Intraperitoneal Administration of Anti–c-fms Monoclonal Antibody Prevents Initial Events of Atherogenesis but Does Not Reduce the Size of Advanced Lesions in Apolipoprotein E–Deficient Mice

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Background—Atherosclerosis results from complex inflammatory-fibroproliferative responses. To elucidate the central role of macrophage and macrophage-colony stimulating factor (M-CSF) during atherogenesis, we used a new strategy to administer to adult apolipoprotein E (apoE)-deficient mice a monoclonal antibody (AFS98) raised against c-fms, the receptor of M-CSF.

Methods and Results—When 6-week-old apoE-deficient mice were fed a high-fat diet and injected with 2 mg of AFS98 intraperitoneally on alternate days for 6 weeks, accumulation of macrophage-derived foam cells in the aortic root was suppressed by 70% compared with that in controls. This preventive effect was associated with neither remarkable decrease of the number of circulating monocytes nor systemic growth retardation. In contrast, when apoE-deficient mice that had been fed a high-fat diet from 6 weeks of age were given AFS98 from 12 to 18 weeks of age, a minimal protective effect on lesion size was observed.

Conclusions—These results suggest that (1) macrophage and M-CSF/c-fms play an essential role in the arterial wall during development of the fatty streak lesion and (2) blockade of the M-CSF/c-fms pathway could act as protection from at least early atherogenesis but could have a less preventive effect on maintenance of the advanced lesions. (Circulation. 1999;99:1740-1746.)

Key Words: antibodies ■ atherosclerosis ■ leukocytes

Atherosclerosis results from complex inflammatory-fibroproliferative responses.1 It is widely accepted that the earliest event of atherogenesis is the adhesion of circulating leukocytes on the surface of vascular endothelial cells and migration into the subendothelial space. Recent investigations suggested that monocyte/macrophage might play the central role in the vast range of atherogenesis, that is, from fatty streak formation to plaque rupture.2–4 Apolipoprotein E (apoE)-deficient mice develop hypercholesterolemia and premature atherosclerotic lesions even on a low-fat diet and are used widely for studying atherogenesis.5–10

Macrophage colony-stimulating factor (M-CSF) and its receptor, c-fms, have been known to play key roles in differentiation and proliferation of the monocyte/macrophage cell lineage.11,12 Macrophages and macrophage-derived foam cells resident within atheroma have been shown to express M-CSF mRNA and secrete active M-CSF.13,14 It has also been reported that c-fms is expressed in atheromatous lesions.15,16 Although these results indicate that M-CSF could be at least associated with atheroma formation, it is yet to be clarified how M-CSF functions in the arterial wall with regard to the development of atherosclerosis. Yoshida et al17 reported that op/op mice homozygous for a recessive osteopetrosis mutation have a genetic defect for M-CSF production, and this observation was confirmed by the report of Naito et al18 that differentiation of the monocyte/macrophage cell lineage was disrupted in these mice. Smith et al19 and Quiao et al20 crossed apoE-deficient mice with op/op mice and found that atherogenesis was delayed in these “double knockout mice.” However, because systemic monocyte counts are decreased markedly in the op/op mice, owing to hematopoietic suppression,21 it remains unanswered whether the slowed atherogenesis in the “double knockout mice” was due to a functional blockade of M-CSF locally in the vessel.
wall during development of atheroma or merely a consequence of reduced systemic monocyte numbers. Complicating matters further, it has been shown that large doses of M-CSF could reduce the severity of atheromatous lesions in rabbits.22

In this study, we addressed the following questions: (1) Could the M-CSF/c-fms signaling pathway have any functional role in the vessel wall during atherogenesis? (2) If so, how and when does it act in the vast stage of atherogenesis? To answer these questions, we invented a new strategy so, how and when does it act in the vast stage of atherogenesis? (2) If macrophage staining, we used the Tyramide Signal Amplification system (NEN Life Science Products) to amplify the weak signal. Endogenous peroxidase activity was blocked by incubating each section in 3% (vol/vol) H2O2 in methanol. An avidin-biotin blocking kit (Vector Labs) was used. BM8 and BA-4001 were used at a dilution of 1:1000 and 1:200, respectively. After horse radish peroxidase–conjugated streptavidin (Vector Labs) was added to the section, antibody binding was visualized with diaminobenzidine (Vector Labs). For smooth muscle cell staining, we used mouse monoclonal anti-human smooth muscle actin antibody 1A4 labeled with horse radish peroxidase (DAKO). Each section was counterstained with Meyer’s hematoxylin solution.

Image Analysis
Each aortic sinus section was evaluated for oil red O staining by capturing images directly from an RGB camera attached to a light microscope (Axioscope, Karl Zeiss) and displayed on a Trinitron RGB monitor. Image analysis was conducted with the use of KS400 software (Karl Zeiss Vision). For each animal, 20 sequential sections as described in “Tissue Preparation” were examined, and the sum of the lesion area was calculated and expressed in square micrometers.

Flow Cytometric Analysis
Mouse peritoneal cells were suspended in Hank’s solution (Nissui) containing 1% BSA. After incubation with mouse serum, AFS98 or control rat IgG2a was added at the concentration of 1 µg/mL. After 15 minutes of incubation, cells were washed twice by the Hank’s/BSA solution. FITC-mouse anti-rat κ-chain mAb was added. Cells were gated to exclude unusual background interference, and the population containing peritoneal macrophages was analyzed by XL (Coulter).

Statistical Analysis
Data are expressed as mean±SD. The lesion area, serum total cholesterol, and hematologic parameters of mice were compared by ANOVA with the use of Abacus Statview software (version 4.5). A value of *P*<0.05 was considered statistically significant.

Results
Administration of AFS98 Prevented Development of Fatty Streaks in ApoE-Deficient Mice
We first conducted flow cytometric analysis to confirm that AFS98 could recognize c-fms on the peritoneal macrophages from apoE-deficient mice (Figure 1). To study animals with early and advanced lesions of atheroma, we designed protocols A and B (Figure 2). As demonstrated in Figure 3a, the mice that had been injected with PBS showed typical fatty streak lesions in the aortic root stained by oil red O. The majority of the cellular component of this lesion was immunolabeled with the rat mAb BM8 against murine macrophage (Figure 3b), demonstrating that these foam cells were derived from monocyte/macrophages. In striking contrast to the PBS control animals, the aortic lesion size in the mice given AFS98 was remarkably smaller (Figure 3, a and c). As shown in Figure 3d, very few cells were immunostained by BM8 in the aortic sinus.
the aorta of the mice given AFS98, showing that the mice injected with AFS98 had a remarkably lower number of macrophages in the arterial wall. When the areas of the aortic lesions were analyzed quantitatively, the total aortic lesion area of mice injected with AFS98 was $2390 \pm 204 \mu m^2$, which was 30% of that in the PBS control mice ($P=0.024$) (Figure 4). To confirm that the preventive effect of AFS98 was caused by selective blockade of the M-CSF/c-fms signal transduction pathway, another experiment was performed. In this experiment, apoE-deficient mice were fed a high-fat diet and were injected with PBS or the isotype-matched rat irrelevant IgG during the same period. As shown in Figure 5, a and b, injection of the irrelevant IgG had no such preventive effect on atherogenesis as seen with AFS98 injection. When the lesion size was examined by quantitative analyses, no significant difference was found between the animals injected with the irrelevant IgG and PBS (data not shown).

**Prevention of Atherogenesis by AFS98 Was Not Associated With Remarkable Changes in Monocyte Differential Count and Body Weight**

Because the observations above suggested that the M-CSF/c-fms signal transduction pathway plays a significant role in the accumulation of monocyte-derived macrophages in the initial atheromatous lesion, we asked whether the preventive effect of AFS98 could be due to the depletion of monocytes...
from the circulation. In contrast to the observation in “double knockout” apoE-deficient op mice,19 AFS98 administration failed to result in a statistically significant reduction of the peripheral blood monocyte count in our study (Table). Furthermore, the mice given AFS98 appeared to be healthy, and their body weight did not differ significantly from that of the control mice during the whole course of the experiments (data not shown). There was no significant difference in serum total cholesterol level between mice given AFS98 and those given irrelevant IgG (981±368, 855±98 mg/dL, respectively). These results suggest that although AFS98 caused drastic prevention of macrophage accumulation in the aortic wall, this effect was not attributed to suppression of differentiation of the monocyte lineage in the hematopoietic tissues.

**AFS98 Administration Did Not Reduce Size of Advanced Atherosclerotic Lesions**

Because AFS98 protected fatty streak formation, we next tested whether the antibody could have a similar preventive effect on the more advanced lesions. For this purpose, we conducted protocol B by preparing 12-week-old mice that had been fed a high-fat diet (Figure 2). From the results of protocol A (Figure 3, a and b), it was deducible that these mice would have aortic lesions filled with a large number of macrophages. To these animals, we administered either AFS98 or PBS intraperitoneally for 6 more weeks and then examined the aortic lesions. In contrast to the results of protocol A, AFS98 had very little effect on the size of aortic lesions in these older animals. As demonstrated in Figure 6, a and b, the aortic lesion stained by oil red O did not differ in size significantly between AFS98-injected mice and PBS-injected mice. The total aortic lesion area of mice injected with AFS98 and PBS was 30 728±2248 and 31 753±17 483 μm², respectively. To exclude the possibility that the antibody could not be delivered to the atheromatous lesions, we stained sections with anti-rat IgG antiserum. As shown in Figure 6g, we could detect the antibody in the lesion of mice given AFS98 in contrast to the mice given PBS (Figure 6h). We further studied whether AFS98 affected the cell numbers of macrophage or vascular smooth muscle cells, the 2 major cellular components in the atheromatous lesion, by using selective antibodies against these cells. As shown in Figure 6, c through f, AFS98 did not reduce the staining of macrophages, nor did it change the staining pattern of vascular smooth muscle cells.

**Discussion**

Thus far, a variety of experimental animal models including rabbit, nonhuman primate, pigeon, and pig have been used to study atherogenesis. Recently, apoE-deficient mice created by genetic engineering enabled us to investigate further the

<table>
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<th>Injection</th>
<th>WBC, cells/μL</th>
<th>Lymphocyte, %</th>
<th>Neutrophil, %</th>
<th>Monocyte, %</th>
<th>Eosinophil, %</th>
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</thead>
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<tr>
<td>Irrelevant IgG</td>
<td>2500±707</td>
<td>62.3±28.1</td>
<td>32.0±22.1</td>
<td>5.0±5.6</td>
<td>0.7±1.2</td>
</tr>
<tr>
<td>AFS98</td>
<td>3000±848</td>
<td>57.6±16.3</td>
<td>39.1±15.6</td>
<td>3.1±2.1</td>
<td>0.1±0.4</td>
</tr>
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Six-week-old mice were fed a high-fat diet and injected with irrelevant IgG (n=4) or AFS98 (n=10) for 6 weeks according to protocol A (Figure 2). Mice were bled by tail-cut into heparinized capillary tubes (Brummond Scientific) at 12 weeks of age. Blood samples were subjected to white blood cell (WBC) count. Dried smears were stained with Wright, and differential leukocyte counts were performed. Data are shown as mean±SD. There was no significant difference between the 2 groups (irrelevant IgG and AFS98).
molecular basis for atherosclerosis formation. To determine the crucial cellular and molecular components of atherogenesis, many experiments with these mice were conducted by crossing them with other mice carrying various genetic defects or activated transgenes. Among the various genes proposed to be involved in atherogenesis, M-CSF and its receptor c-fms are known to regulate several monocyte functions including differentiation in the bone marrow, growth in the peripheral blood, and migration and adhesion in the inflammatory lesions. M-CSF and c-fms are expressed in the atheroma, and modified LDL can induce M-CSF gene transcription in endothelial cells in vitro. Smith et al and Quiao et al crossed apoE-deficient mice with op/op mice and found that atherogenesis was delayed in these “double knockout mice.” However, it might be difficult to inquire about the dynamic response of M-CSF/c-fms and monocytes/macrophages during atherogenesis in these studies because inborn modification in M-CSF gene precedes atherosclerosis initiation.

To circumvent this problem, we used a new approach to administer to adult apoE-deficient mice the anti–c-fms mAb AFS98 and dissected the complicated mechanism of atherogenesis by blocking the M-CSF/c-fms pathway in adult mice (Figure 2). As demonstrated in Figure 3, a and c, continuous AFS98 administration resulted in marked suppression of fatty streak formation in apoE-deficient mice. Quantitative analyses showed that AFS98 caused reduction of atheroma formation by $\approx 70\%$ (Figure 4). This preventive effect was mediated by the decrease in the number of monocyte/macrophages in the intima of the vessel wall (Figure 3d). To confirm that the effect was specific for anti–c-fms antibody, we conducted additional experiments. Flow cytometric analysis reveals that AFS98 could recognize c-fms on the peritoneal macrophages from apoE-deficient mice (Figure 1, a and b). Since the isotype-matched irrelevant rat IgG failed to suppress atherogenesis in apoE-deficient mice (Figure 5, a and b), the preventive effect of AFS98 was due to the selective blockade of the M-CSF/c-fms pathway, which could then decrease the number of monocytes/macrophages in the vessel wall. In this study, we assessed atherosclerosis by examining the aortic root. Although the lesion severity in the aortic root correlates well to that in the entire aorta, mechanical shear stress and other insults could be related to atherogenesis in these mice. Therefore, further investigation should be done with regard to assessment in the entire aortic lesions.

Interestingly, this preventive effect of AFS98 on atherogenesis in apoE-deficient mice was not associated with the depletion of circulating monocytes (Table). It is compatible to the latest report by Rajavashisth et al in which heterozygous op mutation reduces atherosclerosis in LDL receptor–deficient mice without severe reduction of circulating monocytes. We reported previously that administration of 2 mg of AFS98 to mice had no effect on the production of macrophage colony-forming unit in the bone marrow. It is therefore
conceivable that AFS98 at the dose that we used had little suppressive effect on hematopoiesis in the bone marrow, whereas it was still sufficient to block the M-CSF/c-fms pathway in the vessel walls.

We next conducted protocol B to test whether AFS98 had a protective effect once the lesions had been accomplished. After administration of AFS98 to the mice fed a high-fat diet during the “late” period (12 to 18 weeks), little effect on oil red O–stained lesion size was observed (Figure 6a). As far as we could examine, we could not observe a significant difference in staining pattern of macrophages or smooth muscle cells between the mice given AFS98 and control mice (Figure 6, c through f), as was seen in protocol A (Figure 3). As AFS98 staining was found in these atheromatous lesions by immunohistochemistry (Figure 6g), it is unlikely that the antibody could not enter the appropriate site. These observations suggest that antibody AFS98 might have more potent effects on the early events of atherogenesis such as monocyte migration into the vessel wall and their conversion into macrophages.

These results taken together reveal that macrophages are most potently involved in initial or early events of atherogenesis, which would be followed by infiltration of other cells. Our current data suggest that macrophages not only appear in the early atherosclerotic lesion but also give rise to subsequent molecular and cellular chain reactions. Therefore, macrophage-specific cellular ablation in the initial phase of atherogenesis would prevent subsequent fatty streak formation. Recent studies showed that T and B lymphocytes play a minor role in atherothrombosis; plaque formation, the re-endothelialization of injured atherosclerotic plaques, and the migration and entry into the vessel wall of monocytes/macrophages. These data indicate that the pivotal role of the monocyte/macrophage cell lineage in early atherogenesis.

In summary, we demonstrated that the monocyte/macrophage is crucial in initiation and progression of atheromatous lesions and that the M-CSF/c-fms pathway plays a central role in the differentiation, proliferation, and survival of this cell lineage. Provided that c-fms antagonists could be administered and delivered to the vessel walls without considerable effect on the hematopoietic system or bone growth, this could provide us with a unique site-specific therapeutic approach to protect against atherogenesis in the future.

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