, P < 0.05; Figure 2) as determined by immunohistochemistry. The RANKL positive staining was mainly identified in an area populated by so-called chondrocyte-like cells, which were identified by positive staining for type collagen II and aggrecan. These cells did not demonstrate positive staining with antibodies, which were directed against macrophages or smooth muscle cells and were mostly located next to the necrotic cores of the lesions (Figure 3). Expression of osteoprotegerin (OPG) showed no significant difference between the groups (7.4 ± 1.1 × 10³ µm² for GR^{flx} mice versus 7.5 ± 0.6 × 10³ µm² for GR^{LyMCre}, P = 0.94).

Gene Expression Analysis of Aortic Tissues

Real-time RT-PCR analysis of the aortas confirmed the immunocytochemical findings concerning OPG and RANKL expression. In fact, we observed a significant decrease of RANKL expression within the aortas of mice transplanted with GR^{LyMCre} macrophages compared to GR^{flx} mice, whereas no difference was documented in OPG expression (1.0 ± 0.13 relative OPG mRNA expression for control mice and 0.92 ± 0.15 for GR^{LyMCre} mice, P = 0.16). Furthermore, in the aortic tissue of GR^{LyMCre} transplanted mice, we were able to document a significant decrease in the expression of BMP2 and Msx2 (Figure 4).

In Vitro Calcium Deposition

To study a possible paracrine effect of macrophage-derived factors in promoting the acquisition of a procalcifying profile by vascular cells, we treated BSMC cultures with conditioned media from macrophages which have been stimulated with increasing doses of dexamethasone. After 12 days of treatment, we observed a dose-dependent increase in calcium deposition around BSMCs treated with macrophage-stimulated conditioned media compared to controls (Figure 5).

Discussion

Inflammatory mechanisms are involved in the initiation and progression of atherosclerosis and vascular calcification. Macrophages play a pivotal role in this vascular disease. GCs possess a wide variety of antiinflammatory actions, as well as regulatory abilities in cell differentiation and glucose and lipid metabolism. On the other hand, GCs demonstrate severe side effects, such as dyslipidaemia, diabetes, weight gain, hypertension, and endothelial dysfunction, with an increased risk of atherogenesis. One might expect that the...
macrophage-specific inhibition of the glucocorticoid receptor (GR) with the subsequent loss of the antiinflammatory activity of endogenous GC in macrophages might modulate the initiation and progression of atherosclerosis. Using the GR<sub>ΔMc5c</sub> mice, we were able to evaluate the effects of endogenous levels of GCs on macrophages and their role on atherogenesis and vascular calcification without these side effects. Surprisingly, we were not able to demonstrate a significant effect regarding total atherosclerotic lesion size and macrophage content after 15 and 24 weeks of Western diet.

There have been controversial reports of the effects of GCs on atherosclerosis and restenosis. Clinical studies demonstrated that in patients with rheumatoid arthritis, the systemic treatment with GCs resulted in increased carotid plaque volume. Furthermore, the use of systemic and local delivery was also not able to prevent restenosis after coronary stenting. GCs, on the other hand, inhibit the oxidized LDL-induced proliferation of macrophages by suppressing the expression of granulocyte/macrophage colony-stimulation factor. Using an atherosclerotic rabbit model, treatment with dexamethasone inhibited the recruitment and proliferation of macrophages as well as aortic foam cell formation. Recent studies demonstrated antiatherosclerotic effects of dexamethasone incorporated into liposomes. These effects were evaluated by determination of the aortic cholesterol ester level as an initial event in the development of foam cell formation. In addition, Chono et al showed significantly lower cholesterol ester levels in atherogenic mice treated with dexamethasone-liposomes.

This is the first study investigating the macrophage-mediated effect of endogenous GC during atherogenesis. Our findings suggest that potential pro- and antiatherosclerotic effects of corticosteroids are only partially mediated via a glucocorticoid-receptor signaling pathway in macrophages. Reasons for discrepancy with previous reports on the antiatherosclerotic effect of GCs can be identified in different experimental conditions using exogenous GCs.

Experimental studies showed that GCs can play a role in vascular calcification. Stimulation with dexamethasone promotes calcification by vascular smooth muscle cells and pericytes. That might be attributable to an enhanced gene expression of transcription factors involved in osteoblastic differentiation, such as Osf2/Cbfal, or reduced expression of inhibitory molecules of mineralization. In bone tissue, dexamethasone treatment leads to an increase of RANKL expression and decrease of OPG expression by osteoblasts, promoting the osteoclastic differentiation of monocytes. More recently, the RANK/RANKL/OPG axis has been implicated in the pathogenesis of vascular calcification. The original observation that OPG inactivation promotes vascular calcification has been confirmed in the setting of atherosclerosis development, showing that the OPG–Apolipoprotein E (Apo E) double KO mice experience a higher degree of calcium deposition in the vasculature compared to the ApoE<sup>−/−</sup> control mice. In addition OPG injection has been shown to reduce vascular calcification in LDL receptor<sup>−/−</sup> mice, with no significant modification in the lesion size.
our study, whereas OPG expression was unchanged in the lesions and in the aortic tissue of mice transplanted with GR\textsubscript{LysMCre} macrophages compared to GR\textsubscript{flox} mice, the expression of RANKL was significantly reduced in the mice receiving macrophages with a deletion of the GC receptor. Our findings are concordant with the previous observation that hypothesized a potential role of RANKL in vascular calcification.\textsuperscript{35} In fact, although normal vascular tissue does not express RANKL, its presence has been documented during calcification of the arterial wall and the aortic valve.\textsuperscript{19,35} We therefore can speculate that the decrease in the RANKL/OPG ratio (characterized by a relative increase in OPG) observed in mice transplanted with GR\textsubscript{LysMCre} could be responsible for the protective effect on calcium deposition in the lesions.

Mechanisms of vascular calcification now include active cell-driven processes characterized by the acquisition of a chondro/osteoblast-like profile by a specific cell type recruited in the developing lesions. Chondrogenic metaplasia with the presence of chondrocyte-like cells has been described in lesions of both apolipoprotein E \textsuperscript{-/-} and LDL receptor \textsuperscript{-/-} mice.\textsuperscript{19} The presence of these cells could also be observed in our lesions, as documented by aggrecan and type II collagen expression and the absence of specific staining for macrophages and smooth muscle cell markers. The origin of these cells is not defined yet. They could represent a subpopulation of smooth muscle cells resident in the vascular wall that migrates in the neointima during atherogenesis. Alternatively, they could derive from circulating elements that are recruited in the developing plaque. The first hypothesis is endorsed by several studies showing the ability of smooth muscle cells to transdifferentiate in chondro/osteoblast-like cells.\textsuperscript{12,29,36}

The importance of cell-driven processes during vascular calcification has also been recently underlined by studies conducted by Towler’s group. These authors elegantly demonstrated the crucial role played by the BMP2/Msx2 signaling pathway for the activation of an osteogenic program in the vascular wall of LDL receptor \textsuperscript{-/-} mice fed high-fat diet.\textsuperscript{37} In our study, the reduced calcium deposition observed in the lesions of mice transplanted with GR\textsubscript{LysMCre} macrophages is accompanied by a lower expression of both BMP2 and Msx2. These data, besides confirming the relevance of BMP2 - Msx2 signaling in controlling vascular calcification, suggest that macrophages recruited in the developing lesions can produce factors able to influence the activation of this osteogenic program. In fact, in addition to in vitro studies, which demonstrated direct calcifying effects of GCs on vascular cells,\textsuperscript{12,29} our findings demonstrated that GCs can participate in vascular calcification indirectly, via macrophages activation. This was confirmed by preliminary in vitro data showing that GCs could promote the release by RAW cells of molecules able to favor calcium deposition by vascular smooth muscle cells.

Based on our data, we cannot propose a detrimental effect for exogenous GCs on vascular calcification even if osteoporosis, a known consequence of steroid therapy, has been recently linked with increased calcium deposition in the vascular wall.\textsuperscript{38} Whether endogenous GC levels can represent a player in the link between bone metabolism and vascular biology requires further clarification. During bone remodeling, the cross-talk between osteoblasts and osteoclasts appear to be crucial for maintaining the normal homeostasis of the bone and the balance between resorption and synthesis. Recent data suggest that osteoclasts can release anabolic factors promoting bone formation,\textsuperscript{39} and these signals from osteoclasts to osteoblasts could be dependent on glucocorticoid action. Mononuclear cells belonging to the monocyte-macrophage lineage represent a common precursor for both osteoclasts and foam cells. We could speculate that when monocytc cells are recruited in the atherosclerotic lesion and become macrophages/foam cells, they could retain at least in part the ability to produce mediators involved in bone remodeling, maybe under the control of physiological levels of GCs. During atherogenesis, these factors could target resident vascular cells promoting the acquisition of a chondro/osteoblast-like profile.

In conclusion, our data demonstrate that a macrophage-specific inhibition of the GC receptor leads to reduced calcification possibly via a reduced expression of RANKL, BMP2, and Mxs2 in the atherosclerotic lesions of a hyperlipidemic mouse model. These findings point to a central role of macrophages within GC-induced vascular calcification.

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Disclosures
None.

References
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Table II

Total serum cholesterol, LDL, HDL and triglyceride levels in primary LDLr -/- recipients of control GR$^{\text{flo}}$ and GR$^{\text{LysMcRe}}$ bone marrow (in mg/dl) at point one (15 weeks – early lesions) and point two (24 weeks - advanced lesions). Values are mean±SEM, ns = non significant.

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