Differential Expression Patterns of Proinflammatory and Antiinflammatory Mediators During Atherogenesis in Mice

Niels R. Veillard, Sabine Steffens, Fabienne Burger, Graziano Pelli, François Mach

Objective—Recent advances support the current view of atherosclerosis as an inflammatory process that initiates and promotes lesion development to the point of acute thrombotic complications and clinical events. ApoE-deficient mice are a valuable model for studying the involvement of inflammatory mediators during atherogenesis. In this study, we investigated the correlation between atherosclerotic plaque development and expression of important pro- and antiinflammatory mediators during progression of atherosclerosis in ApoE−/− mice.

Methods and Results—Expression of proinflammatory cytokines, chemokines, and chemokine receptors within aortic lesions increased during atherogenesis, as detected by real-time quantitative reverse-transcription polymerase chain reaction. In parallel, the number of inflammatory cells within lesions increased together with serum cholesterol and body weight. Interestingly, the majority of inflammatory mediators investigated reached their maximum expression values at 10 weeks of diet, followed by continuous decrease of their expression levels, whereas atherosclerotic plaque size further increased. We show that the expression pattern of these different inflammatory mediators mainly correlates with the amount of inflammatory cells present within the atherosclerotic lesions.

Conclusion—Atherosclerosis might result from an imbalance between pro- and antiinflammatory mediators in response to endothelial injury induced by cholesterol-rich diet. These data provide important information on the expression kinetics of inflammatory mediators and point out the possible role of antiinflammatory cells during atherogenesis. (Arterioscler Thromb Vasc Biol. 2004;24:2339-2344.)

Key Words: atherogenesis ■ inflammation ■ cytokines ■ chemokines

Atherosclerosis is a disease of the arterial wall that begins early in life and may lead to severe clinical complications later in life with high morbidity and mortality. Our current understanding of the vascular biology of atherogenesis and its clinical manifestations suggests a pathophysiology that is much more complex than simple lipid storage. Atherosclerosis is now recognized as an inflammatory process that initiates and promotes lesion development to the point of acute thrombotic complications and clinical events. Atherosclerotic lesions are characterized by their cholesterol content, immune infiltrates, and fibrosis. This chronic immune-inflammatory disease involves leukocyte migration within the intima of the vessel wall. Hyperlipidemia, hypertension, smoking, and diabetes are the principal risk factors causing endothelial dysfunction that triggers migration of leukocytes, mainly monocyte/macrophages and T lymphocytes, within the intima area. Attraction and further activation of leukocytes to sites of inflammation is caused by proinflammatory cytokines and chemokines. Inflammation appears crucial in all stages of atherosclerosis, from the very initial phases through the progression, and finally to the clinical complications.

During the past few years, extensive studies have reported the implication of various chemokines and chemokine receptors in the pathophysiology of atherosclerosis. Macrophages, known to express high amounts of the chemokine receptor CCR2, have been shown to be of major importance in the development of the disease. Deficiency of the CCR2 receptor leads to a significant reduction of advanced atherosclerotic lesion formation in mice. In addition, several studies have demonstrated that the T-helper lymphocyte type 1 (Th1) subset of CD4+ T-helper cells is the predominant type of lymphocytes found in atherosclerotic lesions. Recently, regulatory T lymphocytes (Tr), a newly described subpopulation of CD4+ T lymphocytes, have been demonstrated to play a protective role against atherosclerosis development. Mallat et al have shown that injection of Tr cells in ApoE−/− could reduce the development of atherosclerosis.

To study the progression of atherosclerosis, murine models have been developed and used extensively. The low-density lipoprotein receptor-deficient and apolipoprotein E (ApoE)-deficient mice are 2 classical models used to study atherosclerotic plaque formation. To induce atherosclerotic plaque formation, low-density lipoprotein receptor-deficient mice...
must be fed with a cholesterol-rich diet (Western-type diet). For the ApoE−/− model, cholesterol-rich diet is not essential to induce atherosclerosis, but rather accelerates its process. The development of atherosclerotic lesions begins, as described by the American Heart Association classification,12,13 with isolated fatty dots through stages of fatty streaks, atheromas, and fibroatheromas, to the complicated lesions. In mice, several studies have described the development of atherosclerotic lesions, which progress from an initial appearance within the aortic sinus to subsequently involve the proximal portion of the coronary arteries, carotid arteries and abdominal aorta.14-15 Nakashima et al have shown that lesion progression in ApoE−/− mice, fed 15 weeks with a cholesterol-rich diet, had advanced fibrous plaques at site of the aortic sinus, aortic arch, and at the principal branches of the thoracic aorta, and earlier stages of lesion development at sites such as the lower abdominal aorta and the lower thoracic aorta.16 Recently, Murphy et al reported that ApoE−/−Leiden mice had increased circulating JE/MCP-1 and KC concentrations after 2 to 4 weeks of high-fat diet consumption, and stabilized throughout the study.17 In another recent report, Martin et al showed dynamic changes in some gene expression in ApoE-deficient mice on a regular chow diet.18 However, none of these studies has yet demonstrated how and when pro- and antiinflammatory molecules such as cytokines, chemokines, and their receptors, as well as immune cell markers, are expressed during atherogenesis within the vascular wall of ApoE−/− mice fed a cholesterol-rich diet.

In this article, we show that the expression of inflammatory mediators, such as interferon (IFN)-γ, IL-12, monocyte chemoattractant protein-1 (MCP-1/CC122), and CCR2, increased in parallel to atherosclerotic plaque formation, reaching highest levels after 10 weeks of cholesterol-rich diet and reduced after 12 weeks despite further progression of the lesions. Expression patterns varied together with the content of inflammatory cells present within lesions. These findings suggest a causal relation between inflammatory mediators and the stages involved in the progression from local inflammation through atherosclerotic lesions formation.

Materials and Methods

Animals
As a model of in vivo atherosclerosis, male ApoE−/− C57BL/6J mice were fed a high-cholesterol diet (1.25% cholesterol, 0% cholate; product #D12108; Research Diets, New Brunswick, NJ) in conventional housing. Littermate mice (10 weeks old) were fed high-cholesterol diet for 0, 2, 4, 6, 8, 10, 12, and 14 weeks (n=12 per group), euthanized, and analyzed for vascular lesions within the aorta. For histological analysis (n=6 per group), atherosclerotic lesions were measured on the aortic sinus and on the thoraco-abdominal aorta, as previously described.19 Aortas (including the aortic arch and the thoraco-abdominal aorta) were used for mRNA analysis (n=6 per group). Rabbit anti-mouse Mac-3 (Pharmingen, clone M3/84) and rabbit anti-mouse CD4 (Pharmingen, clone H129.19) antibodies were used for immunohistochemistry analysis on acetone fixed sections.

Quantification of Atherosclerotic Lesions
The extent of atherosclerosis was assessed on the aortic sinus and the thoraco-abdominal aorta by quantified by computer image analyses using KS400 software (Feldbuch, Switzerland). Atherosclerotic lesions were measured by lipid deposition stained with oil red O. To quantify lipid depositions within the aortic sinus, 5-μm sequential sections were cut through the aortic root. We calculated for each aortic root an average of lipid deposition from 6 sections separated by 50 μm from each other. We then divided the area of atherosclerotic lesions by the total valve surface of each section. Aortic valve leaflets were used as an anatomic reference point. The first section that displayed the entire 3-aortic valve leaflets counted as for the fourth section over the 6 selected. Normalization to the valve surface allowed correction of the section angle through the tissue. Knowing that lesions are abundant around the leaflets themselves, such a ratio permitted to correct the quantification. This ratio also allowed a more precise location of the section within the tissue and ameliorated the comparison between samples. Quantification on the abdominal aorta was performed as described elsewhere.19

Analysis of Gene Expression by Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction
For mRNA analysis, aortas were cleaned on ice and snap-frozen in liquid nitrogen (LN2). Total murine mRNA was extracted from the aorta (from the beginning of the aortic arch, just after the aortic roots, to the iliac bifurcation) and prepared with TRI reagent (MRC Inc, Cincinnati, Ohio) according to the manufacturer’s instructions.

Real-time quantitative reverse-transcription polymerase chain reaction (ABI Prism 7000 Sequence Detection System; Applied Biosystems, Foster City, Calif) was used to determine the mRNA levels of CCR1, CCR3, CCR4, CCR5, CCR8, CXCR3, RANTES, MCP-1, MIP-1α, MIP-1β, IFN-γ, and IL-10 (ABI Prism, Pre-Developed TaqMan Assay Reagents) 6-carboxyfluorescent (6-FAM)-labeled. Each sample was analyzed in triplicate, normalized in multiplex reactions using TaqMan eukaryotic 18S Control (TaqMan Reagent; Applied Biosystems) VIC-labeled. The fold inductions in mRNA expression of inflammatory mediators at each time point relative to the reference week 0 were analyzed by the comparative computed tomography method. For each mediator, statistical comparisons were made between 10 weeks and 14 weeks of diet. Foxp3 and TIM-3 primers and probes were designed as described.19 Primers (800 nM) and probes (200 nM) for CD4 (Gene Bank BC039137; 5′-start: 143), CD25 (Gene Bank NM008367; 5′-start: 54), endothelial nitric oxide synthase (Gene Bank BC052636; 5′-start: 3328), and IL-18BP (Gene Bank NM010531; 5′-start: 286) labeled with FAM dye used in this study are presented elsewhere (see online reference I, available at see www.atvb.ahajournals.org). These primers and probes were designed using Primer Express software (Perkin-Elmer, Foster City, Calif), IL-12p40 primers and probe were used according to Bruemmer et al.22

Blood Analysis and Inflammatory Cells Quantification
Blood samples were collected at beginning and end of the study for each mouse. Hematocrit and leukocyte counts were measured, and sera were used for measurement of cholesterol and triglycerides content.24 Presence of macrophages and T lymphocytes within atherosclerotic lesions was determined by quantification (KS400) of immunohistochemistry stained on aortic arches for Mac-3 (1:50 dilution) and CD4 (1:50 dilution).

Statistical Analysis
All results are expressed as mean±SEM. Differences between the groups were considered significant at P<0.05 using the 2-tailed Student t test or the Mann–Whitney U Wilcoxon sum test.

Results
General features of ApoE-deficient mice fed a cholesterol-rich diet. To study the kinetics of pro- and antiinflammatory mediators during the development of atherosclerosis, we fed ApoE−/− mice a cholesterol-rich diet for the following time periods: 0, 2, 4, 6, 8, 10, 12, and 14 weeks. After each diet
treatment period, we determined values for the body weight, total cholesterol, triglycerides, and circulating T lymphocytes. Body weight increased during the first 8 weeks and reached a plateau between 10 and 14 weeks of treatment with a stabilized weight \( \pm 30 \text{ grams} \) (reference IIA). Total cholesterol increased in a linear manner up to 14 weeks (reference IIB). Triglycerides concentrations and the number of circulating T lymphocytes did not significantly change during the whole period of the diet treatment (IIC and IID).

**Development of Atherosclerosis Is Delayed Between Aortic Sinus and Abdominal Aorta**

Atherosclerotic lesions were measured by lipid deposition stained with oil red O on the aortic sinus as well as the thoraco-abdominal aorta. As shown in Figure 1A and 1B, lesions developed relatively faster on the aortic origin. Atherosclerotic plaque formation on the aortic sinus was already detectable after 2 weeks of cholesterol-rich diet. Subsequently, the extent of atherosclerosis as measured by lipid deposition increased nearly in a constant manner up to 12 weeks and stabilized after this time point. In contrast, compared with the aortic sinus, atherosclerotic lesion progressed much slower within the abdominal aorta (Figure 2A and 2B). Mice did not show relevant levels of lipid deposition on the abdominal aorta up to 6 weeks. Significant values of lesion size were detectable after 8 weeks of diet with 5.1% of lesion area within the abdominal aorta, and lesion size further increased to a maximum of 19.3% after 14 weeks of diet.

**Kinetics of Inflammatory Mediators During Atherogenesis**

To understand when and how different inflammatory cells are implicated during atherogenesis, we analyzed the kinetics of their mRNA levels isolated from thoraco-abdominal aortas using real-time polymerase chain reaction (Figure 3A). As reported by the mRNA level for CD68, the number of monocyte/macrophages within the vascular wall of the abdominal aorta increased significantly from the early stages of inflammation until week 10, and reduced after 12 and 14 weeks of diet. Similar results were obtained for the expression of the T-helper lymphocytes (Th) marker CD4. However, the amount of Th cells reached their highest value after 8 weeks of diet, an amount that did not significantly change during the next 2 weeks, but decreased after 12 weeks. These results were confirmed by staining quantification for Mac-3 (macrophages) and CD4 (data not shown). Interestingly, when normalized to atherosclerotic plaque size, we observed that macrophage and T cell contents decrease as lesions progress (Figure 3B and 3C). Analysis of the chemokine receptor CCR4 expression, a T-helper lymphocyte type 2 (Th2) marker, revealed that Th2 cells are recruited into the vessel wall already after 4 weeks of diet. Recruitment of the Th2 cells appears to be transient because CCR4 expression...

![Figure 1. Development of atherosclerotic lesions on the aortic sinus. Atherosclerotic lesions were measured by lipid deposition detected with oil red O staining represented here in red within aortic sinus (A, B). Scale bar represents 500 \( \mu \text{m} \). Similar results were obtained in separate experiments using six different mice. *\( P < 0.05 \).](image1)

![Figure 2. Development of atherosclerotic lesions on the thoraco-abdominal aorta. Atherosclerotic lesions were measured by lipid deposition detected with oil red O staining represented here in red within thoraco-abdominal aortas (A, B). Similar results were obtained in separate experiments using 6 different mice. *\( P < 0.05 \).](image2)
markedly decreased after 10 weeks. Similarly, the amount of Th1 cells, as detected with the TIM3 probe (T-cell immunoglobulin domain, mucin domain), accumulated constantly up to 10 weeks. But, in contrast to the Th2 cells, the proportion of Th1 cells remains at high levels under further progression of atherosclerotic lesion. Interestingly, the amount of regulatory T cells, as measured with the forkhead/winged helix transcription factor (Foxp3) probe, strongly increased between 4 and 6 weeks of diet, with continuously high expression up to 12 weeks.

In a second set of experiments, we analyzed the expression of pro- and antiinflammatory mediators. The proinflammatory factors inducible nitric oxide synthase and IL-12, but not IFN-γ, strongly increased during 10 weeks of diet. Although IL-12 could only be detected after 4 weeks of diet, iNOS expression was already detectable after 2 weeks. Surprisingly, only a minor increase of IFN-γ expression was detectable after 6 and 10 weeks of diet. Expression pattern of the antiinflammatory molecules IL-10, endothelial nitric oxide synthase, and IL-18BP did not strongly increase, showing only a slightly enhanced expression after 4 weeks of diet (Figure 4).

We further investigated the expression of the chemokine receptors CCR1, CCR2, CCR3, CCR4, CCR5, and CXCR3. Same expression patterns to those described were observed, with increased expression up to 10 weeks of diet, followed by decreased expression after 12 and 14 weeks (Figure 5A). Analysis of the chemokines MCP-1 and macrophage inflammatory protein-1α/β (MIP-1α/CCL3 and MIP-1β/CCL4) also revealed similar expression patterns as their corresponding receptors, with maximum values reached after 10 weeks (Figure 5B). In contrast, highest expression of the chemokine regulated on activated normal T-cell expressed and secreted (RANTES/CCL5) appears already after 6 weeks of diet and markedly decreases after 10 weeks.

**Discussion**

Development of atherosclerotic lesions involves inflammatory infiltrates, mainly composed of T lymphocytes and mononuclear phagocytes that are recruited and activated by a network of proinflammatory cytokines, chemokines, and chemokine receptors secreted by leukocytes, as well as vascular cells.24 Development of atherosclerosis in the ApoE−/− mice model has been extensively studied. As described by several authors, vascular lesions in young mice are more likely to occur in the aortic sinus, at the lesser curvature of the aortic arch, the principal branches of the aorta, and in the pulmonary and carotid arteries. Together with increment of age, distribution and size of lesions also increase in the descending thoracic, lower abdominal, proximal coronary, common iliac, and femoral arteries.14–16 The aim of our study was to investigate the correlation between morphological and molecular components implicated during the development of atherosclerosis and to measure expression kinetics of important pro- and antiinflammatory mediators within the aortic vascular wall.

The analysis of lesion size progression, based on lipid quantification of aortic sinus as well as abdominal aorta, confirmed previous studies mentioned. A more advanced
by several studies. Programmed cell death is apoptotic events induced within lesions of advanced stages, especially in very advanced stage of the lesions after 12 weeks of cholesterol-rich diet. These results might also reflect the enhanced expression of inflammatory mediators did not further increase or even decreased. A possible explanation for this finding might be the already high expression of proinflammatory cytokines correlated with the kinetics of Foxp3 and CCR4, the markers of Tr and Th2, respectively. Th2 cells are known to exhibit antinflammatory properties via secretion of IL-10. In addition, Tr cells have also been shown to secrete high levels of IL-10. In contrast, the proinflammatory cytokine IL-12, which has been shown to play an important role during Th1 polarization, might be secreted in response to the recruitment of Th2 lymphocytes within the vascular wall. These results could indicate that the recruitment of Th1 and Th2 cells would act as antinflammatory regulators by compensating the effects of proinflammatory mediators only at the early stages of atherogenesis. After 4 weeks of diet, expression of the proinflammatory cytokines increased much more importantly compared with the antinflammatory mediators. This imbalance between pro- and antinflammatory cytokines might result in the progression of atherosclerosis under a cholesterol-rich diet. Lesions were clearly detectable, even though the amount of Tr cells did not significantly change between week 10 and the end of the diet treatment. Secretion of chemokines, such as MCP-1, might be responsible for the recruitment of the Tr and Th2 cells. Sebastiani et al have shown that Tr cells express high amounts of CCR2 and CCR5, and that intracellular calcium mobilization within Tr cells was highly induced under stimulation with MCP-1, MIP-1α, and MIP-1β. The chemokine receptor CCR4 is highly expressed on Th2 lymphocytes and is known to bind RANTES, MCP-1, and MIP-1α. Thus, secretion of these chemokines, responsible for the chemotaxis of inflammatory cells at site of inflammation, also seems to chemo-attract the antinflammatory T lymphocytes. These findings are in accordance with the recent article by Martin et al. Both studies, using 2 different atherosclerotic mouse models, demonstrate that chemokines and chemokine receptors increased rapidly to reach a plateau, even followed by a decrease in expression for some of these molecules. In conclusion, our results indicate that inflammatory mediators expressed within atherosclerotic lesions are mainly related to the amount of inflammatory cells present in these lesions. Atherosclerosis might result from an imbalance between pro- and antinflammatory mediators crucial during atherogenesis.

In conclusion, our results indicate that inflammatory mediators expressed within atherosclerotic lesions are mainly related to the amount of inflammatory cells present in these lesions. Atherosclerosis might result from an imbalance between pro- and antinflammatory mediators in response to endothelial injury induced by cholesterol-rich diet. These data provide important information on the expression kinetics of inflammatory mediators expressed during atherogenesis.
inflammatory mediators and point out the possible role of antiinflammatory cells during atherogenesis.

Acknowledgments
This work was supported by grants from the Swiss National Science Foundation (#32000-065121.01/1) to François Mach, and by a grant from the Foundation for Medical Research (Geneva) to Niels Veillard.

References
Differential Expression Patterns of Proinflammatory and Antiinflammatory Mediators During Atherogenesis in Mice

Niels R. Veillard, Sabine Steffens, Fabienne Burger, Graziano Pelli and François Mach

Arterioscler Thromb Vasc Biol. 2004;24:2339-2344; originally published online September 30, 2004;
doi: 10.1161/01.ATV.0000146532.98235.e6

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/24/12/2339

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Figure I (online data supplement) Evolution of body weight, cholesterol, triglycerides and circulating leukocytes.

A, Evolution of the body weight during 14 weeks of cholesterol-rich diet (n=12).

B, Evolution of cholesterol during 14 weeks of cholesterol-rich diet (n=12).

C, Evolution of triglycerides during 14 weeks of cholesterol-rich diet (n=12).

D, Evolution of circulating lymphocytes during 14 weeks of cholesterol-rich diet (n=12).
<table>
<thead>
<tr>
<th>Assay</th>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>(+)-AGATCACAGTCTTCACCTGGGAAGTT-&lt;br&gt;(-)-TGCCCCCTTTTTTGGAATCAA-&lt;br&gt;-TTAATTAGAGGAGGTTCGCC-&lt;br&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD25</td>
<td>(+)-TTGTCGGCAGAACTGTGTCTC&lt;br&gt;(-)-GGCTTTGAATGTGGCATTGG&lt;br&gt;-ACCCACCCGAGGTC-&lt;br&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>(+)-TTGTCTGCGGCGATGTCA&lt;br&gt;(-)-GAATTCTCTGACCGTTTAGCA&lt;br&gt;-TATGGCAACCAGCAGTC-&lt;br&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18BP</td>
<td>(+)-CTGGGCAATGGTGCCCTTTCA&lt;br&gt;(-)-TTGTGTGGCCCTCTTCAGC&lt;br&gt;-TGAGCACCTCCCAGGC-&lt;br&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>