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Absence of Monocyte Chemoattractant Protein-1 Reduces Atherosclerosis in Low Density Lipoprotein Receptor–Deficient Mice

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Summary

Recruitment of blood monocytes into the arterial subendothelium is one of the earliest steps in atherogenesis. Monocyte chemoattractant protein-1 (MCP-1), a CC chemokine, is one likely signal involved in this process. To test MCP-1's role in atherogenesis, low density lipoprotein (LDL) receptor–deficient mice were made genetically deficient for MCP-1 and fed a high cholesterol diet. Despite having the same amount of total and fractionated serum cholesterol as LDL receptor–deficient mice with wild-type MCP-1 alleles, LDL receptor/MCP-1-deficient mice had 83% less lipid deposition throughout their aortas. Consistent with MCP-1's monocyte chemoattractant properties, compound-deficient mice also had fewer macrophages in their aortic walls. Thus, MCP-1 plays a unique and crucial role in the initiation of atherosclerosis and may provide a new therapeutic target in this disorder.

Introduction

Atherosclerosis is a progressive disease in which accumulation of cells, lipid, and extracellular matrix in the wall of an artery can result in occlusion of the vessel lumen either by the atherosclerotic plaque itself or by thrombus formation due to plaque rupture (Fuster et al., 1992; Ross, 1995). The complex atheromatous plaque contains a variety of cell types, and its evolution proceeds in well described stages. Early lesions, so-called fatty streaks, are composed primarily of lipid-laden macrophages in the subendothelium. Later lesions, the fibrous plaques, show evidence of smooth muscle cell proliferation and deposition of extracellular matrix. Fibrofatty plaques often have large, central, lipid-rich cores, and have a particular propensity to rupture and cause thrombosis especially when they contain many macrophages.

Experimental analyses have shown that adhesion of circulating monocytes to the vascular endothelium occurs very early in atherogenesis (Gerrity, 1981). These cells diapedese into the subendothelium where they ingest lipid to become the foam cell macrophages of the fatty streak (Aqel et al., 1985; Gown et al., 1986; Jonasson et al., 1986). Although it is clear that blood monocytes are the source of arterial wall macrophages, the mechanism underlying their recruitment has been obscure. It has been presumed that arterial cells elaborate monocyte attractant signals in response to atherogenic injuries such as turbulent blood flow, high blood pressure, or hyperlipidemia, but the molecular signals have not been identified.

Chemokines are excellent candidates for the chemoattractants that recruit monocytes into the subendothelium of damaged arteries (Rollins, 1997; Baggiolini, 1998). In particular, extensive circumstantial evidence supports a role for the CC chemokine monocyte chemoattractant protein-1 (MCP-1) in this process. For example, all cellular elements of the arterial wall, including endothelial cells, smooth muscle cells, and fibroblasts, secrete MCP-1 in response to many physiologically relevant signals (Strieter et al., 1989; Rollins et al., 1990; Taubman et al., 1992). Inducers of MCP-1 expression include minimally modified low density lipoprotein (Berliner et al., 1990) and fluid shear stress (Shyy et al., 1994), two well documented atherogenic stimuli. Furthermore, in vivo, MCP-1 expression occurs in the arterial wall in response to hypercholesterolemia in rabbits (Clinton and Libby, 1992), in nonhuman primates (Yu et al., 1992), and in response to balloon injury in rabbits (Taubman et al., 1992). Human atherosclerotic plaques also contain MCP-1 (Nelken et al., 1991; Yla-Herttuala et al., 1991a).

MCP-1's properties are consistent with a monocyte chemoattractant role in atherogenesis. In vitro, MCP-1 specifically attracts monocytes, memory T lymphocytes, and NK cells. In vivo, several transgenic models of MCP-1 overexpression confirm that this chemokine can attract blood monocytes into the parenchyma of expressing organs (Fuentes et al., 1995; Nakamura et al., 1995; Grewal et al., 1997; Gunn et al., 1997). The physiological relevance of this activity has been documented by *MCP-1*$^{-/-}$ mice carrying targeted null alleles. (Although the gene encoding MCP-1 in the mouse is named *ScyA2*, the genotype of these mice will be denoted *MCP-1*$^{-/-}$ for convenience.) These mice demonstrate severe defects in monocyte recruitment to sites of inflammatory damage or in response to immunological signaling (Lu et al., 1998). Like all chemokines, MCP-1's receptors are 7-transmembrane segment G-protein coupled receptors. MCP-1 binds both to CCR2 and CCR9 but appears to signal only through the former (Charo et al., 1994; Nibbs et al., 1997). CCR2-deficient mice have a phenotype similar to that of MCP-1-deficient mice, suggesting that CCR2 is in fact MCP-1's primary receptor in vivo (Boring et al., 1997; Kurihara et al., 1997; Kuziel et al., 1997).

To determine whether MCP-1 is pathogenetically involved in atherogenesis, we crossed MCP-1-deficient mice with mice deficient for the low density lipoprotein receptor (LDL-R) which removes cholesterol-rich intermediate density lipoproteins (IDL) and LDL from the blood (Ishibashi et al., 1993). When fed high cholesterol diets, LDL-R-deficient mice have extremely high levels

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Table 1. Plasma Lipids in *LDL-R*$^{-/-}$/MCP-1$^{+/+}$ and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ Mice

	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
LDL-R$^{-/-}$/*MCP-1*$^{+/+}$	1202 ± 72	156.0 ± 20.6
LDL-R$^{-/-}$/*MCP-1*$^{-/-}$	1501 ± 503	279.2 ± 36.4
p value	0.375	0.05

Four male *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and five male *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice aged 6–8 weeks were fed a diet consisting of 1.25% cholesterol without cholate for 12 weeks. Mice were fasted for 12 hr, after which plasma cholesterol and triglycerides were determined as described in the Experimental Procedures. Standard errors of the mean are indicated. p values were derived using Student's t test.

of IDL and LDL and develop extensive fatty streaks throughout their aortas. Although their lesions do not evolve into fibrous plaques, this murine model recapitulates many of the features of fatty streak formation found in humans (Breslow, 1996). Therefore, we fed high cholesterol diets to compound LDL-R/MCP-1-deficient mice and found that despite their having equivalent levels of plasma cholesterol, IDL, and LDL, *MCP-1*$^{-/-}$ mice had far less lipid deposition in their aortas than their counterparts with wild-type MCP-1 alleles. Furthermore, *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice had fewer macrophages in their aortic walls than did *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice, suggesting that recruitment of fewer macrophages results in less lipid deposition even in the face of high blood cholesterol levels.

Results

Mice homozygous for null alleles of *MCP-1* in a 129/SvJ × C57Bl/6 strain background (Lu et al., 1998) were mated to mice homozygous for null alleles of *LDL-R* in a C57Bl/6 background (Ishibashi et al., 1993). Their progeny, which were heterozygous at both loci, were backcrossed to the parental *LDL-R*$^{-/-}$ strain. Progeny with the genotype *LDL-R*$^{-/-}$/*MCP-1*$^{+/-}$ were crossed with each other, and littermates having the genotypes *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$, respectively, were used to generate control and experimental lines with the same strain background.

Male mice of both genotypes were fed a standard chow diet until 6–8 weeks of age and then switched to a diet containing 1.25% cholesterol without cholate. After 12 weeks on a high cholesterol diet, mice were fasted for 12 hr, and plasma lipid analyses were performed. Total plasma cholesterol was the same in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice and *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice, indicating that MCP-1 had no net influence on cholesterol metabolism (Table 1). Fractionation of plasma lipids by FPLC showed a statistically insignificant increase in VLDL in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice, but otherwise the lipoprotein profiles of the two genotypes were nearly superimposable (Figure 1). In contrast, *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice had nearly twice the plasma concentration of triglycerides compared to *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice (Table 1), perhaps accounting for their increased VLDL. The reason for this difference is unknown, but its implications for atherogenesis are discussed below.

After 12–14 weeks on a high cholesterol diet, mice were sacrificed, and their aortas were dissected and

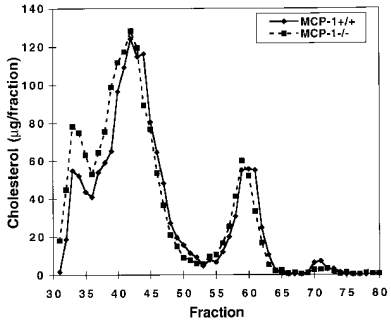

Figure 1. FPLC Fractionation of Plasma Lipids from *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ Mice

Mice were fed a 1.25% cholesterol, cholate-free diet for 12 weeks and then fasted overnight. Blood was obtained from four *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and two *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice in EDTA-coated tubes, and equivalent amounts of plasma from individuals of each genotype were combined to yield 0.2 ml. Plasma was fractionated by FPLC through two Superose-6 columns in series as described in the Experimental Procedures, and cholesterol concentrations in each 0.5 ml fraction were determined. This procedure was performed twice on two independent groups of mice and the mean cholesterol amounts in each fraction are shown. Solid lines indicate *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice; dotted lines indicate *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice.

stained en face with Oil Red O to reveal lipid deposition. The extent of Oil Red O staining was variable among *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice and ranged from 8% to 63% of the aortic tree from the root to the iliac bifurcation. In contrast, no *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mouse had involvement of more than 4% of the aorta. Figure 2 shows representative examples taken from the mid range of aortic involvement in both genotypes. Image analysis revealed that the average proportion of the aorta stained by Oil Red O in *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice was 21.8 ± 6.0% (n = 9, mean ± s.e.m.) compared to 3.8 ± 0.3% (n = 5) in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice (Table 2). This 83% reduction was highly significant (two-sided p = 0.001 by Wilcoxon's rank sum test).

Additional mice were analyzed after a total of 20–25 weeks on a high cholesterol diet (Table 2). Despite more prolonged hypercholesterolemia, lipid deposition in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice was 6.0 ± 2.0% of the aortic surface (n = 4), not significantly greater than the amount of disease at 12–14 weeks. However, this value remained significantly lower than the 27.6 ± 2.0% aortic involvement in *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice (n = 3, p = 0.005). This indicates that lipid deposition in MCP-1$^{-/-}$ mice did not "catch up" to that in *MCP-1*$^{+/+}$ mice over a 25-week time period.

Some anatomical areas of the aorta may be more susceptible to atherosclerosis than others. For example, disturbed blood flow at the aortic root has been invoked to explain the predisposition of this region to plaque formation in many mouse models (Paigen et al., 1987; Nakashima et al., 1994). However, in LDL-R-deficient mice, it has been shown that the amount of disease in the aortic tree correlates closely with disease in the aortic root (Tangirala et al., 1995). Nonetheless, to confirm the differences observed in the en face aorta preparations, we analyzed the aortic root by staining serial sections with Oil Red O and determining the average cross-sectional area of lipid deposition (Figure 3). In the

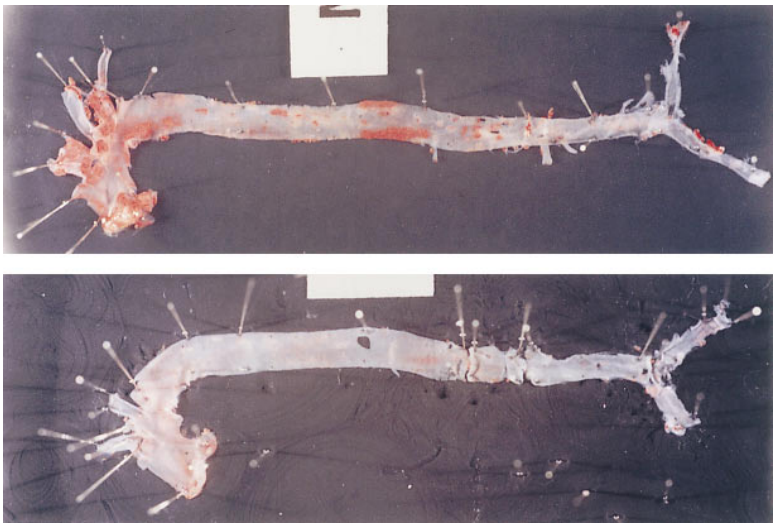

Figure 2. Lipid Deposition in Aortas of *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ Mice

After 12–14 weeks on a 1.25% cholesterol, cholate-free diet, mice were sacrificed and their aortas perfused with PBS. Aortas were dissected and opened along the ventral surface, pinned on a black wax background, fixed, and stained for lipids using Oil Red O. Red stain indicates lipid deposition. These are representative examples from the mid range of each genotype (upper, *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$; lower, *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$).

first 300 μm distal to the origin of the aortic valve leaflets, lipid staining involved 668,736 ± 136,746 μm^2/section in *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice and 140,723 ± 48,123 μm^2/section in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice (Table 3). This 79% reduction in lesion area was highly significant ($p = 0.015$ by Student's t test) and was quantitatively similar to the reduction in disease determined for the entire aorta. The histologic examinations of the lesions in both genotypes were consistent with the descriptions of the lesions in the LDL-R-deficient mice (Ishibashi et al., 1994), where lipid-laden macrophages line the subintimal space surrounded by pools of lipid. Thus, by two measures, disease severity was greatly reduced in *MCP-1*$^{-/-}$ mice.

Based on MCP-1's chemoattractant properties, the mechanism for its atherogenic effects is likely to involve recruitment of monocytes into the arterial wall. To evaluate this possibility, we examined the extent of macrophage infiltration in the aortic arch by immunohistochemical staining with MOMA-2, an antibody specific for mature murine macrophages that is useful because of its intense cytoplasmic staining properties (Kraal et al., 1987; Leenen et al., 1994). It is difficult to discern individual macrophages using this antibody (Figure 4), as it is with all other macrophage-specific antibodies that we tested (data not shown). Therefore, macrophage infiltration was quantified by determining the area of MOMA-2 staining as a percentage of aorta cross-sectional area. Aortic arches from *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice had 54% less MOMA-2 staining than arches from *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice, a highly significant difference (Table 3).

Table 2. Effect of MCP-1 on Lipid Deposition in the Aortic Tree

Weeks on High Cholesterol Diet	Aorta Area Stained by Oil Red O[a] (%)	
	12–14	20–25
LDL-R$^{-/-}$/*MCP-1*$^{+/+}$	21.8 ± 6.0 (9)	27.6 ± 0.4 (3)
LDL-R$^{-/-}$/*MCP-1*$^{-/-}$	3.8 ± 0.3 (5)	6.0 ± 2.0 (4)
p value	0.001	0.005

Male mice of the indicated genotype were fed a diet containing 1.25% cholesterol for the indicated time, after which aortas were isolated and stained en face as described in the Experimental Procedures.

[a] Area of Oil Red O Staining was determined by image analysis and is indicated as a proportion of the area of the entire aortic tree ± standard error of the mean. Figures in parentheses represent the numbers of mice analyzed. The range for the experiments performed at 20–25 weeks was 27%–30% for the controls and 2.2%–10.8% for the MCP-1/LDL-R double knockout. p values were derived using Wilcoxon's rank sum test (12–14 weeks) and Student's t test (20–25 weeks).

Discussion

Macrophages derived from circulating monocytes feature prominently in atherosclerotic plaques (Gerrity,

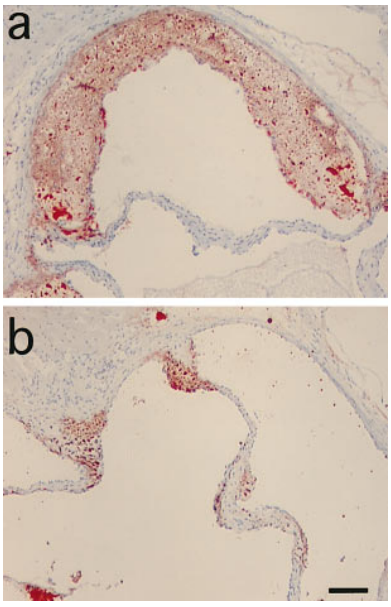

Figure 3. Lipid Deposition in the Aortic Roots of *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ Mice

Frozen sections from the aortic roots were stained with Oil Red O as described in the Experimental Procedures. These are representative samples of the same anatomic level from *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice (A) and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice (B). Bar is 100 microns.

Table 3. Effect of MCP-1 on Lipid Deposition in the Aortic Root and Macrophage Accumulation in the Aortic Arch

	Cross-Sectional Area of Aortic Root Stained by Oil Red O (μm^2)	Percentage of Aortic Arch Area Stained by MOMA-2
LDL-R$^{-/-}$/*MCP-1*$^{+/+}$	668,736 ± 136,746 (4)	6.22 ± 0.01 (4)
LDL-R$^{-/-}$/*MCP-1*$^{-/-}$	142,073 ± 48,123 (4)	2.86 ± 0.01 (4)
p value	0.015	0.016

Male mice of the indicated genotype were fed a diet containing 1.25% cholesterol for 14–25 weeks, after which aortic roots were isolated, sectioned, and stained for lipid using Oil Red O as described in the Experimental Procedures. Area of Oil Red O staining was determined in each section by image analysis, and the average cross-sectional area for 18 sections is indicated in μm^2 ± standard error of the mean. Aortic arches from the same animals were sectioned and stained for macrophage infiltration using MOMA-2. Area of MOMA-2 staining was determined by image analysis in six sections and is shown as the average percentage of the aortic cross-sectional area stained ± standard error of the mean. Figures in parentheses indicate the number of animals analyzed. p values were determined using Student's t test.

1981). Early fibrofatty lesions in humans are filled with macrophages, suggesting that monocyte infiltration into the intima is one of the first steps in atherogenesis (Aqel et al., 1985; Gown et al., 1986; Jonasson et al., 1986). These observations have led to the hypothesis that atherogenesis is a special case of chronic inflammation initiated by vascular insults such as hypercholesterolemia or disturbed blood flow (Libby et al., 1992; Ross, 1995). While macrophage accumulation might result from, rather than cause, arterial disease, the pathogenetic importance of monocytes is indicated by the near absence of diet-induced atherosclerosis in *op*$^{-/-}$ mice that have reduced numbers of monocytes and tissue macrophages due to the genetic absence of monocyte colony stimulating factor-1 (Smith et al., 1995; Qiao et al., 1997). However, these observations do not address the question of what signals initiate trafficking of monocytes into the subendothelium in mice that have a normal complement of monocytes.

Since chemokines control the migration of specific leukocyte subtypes in inflammation, members of this class of intercellular signaling molecules likely contribute to attracting monocytes to sites of endothelial dysfunction. MCP-1 has been the leading candidate among chemokines both because of its target cell specificity and because of an extensive literature pointing to its expression in atherogenesis (Cushing et al., 1990; Nelken et al., 1991; Yla-Herttuala et al., 1991b; Clinton and Libby, 1992) and in response to oxidized lipid (Berliner et al., 1990). However, testing MCP-1's pathophysiological role in this disease required the development of reproducible murine models both of atherosclerosis and chemokine deficiency. The approaches used in this report now directly establish a central role for MCP-1 in atherogenesis, placing leukocyte trafficking in this disease squarely within the paradigms established for other inflammatory processes. While the LDL-R-deficient mouse model may not faithfully replicate all features of human atherosclerosis (Ishibashi et al., 1993), the extensive fatty deposition observed in LDL-R-deficient mice does mimic early atherogenesis in humans, when monocyte recruitment and macrophage accumulation figure prominently. After longer periods of hypercholesterolemia than those used in this report, LDL-R-deficient mice also develop diffuse xanthomata (Ishibashi et al., 1994). Absence of these lesions in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice might indicate that mechanisms of macrophage recruitment in the vasculature could be similar to those that occur elsewhere.

We observed fewer macrophages infiltrating the aortic wall of *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice, suggesting that the decreased severity of their atherosclerotic disease was

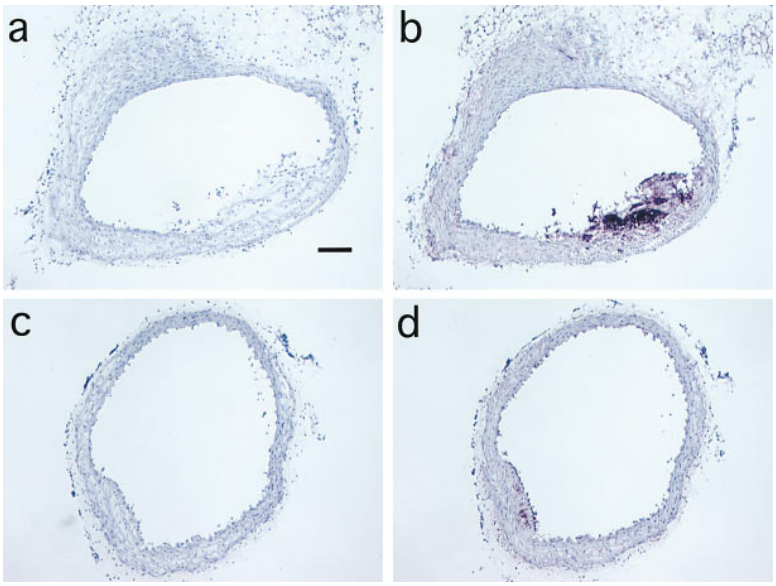

Figure 4. Macrophage Infiltration into the Aortic Walls of *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ Mice

Frozen sections from the aortic arches of *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ (A and B) and *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ (C and D) mice were stained with control rat IgG_{2b} (A and C) or MOMA-2 (B and D) to identify macrophages. Bar is 100 microns.

due to diminished monocyte recruitment. While this mechanism is consistent with MCP-1's in vivo properties, *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice also had twice the plasma triglycerides of *LDL-R*$^{-/-}$/*MCP-1*$^{+/+}$ mice. On one hand, lipoprotein lipase activity has been implicated as a risk factor in human atherosclerosis (Goldberg, 1996). Increased triglycerides suggest decreased lipoprotein lipase activity which, in turn, may imply that *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice were protected from disease on this basis. On the other hand, a major source of lipoprotein lipase activity is the vessel wall macrophage (Yla-Herttuala et al., 1991b; O'Brien et al., 1992). Since *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice had fewer macrophages in their aortic walls, their increased triglyceride may be a result of the more proximal protective effect of decreased macrophage recruitment. Consistent with this idea is the finding that the quantitative diminution in lipid deposition, both by en face and aortic root measurements, greatly exceeded the doubling of plasma triglyceride concentrations.

Despite their lack of MCP-1, *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice could still mount a modest macrophage infiltrate. As with all models involving targeted gene disruption, potential exists for compensatory activity by the products of other genes. To date, however, the severity of the phenotype in chronically MCP-1-deficient mice, i.e., *MCP-1*$^{-/-}$, resembles that in an acutely MCP-1-deficient mouse, i.e., after neutralizing antibody treatment (Chensue et al., 1996; Lu et al., 1998). Thus, a more likely explanation for the residual infiltrate in *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice may be found in overlapping specificity among chemokine ligands and receptors. For example, although MCP-1 binds only to CCR2 with high affinity, CCR2 binds several other chemokines, including MCP-3 and -5 in the mouse (Kurihara and Bravo, 1996; Sarafi et al., 1997) and MCP-3 and -4 (and perhaps MCP-2) in humans (Sozzani et al., 1994; Combadiere et al., 1995; Franci et al., 1995; Garcia-Zepeda et al., 1996; Moore et al., 1997; Stellato et al., 1997). Any of these could account for residual macrophage infiltration observed in the *LDL-R*$^{-/-}$/*MCP-1*$^{-/-}$ mice. Alternatively, ligands for other chemokine receptors expressed by monocytes could be expressed in the arterial wall and result in monocyte accumulation. Still, in the absence of MCP-1 alone, lipid deposition was reduced by approximately 80%, suggesting a major role for this chemokine in atherogenesis under these conditions. Finally, we note that the mice described in this study are F3 backcrosses into the C57BR/6 background, with ~12.5% contribution from the 129/SvJ genotype derived from the ES cells used for the MCP-1 gene targeting. This genetic background is esssentially identical to that reported in the original studies of the LDL receptor–deficient atheroma model (Ishibashi et al., 1993, 1994). Thus, it is unlikely that strain differences between mice other than at the *MCP-1* locus contributes to the phenotype we describe.

The present demonstration of a crucial role for MCP-1 in the initiation of monocyte accumulation and lipid deposition in atherosclerosis suggests that chemokines and their receptors can serve as new targets for antiatherosclerotic drugs that exert their effects in a manner distinct from lipid-lowering agents. These observations suggest the potential utility of drugs with a mechanism of action that complements currently available therapy.

Experimental Procedures

Mice

MCP-1$^{-/-}$ mice (Lu et al., 1998), *LDL-R*$^{-/-}$ mice (Ishibashi et al., 1993) from Jackson Laboratories (Bar Harbor, ME), and their progeny were genotyped using PCR. To identify the disrupted *MCP-1* allele, DNA extracted from tail snips was amplified using primers designated as MCP-1-F1 (GGAGCATCCACGTGTTGGC) and IMR060 (AGGATCT CGTCGTGACCCATGGCGA), which recognize sequences in *MCP-1* and in the PGK-*neo* cassette that interrupts *MCP-1*, respectively. Wild-type alleles were identified using MCP-1-F1 and MCP-1-R2 (ACAGCTTCTTTGGGACACC). (Using standard PCR conditions, this primer pair does not generate a band from the disrupted allele, presumably because of difficulty amplifying across the PGK promoter.) The disrupted *LDL-R* allele was identified by PCR using the primers suggested by Jackson Laboratories (Bar Harbor, ME). The 1.25% cholesterol/cholate-free diet was prepared by Research Diets, Inc. (New Brunswick, NJ).

Lipid Analysis

Plasma was prepared from EDTA-treated blood, and total cholesterol and triglycerides were determined enzymatically (Sigma, St. Louis, MO). Lipoproteins were fractionated by FPLC in 1 mM NaEDTA (pH 7.2) and 150 mM NaCl using two Superose-6 columns in series (Pharmacia Biotech, Inc., Piscataway, NJ). Flow rate was 0.3 ml/min, and 0.5 ml fractions were collected. Cholesterol concentration was determined for each fraction enzymatically.

Lesion Analysis

Lipid deposition was quantified as described (Tangirala et al., 1995). In brief, for en face aorta analyses mice were sacrificed by ether overdose and the aortic tree was perfused with PBS. The aorta was isolated by severing minor branching arteries and dissecting the adventitia. It was removed en bloc from the root to the iliac bifurcation and then opened by a longitudinal cut along the ventral surface and pinned on a black wax surface. After 12 hr of fixation in 4% paraformaldehyde, 5% sucrose, and 20 μmol EDTA (pH 7.4), lipids were stained with Oil Red O (Fisher Scientific, Pittsburgh, PA). Photographs were taken, and the percentage of aortic area stained red was determined using image analysis software (Image Pro, Media Cybernetics, Silver Spring, MD). For analysis of the aortic root, aortas were perfused with PBS and frozen in OCT embedding medium. A total of 50 serial 6 μ sections were obtained, and 18 representative sections were stained with Oil Red O. Images were captured directly using a three-color video camera and frame grabber, and stained areas were quantified using image analysis software (Image Pro, Media Cybernetics, Silver Spring, MD).

Immunohistochemistry

Aortas were perfused with PBS and then removed and frozen in OCT embedding medium. Serial 6 μ sections were obtained from the root to the origin of the left subclavian artery. Every sixth section was fixed in cold acetone and stained using MOMA-2 (Serotec, Raleigh, NC) or control rat IgG_{2b} (PharMingen, San Diego, CA), followed by biotinylated rabbit anti-rat IgG adsorbed against mouse antigens (Vector Labs, Burlingame, CA). Sections were developed with horseradish peroxidase-derivatized avidin–biotin complex and 3-amino-9-ethylcarbazole (Vector Labs, Burlingame, CA) and counterstained with hematoxylin. Images were captured and stained areas quantified as described above.

Acknowledgments

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