Endothelial Responses to Oxidized Lipoproteins Determine Genetic Susceptibility to Atherosclerosis in Mice

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Background—Oxidized LDL has been found within the subendothelial space, and it exhibits numerous atherogenic properties, including induction of inflammatory genes. We examined the possibility that variations in endothelial response to minimally modified LDL (MM-LDL) constitute one of the genetic components in atherosclerosis.

Methods and Results—By a novel explant technique, endothelial cells (ECs) were isolated from the aorta of inbred mouse strains with different susceptibilities to diet-induced atherosclerosis. Responses to MM-LDL were evaluated by examining the expression of inflammatory genes involved in atherosclerosis, including monocyte chemotactic protein-1 (MCP-1) and macrophage-colony–stimulating factor (M-CSF), an oxidative stress gene, heme oxygenase-1 (HO-1), and other, noninflammatory, genes. ECs from the susceptible mouse strain C57BL/6J exhibited dramatic induction of MCP-1, M-CSF, and HO-1, whereas ECs from the resistant strain C3H/HeJ showed little or no induction. In contrast, ECs from the 2 strains responded similarly to lipopolysaccharide.

Conclusions—These data provide strong evidence that genetic factors in atherosclerosis act at the level of the vessel wall. **(*Circulation*. 2000;102:75-81.)**

Key Words: endothelium ■ cells ■ atherosclerosis ■ lipoproteins ■ mice

Atherosclerosis is a multifactorial disease with a strong heritable component. Epidemiological studies have revealed a number of genetically determined systemic factors that influence disease susceptibility, such as quantitative variations in plasma lipoproteins, blood pressure, homocysteine, and certain hemostatic factors, but together, the known risk features appear to be insufficient to explain the hereditary propensity to atherosclerosis.[1,2]

See p 5

There is considerable evidence that oxidized LDLs (oxLDL) contribute to the development of atherosclerosis. OxLDLs or their products have been found in atherosclerotic lesions in both human and animal models.[3–5] OxLDLs or their components have been shown to stimulate endothelial cells (ECs) to express several proteins that contribute to atherosclerosis, including monocyte chemotactic protein-1 (MCP-1), macrophage-colony–stimulating factor (M-CSF), vascular cell adhesion molecule-1, and other adhesion molecules.[6–10] OxLDLs are avidly taken up by macrophages, resulting in foam cell formation.[11,12] In addition, antioxidants that inhibit LDL oxidation reduce the development of atherosclerotic lesions in animal models.[13,14]

Inbred strains of mice differ strikingly in susceptibility to atherosclerosis,[15,16] and we have previously observed that a high-fat diet induced considerably greater hepatic levels of oxidized lipids and expression of several inflammatory and oxidative stress–responsive genes in atherosclerosis-susceptible C57BL/6J mice than in atherosclerosis-resistant C3H/HeJ mice.[17,18] Because of the small size of arteries in mice, differences between the 2 strains in the formation of oxLDLs and the subsequent induction of inflammatory genes in the arterial wall have not been reported.

In the present study, we developed a method to culture mouse aortic ECs and examined their responses to minimally modified LDL (MM-LDL). Our results indicate that ECs from the susceptible strain C57BL/6J exhibited dramatic induction of MCP-1, M-CSF, and heme oxygenase-1 (HO-1) in response to MM-LDL, whereas ECs from the resistant strain C3H/HeJ showed little or no induction. Thus, these data provide the first strong evidence that genetic factors influencing the responsiveness of vascular cells to oxLDLs contribute to the genetic component in atherosclerosis.

Methods

Reagents

DMEM, FBS, trypsin-EDTA, penicillin-streptomycin, fungizone, and all primers for reverse transcription–polymerase chain reaction (RT-PCR) were obtained from Gibco. Matrigel, dispase, and EC growth supplements (a growth stimulator of ECs; catalogue No.

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40006B) were from Collaborative Research. Heparan sulfate, lipopolysaccharide (LPS), rabbit anti-human von Willebrand factor antibody, and rhodamine-conjugated rat anti-rabbit immunoglobulin were from Sigma Chemical Co. Acetylated LDL labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) was purchased from Biomedical Technologies. Murine cDNA clones were purchased from ATCC.

Mice

Mice were purchased from the Jackson Laboratory, Bar Harbor, Me, and maintained on a regular chow diet. All mice were females 3 to 6 months of age at the time of experiments. All procedures were in accordance with current NIH guidelines and were approved by the UCLA Animal Research Committee.

Isolation and Culture of ECs

ECs from the thoracic aorta were isolated by an explant technique. The mice were anesthetized with isoflurane and euthanized by cervical dislocation. Under sterile conditions, their anterior chest walls, lungs, and esophagi were removed. The thoracic aorta was gently cleaned of periadventitial fat and connective tissue and was cut into rings ≈3 mm long. The aortic segments of each mouse were placed on Matrigel in a 35-mm tissue culture plate and incubated in DMEM supplemented with 15% FBS, 1% penicillin-streptomycin, 90 μg/mL heparin, 60 μg/mL EC growth supplements, and 100 U/mL fungizone at 37°C in a 95% air/5% CO_2 incubator. The vessel rings were removed once cell outgrowth was observed. Approximately 4 days later, the cells were passaged with Dispase and then plated onto 0.1% gelatin–coated 60-mm culture dishes. The subsequent passages were performed with 0.25% trypsin-EDTA, and cells were split in a 1:4 ratio.

Cell Characterization

The identity of cells was confirmed by staining for von Willebrand factor[19] and by DiI-Ac-LDL uptake experiments.[20] Cells were passaged onto sterile glass coverslips and allowed to grow for >4 hours. For von Willebrand factor staining, cells were fixed with acetone for 10 minutes at 4°C. After a washing with PBS, the coverslips were incubated with anti-human von Willebrand factor polyclonal antibody (1:100 dilution) for 1 hour at room temperature. After extensive washing, the cells were incubated with rhodamine-conjugated rat anti-rabbit IgG (1:100) for 1 hour, after which they were washed and visualized with an epifluorescence microscope. For the acetylated LDL uptake experiments, the cells were incubated with 10 μg/mL DiI-Ac-LDL in DMEM for 1 hour at 37°C. The medium was then removed, and the cells were rinsed with PBS. The DiI-Ac-LDL was visualized with an epifluorescence microscope.

Lipoprotein Isolation and Modification

LDL (d=1.019 to 1.069 g/mL) was isolated from the serum of healthy human donors by density-gradient ultracentrifugation as described,[21] dialyzed with PBS containing 0.3 mmol/L EDTA, filtered through 0.22-μm filters, and stored at 4°C. Lipoprotein concentrations were expressed as protein content. MM-LDL was prepared by incubating LDL at a concentration of 5 mg/mL with 7 μmol/L $FeSO_4$ or 4 μmol/L $CuSO_4$ as described.[22] Iron and copper oxidation of LDL produced 1.8 and 1.2 nmol thiobarbituric acid–reactive substances per mg protein, respectively, after dialysis. The concentration of LPS in the lipoprotein solutions was <0.20 ng/mg of protein, which was 50-fold less than required to induce inflammatory genes.

Treatment Conditions

Confluent cells at passage 4 were incubated overnight in DMEM containing 1% FBS. The cells of each mouse were then treated in duplicate with medium containing 100 μg/mL native LDL, 100 or 200 μg/mL Fe^{2+}-modified LDL, 100 μg/mL Cu^{+2}-modified LDL, 2 μg/mL LPS, or medium only. After 4 hours of incubation, the medium was collected and centrifuged at 500g for 10 minutes. The supernatant was stored at −70°C and used for determination of MCP-1 by ELISA.

RNA Extraction and Northern Blot Analysis

After treatment of confluent ECs with the indicated stimuli, total RNA was isolated with TRIzol reagents according to the protocol from the manufacturer. For Northern blot analysis, total RNA was fractionated on 1% agarose-formaldehyde gel, transferred onto nylon membranes, and covalently linked by UV radiation. The blots were prehybridized for 30 minutes and hybridized with ^{32}P-labeled mouse cDNA probes overnight at 65°C. The blots were washed in 2× SSC, 0.1% SDS at room temperature for 20 minutes and then twice in 0.5× SSC, 0.1% SDS at 65°C for 20 minutes. Blots were exposed to Hyperfilm-ECL (Amersham Corp) at −70°C. The density of the bands was quantified with a densitometer and standardized with GAPDH.

Quantitative RT-PCR

mRNA levels of scavenger receptors class A (SRA), CD36, and peroxisome proliferator–activated receptor-γ (PPARγ) were determined by quantitative RT-PCR. Briefly, the first-strand cDNA was reverse-transcribed from total RNA with the SuperScript Preamplification System (Gibco/BRL). The cDNA product was amplified by PCR for 25 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 9 minutes at 72°C. The RT-PCR products were separated in 1.5% (wt/vol) agarose gels and visualized with SYBR Green (Molecular Probes). GAPDH was amplified simultaneously in a separate set of tubes under the same conditions.

Quantification of MCP-1 Protein

MCP-1 in culture media was quantified with a sandwich ELISA technique and a curve calibrated from MCP-1 standards according to the manufacturer's instructions (R&D Systems). Each assay was performed in duplicate.

Monocyte Chemotaxis Assay

For the study of chemotactic effects of conditioned medium on human monocytes, DMEM containing 1% FBS was incubated with confluent ECs for 4 hours. This conditioned medium was centrifuged at low speed to remove the cells and used for test of chemotactic activity in a 48-well modified Boyden micro–chemotaxis chamber (Neuro Probe) as described by Syrovets et al.[23] Positive controls using the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) and negative controls using medium not exposed to ECs were run simultaneously with the test samples. The number of transmigrated monocytes was determined by counting of 5 fields per well with light microscopy.

Statistical Analysis

Data are presented as mean±SEM, with n indicating the number of mice from which ECs were obtained. These means were obtained by averaging data from each animal, and only this average was used for statistical analyses. To compare differential induction of ECs by the indicated treatments between C57BL/6J and C3H/HeJ mice, we used 2-way ANOVA. When only 2 means were compared, Student's t test was used. Differences were considered statistically significant at P<0.05.

Results

EC Cultures

The cells isolated from the mouse aorta were examined for the presence of the endothelium-specific marker von Willebrand factor. As shown in Figure 1, the staining was observed in all of the cells present. Acetylated LDL has also been used as a probe to identify ECs.[20] When incubated with DiI-Ac-LDL for 1 hour, the cells showed fluorescent deposits throughout the cytoplasm. ECs from both strains grew in

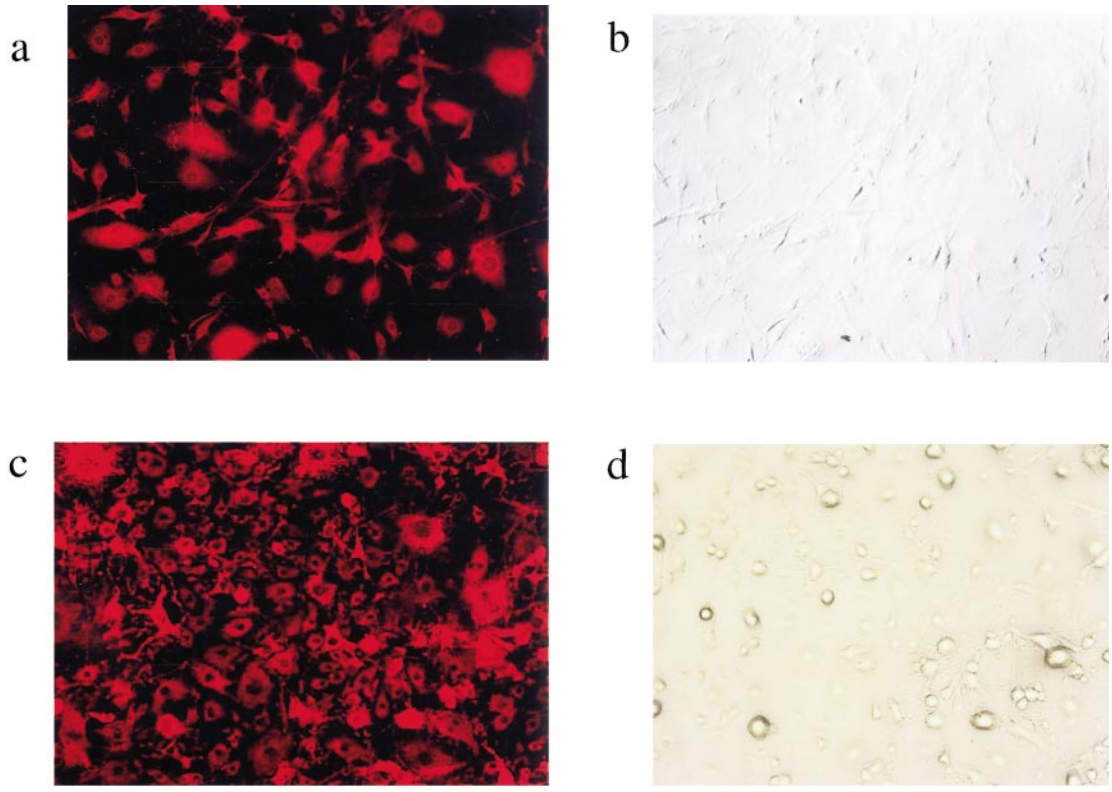

Figure 1. Characterization of mouse ECs. a (Fluorescence) and b (phase): expression of endothelium-specific marker von Willebrand factor. c (Fluorescence) and d (phase): uptake of DiI-Ac-LDL.

monolayers and exhibited contact inhibition. The cell doubling time was 2.4±0.3 days (n=4) in C57BL/6J mice and 2.9±0.5 days (n=4) in C3H/HeJ mice.

Induction of Inflammatory Genes and Other Genes Relevant to Atherosclerosis

As shown in Figure 2, both Fe^{2+}- and Cu^{2+}-modified LDL induced significant production of MCP-1 (Figure 2A and 2B), M-CSF (Figure 2C), and HO-1 (Figure 2D and 2E) in ECs from C57BL/6J mice. In contrast, ECs from C3H/HeJ mice failed to show significant induction of MCP-1 and M-CSF mRNA, although HO-1 was significantly increased. It is noteworthy that the baseline level of HO-1 mRNA was significantly higher in C3H/HeJ than in C57BL/6J mice. Native LDL had no effect on gene induction in ECs from either strain. Interestingly, LPS induced prominent but similar expression of MCP-1 and M-CSF gene in both strains. Unlike oxLDL, LPS had little effect on HO-1 expression.

Vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and antioxidant protein 2 expression was also examined by Northern blot analysis. We found that vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 mRNA was significantly induced by Fe^{2+}- or Cu^{2+}-modified LDL in C57BL/6J but not in C3H/HeJ mice and that antioxidant protein 2 mRNA was not induced in either strain (data not shown).

SRA, CD36, and PPARγ mRNA levels of ECs after treatment with native LDL (control) and Fe^{2+}-modified LDL for 4 hours were measured by quantitative RT-PCR (Figure 3). SRA mRNA levels were low at baseline but were significantly induced by Fe^{2+}-modified LDL in C57BL/6J mice. In C3H/HeJ mice, in contrast, SRA mRNA levels were high at baseline but were not induced by Fe^{2+}-modified LDL. The mRNA levels of CD36, PPARγ, and GADPH did not differ between the 2 strains either at baseline or after stimulation with Fe^{2+}-modified LDL.

Induction of MCP-1 Protein and Chemotactic Activity

MCP-1 protein levels were measured by ELISA. As shown in Figure 4, both Fe^{2+}- and Cu^{2+}-modified LDL induced significant production in C57BL/6J mice, whereas in C3H/HeJ mice, the induction was not significant. Native LDL had no effect on MCP-1 production in either of the strains. We also tested the chemotactic effects of conditioned medium that had been incubated with confluent ECs for 4 hours and found that the EC-conditioned medium of C57BL/6J mice had a greater effect on human monocyte transmigration than that of C3H/HeJ mice ($P<0.05$; Figure 5).

Discussion

The goal of this study was to address whether genetic factors act at the level of the vessel wall. We have now tested this using ECs isolated from 2 mouse strains that differ strikingly in susceptibility to atherosclerosis. The results show that cultured ECs from C57BL/6J mice are much more responsive to MM-LDL, as judged by the induction of MCP-1, M-CSF, and HO-1, than ECs from C3H/HeJ mice. These data provide direct evidence that strain C57BL/6J mice are more suscep-

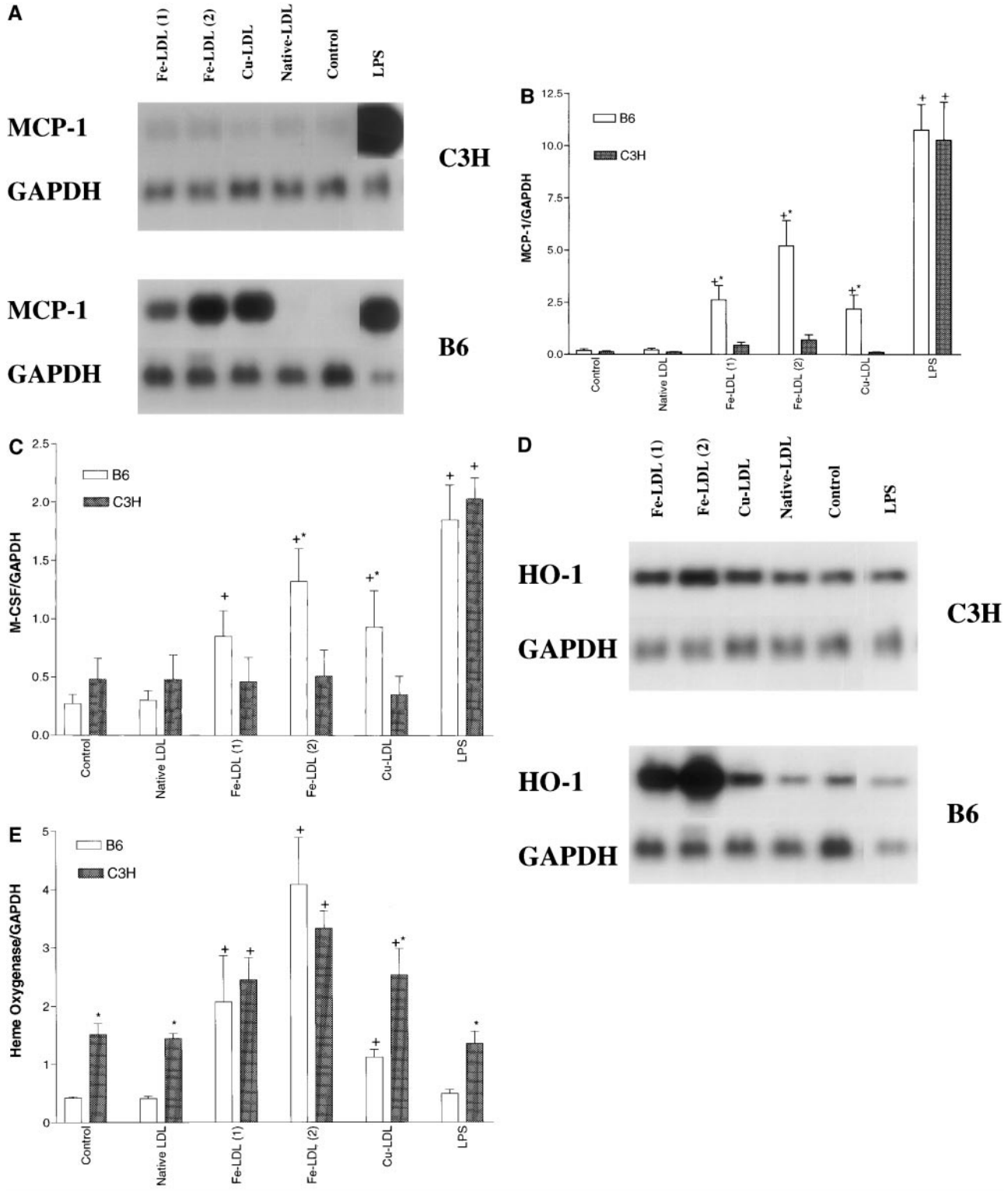

Figure 2. MCP-1 (A, B), M-CSF (C), and HO-1 (D, E) mRNA expression of aortic ECs from C57BL/6J (B6) and C3H/HeJ (C3H) mice after incubation with Fe^{2+}- or Cu^{2+}-modified LDL, native LDL, medium (control), or LPS for 4 hours. A and D, Representative northern blots of MCP-1 and HO-1 mRNA. B, C, and E, Ratio of densitometry units of MCP-1, M-CSF, or HO-1 mRNA to GAPDH mRNA. Values are mean±SEM of 4 to 6 individual mice. *$P<0.05$ vs C3H with same treatment; +$P<0.05$ vs control.

tible to atherosclerosis than C3H/HeJ, at least in part because of genetic differences in the response of ECs to MM-LDL.

Our studies required the isolation of ECs from mice, and we developed a reliable and simple procedure using aortic ring explants. The resulting cells were identified as ECs by the fact that all of the cells were positive for the expression of the von Willebrand factor, a specific marker of ECs.[24] Moreover, all of the cells rapidly internalized DiI-Ac-LDL, a

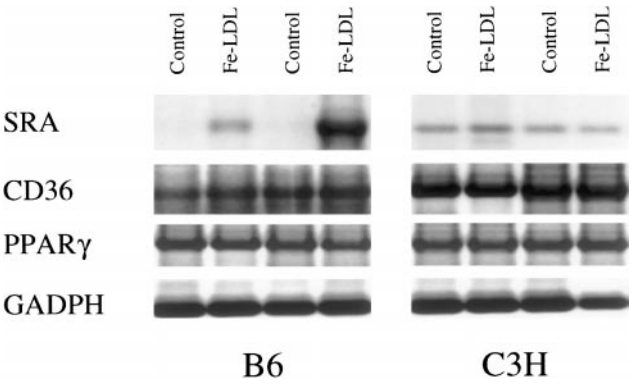

Figure 3. Expression of SRA, CD36, PPARγ, and GADPH mRNA in ECs of C57BL/6J and C3H/HeJ mice analyzed by RT-PCR. Cells were treated with native LDL (control) and Fe^{2+}-modified LDL for 4 hours.

characteristic of ECs.[20] Multiple independent isolates from strains C57BL/6J and C3H/HeJ by our present methods gave reproducible results for the expression of endothelium-specific markers.

Previously, we observed that challenge with an atherogenic diet induced much greater expression of MCP-1, M-CSF, and HO-1 in the liver of C57BL/6J mice than C3H/HeJ mice.[17,18] Our present study shows a similar difference between 2 strains in gene induction by MM-LDL in arterial cells. Presumably, the feeding of the atherogenic diet results in oxLDL accumulation in the arterial walls, and the variations between the strains in endothelial responses to MM-LDL lead to differences in monocyte attachment, transmigration, maturation, and transformation into foam cells, all of which affect lesion formation.

A substantial body of evidence shows that minimally or mildly oxidized species of LDL are potent inducers of a variety of inflammatory genes involved in atherogenesis.[25–27] Among them, it is clear that M-CSF and MCP-1 are significant in atherogenesis. Both are highly elevated in atherosclerotic lesions, and both are highly induced in ECs by oxLDL.[6,7,28,29] Furthermore, genetic mutations resulting in

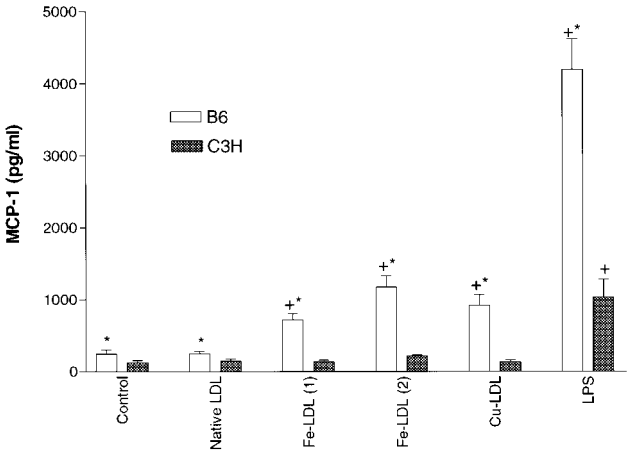

Figure 4. Induction of MCP-1 protein. Confluent cells were treated as in Figure 2. Conditioned medium was analyzed for MCP-1 proteins by ELISA. Results are mean±SEM of 6 mice. *$P<0.05$ vs C3H with same treatment; +$P<0.05$ vs control.

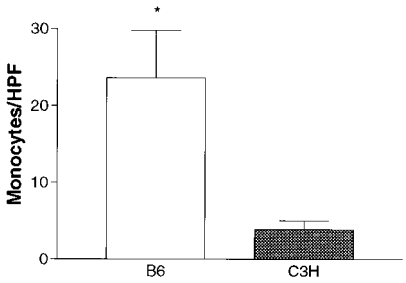

Figure 5. Chemotactic effects of EC-conditioned medium on human monocytes. Medium that had been incubated with ECs for 4 hours was used to test chemotactic effects. Data are average numbers of transmigrated monocytes per high-power field±SEM of 3 separate experiments. *$P<0.05$ vs C3H.

deficiencies of the 2 proteins or the MCP-1 receptor significantly reduce atherogenesis in mice.[30–33]

Our present data clearly show that ECs from susceptible C57BL/6J mice exhibit activation of both MCP-1 and M-CSF in response to MM-LDL, whereas ECs from C3H/HeJ mice exhibit little or no induction (Figure 2). We observed differences in MCP-1 induction not only at the level of mRNA but also at the level of protein (Figure 4). In contrast to MM-LDL, LPS resulted in similar responses of ECs from the 2 strains with regard to MCP-1 and M-CSF mRNA induction (Figure 2). This finding suggests that there are differences in the signal pathways for LPS and MM-LDL, consistent with the observation that in ECs, MM-LDL specifically induces adhesion molecules for monocytes, whereas LPS induces adhesion molecules for both neutrophils and monocytes.[34]

Our data also indicate that endothelial HO-1 expression in C57BL/6J mice was significantly lower under the basal condition but was more dramatically induced by MM-LDL than in C3H/HeJ mice. The significance of the differential HO-1 induction by MM-LDL between the 2 strains is unclear. Because HO-1, an inducible isoform of heme oxygenases, is considered one of the most sensitive and reliable indicators of cellular oxidative stress,[35] our data suggest that MM-LDL induced a greater oxidative stress response in C57BL/6J than in C3H/HeJ mice. Although LPS injection induced HO-1 gene expression in peritoneal macrophages and hepatic cells of mice,[36] it failed to induce HO-1 expression of ECs in our study. This may be due to the possibility that LPS induces HO-1 in vivo through an indirect pathway or to differences in cell types. The higher HO-1 basal level in ECs of C3H/HeJ mice could protect against atherosclerosis, because the production of HO-1 inhibits monocyte transmigration.[37]

In C3H/HeJ mice, a single defective allele on chromosome 4 renders most cells, including lymphocytes and macrophages, insensitive to LPS-induced cytokine release.[38] A recent study showed that the LPS allele of C3H/HeJ mice corresponds to a missense mutation in exon 3 of the Toll-like receptor-4 gene.[39] The Toll-like receptor-4 has been suggested to transduce the LPS signal across the plasma membrane. However, our present study showed that ECs from C3H/HeJ mice were as responsive to LPS as those from C57BL/6J mice with respect to MCP-1 and M-CSF mRNA induction. These results suggest that receptor subtypes on ECs that mediate the effect of LPS differ from those on

lymphocytes and macrophages. Moreover, because the expression of MCP-1 was similar at the mRNA level in 2 strains but ≈4-fold less in C3H/HeJ than C57BL/6J mice at the protein level, our data suggest that a posttranscriptional mechanism may be involved in the defective response to LPS of C3H/HeJ mice.

In the present study, we observed that CD36 and PPARγ genes were not induced by MM-LDL in ECs from C57BL/6J and C3H/HeJ mice, although SRA mRNA levels were elevated in C57BL/6J mice. ECs express scavenger receptors, such as SRA and CD36, that recognize oxLDL.[40,41] Previous studies have suggested that scavenger receptors are unlikely to be the receptors that mediate MM-LDL–induced inflammatory responses in ECs.[34,42,43] Because they bind, internalize, and degrade oxLDL, scavenger receptors may influence accumulation of oxLDL in arterial walls.

The reason why C57BL/6J mice tend to accumulate more oxidized lipids and to be more responsive to them than C3H/HeJ mice is unknown. However, it is known that on the atherogenic diet, C57BL/6J mice show reduced levels of HDL[15] and serum paraoxonase.[44] HDL and paraoxonase play important roles in preventing lipid accumulation and oxidation in arterial walls. Liao et al[45] reported that inflammation induced by feeding of atherogenic diets or injection of MM-LDL markedly elevated the level of serum amyloid A protein, an acute-phase reactant, in C57BL/6J but not in C3H/HeJ mice and that the increased serum amyloid A levels altered the ability of HDL to protect against LDL oxidation. There may be other links between the accumulation of oxidized lipids and responsiveness to oxLDLs as well.

The demonstration that the differences in atherosclerosis susceptibility between inbred mouse strains C57BL/6J and C3H/HeJ are due, at least in part, to genetic differences in endothelial responses provides the first clear evidence for genetic factors in atherosclerosis that act at the level of vascular cells. It seems likely that this will be the case for humans as well as mice. The finding that levels of certain inflammatory gene products are elevated in patients with clinical evidence of atherosclerosis[46] supports this possibility.

Acknowledgments

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