Anti-inflammatory HDL Becomes Pro-inflammatory during the Acute Phase Response

Loss of Protective Effect of HDL against LDL Oxidation in Aortic Wall Cell Cocultures

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Abstract

We previously reported that high density lipoprotein (HDL) protects against the oxidative modification of low density lipoprotein (LDL) induced by artery wall cells causing these cells to produce pro-inflammatory molecules. We also reported that enzyme systems associated with HDL were responsible for this anti-inflammatory property of HDL. We now report studies comparing HDL before and during an acute phase response (APR) in both humans and a croton oil rabbit model. In rabbits, from the onset of APR the protective effect of HDL progressively decreased and was completely lost by day three. As serum amyloid A (SAA) levels in acute phase HDL (AP-HDL) increased, apo A-I levels decreased 73%. Concomitantly, paraoxonase (PON) and platelet activating factor acetylhydrolase (PAF-AH) levels in HDL declined 71 and 90%, respectively, from days one to three. After day three, there was some recovery of the protective effect of HDL. AP-HDL from human patients and rabbits but not normal or control HDL (C-HDL) exhibited increases in ceruloplasmin (CP). This increase in CP was not seen in acute phase VLDL or LDL. C-HDL incubated with purified CP and re-isolated (CP-HDL), lost its ability to inhibit LDL oxidation. Northern blot analyses demonstrated enhanced expression of MCP-1 in coculture cells treated with AP-HDL and CP-HDL compared to C-HDL. Enrichment of human AP-HDL with purified PON or PAF-AH rendered AP-HDL protective against LDL modification. We conclude that under basal conditions HDL serves an anti-inflammatory role but during APR displacement and/or exchange of proteins associated with HDL results in a pro-inflammatory molecule. (*J. Clin. Invest.* 1995. 96:2758–2767.) Key words: apo A-I • atherosclerosis • ceruloplasmin • PAF acetylhydrolase • paraoxonase • SAA

Introduction

The acute phase response (APR)[1] is a systemic reaction to infectious and to non-infectious tissue destructive processes. Multiple physiologic adaptations occur including changes in the hepatic synthesis of a number of plasma proteins termed acute phase reactants (1). Two acute phase reactants, C-reactive protein (CRP) and serum amyloid A protein (SAA) are known to interact with lipoproteins (2, 3). CRP binds to apolipoprotein B-containing lipoproteins, whereas SAA circulates primarily with high density lipoproteins (HDL). SAA is in fact a family of proteins some of which appear in plasma as major acute phase reactants after a variety of stimuli including surgery, myocardial infarction, infection, and even in more chronic illnesses such as arthritis (4). During inflammation SAA expression is increased by up to a 1000-fold as a result of a markedly increased gene transcription (5). Ceruloplasmin is another acute phase reactant that normally carries ~ 95% of the plasma copper (6), however, the physiological functions of ceruloplasmin appear to be both varied and complex. Antioxidant properties of ceruloplasmin have been described by a number of laboratories (7–9). In part, this antioxidant nature may derive from the ability of ceruloplasmin to inhibit metal-catalyzed lipid peroxidation by reincorporating reductively mobilized iron back into ferritin (9). Thus, ceruloplasmin may provide the major link between copper and iron metabolism. But a different role for ceruloplasmin as an independent risk factor in coronary heart disease was proposed from studies nearly 20 years ago, demonstrating an elevated serum ceruloplasmin in arteriosclerotic patients (10). Supporting this concept is recent evidence from Ehrenwald et al who demonstrated that ceruloplasmin can exhibit pro-oxidant activity, dependent on its structural integrity (11). Additional evidence for this pro-oxidant nature of ceruloplasmin has shown that an acidic pH, similar to what might be expected in an area of inflammation, was shown to promote the ceruloplasmin-catalyzed modification of low density lipoprotein LDL (12).

Originally it was demonstrated that cytotoxicity of oxidized low density lipoprotein (LDL) for endothelial (EC) and smooth muscle cells (SMC) was prevented by HDL (13). Subsequently, other studies confirmed and extended these observations demonstrating that HDL protected LDL against oxidation

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1. *Abbreviations used in this paper:* AP, acute phase; APR, acute phase response; BHT, butylated hydroxytoluene; CP, ceruloplasmin; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cell; MCP-1, monocyte chemotactic protein 1; MM-LDL, minimally oxidized low density lipoprotein; PAF-AH, platelet activating factor acetylhydrolase; SAA, serum amyloid A.

in vitro (14–16). Enzyme systems associated with HDL have been shown to have a role in protecting LDL against oxidation in vitro (18–22). In the studies reported here we demonstrate that HDL is converted from an anti-inflammatory molecule to a pro-inflammatory molecule during an APR and that (*a*) ceruloplasmin is a constituent of human and rabbit acute phase high density lipoprotein (AP-HDL) and the in vitro enrichment of HDL with purified ceruloplasmin alters the ability of HDL to inhibit LDL modification in an aortic wall cell coculture system; (*b*) AP-HDL exhibits a marked increase in SAA protein with a concomitant loss of apo A-I; and (*c*) two enzyme systems associated with HDL, platelet activating factor acetylhydrolase (PAF-AH) and paraoxonase, are readily displaced from HDL during the APR both in rabbits and in humans.

Methods

Materials

Tissue culture media, serum and supplements were obtained from sources previously reported (17, 23). Gelatin, endotoxin-free, tissue culture grade, No. G 9391 was obtained from Sigma Chemical Co. (St. Louis, MO). Transwells and Chamber Slides were obtained from Costar (Cambridge, MA). The fluorescent probe DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate) was purchased from Molecular Probes (Eugene, OR). Centricons were purchased from Amicon Corp. (Beverly, MA). Human plasma paraoxonase was purified as previously reported (24). PAF-AH was isolated from human erythrocytes as described (25). Serum amyloid A (SAA) was purified from the sera of patients during the APR as described previously (26). Purified ceruloplasmin was obtained from Boehringer Mannheim and was shown on 3–20% SDS PAGE gel analysis to be composed of > 85% 132-kD ceruloplasmin. Re-constituted ceruloplasmin samples were stored at 4°C and used within 72 h.

Rabbit lipoproteins

New Zealand white male rabbits, 2–3 kg each, were injected with croton oil based on the protocol of Cabana et al. (27). A 1% (vol/vol) emulsion of the mineral oil in saline was prepared in a sterile manner and 2.0 ml per kg was injected into five sites in the large lower back muscle. Blood samples were drawn from the central ear artery before and at various times after injection of croton oil. Thus, each rabbit served as its own control. The serum samples were subjected to ultracentrifugation for the isolation of HDL (d = 1.063–1.210 g/ml) (28). Alternatively, plasma samples were fractionated by an FPLC system equipped with two Superose 6 columns connected in series as described (29). Samples were eluted with a buffer containing NaCl (154 mM) and EDTA (100 μM), pH 8.0, at a flow rate of 0.5 ml/min. Lipoproteins were isolated in the absence of EDTA to avoid a loss of paraoxonase. Instead, BHT at 20 μM was present throughout the isolation.

Human lipoproteins

Low density lipoproteins (LDL d = 1.019–1.063 grams/ml) and high density lipoprotein (HDL, d = 1.069–1.210 grams/ml) were isolated from the sera of normal blood donors by density gradient ultracentrifugation as described (28) and used within 2–4 d of isolation. LDL isolation was accelerated by the use of 300,000 mol wt cut off Centricons for desalting and was completed in 48 h. HDL was isolated from the sera of five subjects before surgery using 20 μM BHT (30) in place of EDTA to avoid a loss of paraoxonase. The BHT was removed by dialysis before the addition to the cocultures. Acute phase HDL was obtained from the sera of the same subjects 2–3 d after cardiac surgery using the protocol for isolation of normal HDL. The concentration of lipoproteins is expressed in terms of protein content throughout this report. The concentration of endotoxin in the lipoprotein solutions was less than 100 pg/ml (determined by a chromogenic assay) which is 20-fold less than that required to induce monocyte binding to endothelium or transmigration.

Cocultures

Human aortic endothelial cells (HAEC), and smooth muscle cells (HASMC) were isolated as previously described (15, 21). Transwells or Chamber Slides were used for formation of cocultures and for the study of monocyte transmigration. The wells were treated with 0.1–0.5% gelatin at 37°C overnight. HASMC were seeded in units or on membranes at a confluent density of 1×10^5 cells/cm^2 and were cultured for 3 d at which time they had covered the entire surface of the well and had produced a substantial amount of extracellular matrix. HAEC, were subsequently seeded at 2×10^5 cells/cm^2 and were allowed to grow forming a complete monolayer of confluent EC in 2 d. In all experiments, HAEC and autologous HASMC (from the same donor) were used at passage levels of four to six. Identical results were obtained with multilayer cocultures produced in the different systems (i.e., Transwells or Chamber Slides). Blood monocytes were obtained from a large pool of healthy donors by modification of the Recalde procedure as previously described (31).

Monocyte transmigration assay. The cocultures were treated with native LDL (250–350 mg/ml) in M199 media containing 2% pooled human serum in the absence or presence of various test compounds for 24 h. The culture supernatants were subsequently transferred to untreated cocultures and were incubated for an additional 24 h (17, 23). Monocytes were labeled with the fluorescent probe DiI or carboxyfluorescine at 4°C for 10 min, were washed and the cell pellet was resuspended in medium 199 at the desired monocyte density. At the end of the second 24-h treatment, labeled monocytes were added to cocultures at 2.5×10^5 cells/cm^2 and were incubated for 60 min at 37°C. The medium containing nonadherent cells was then removed and the cell layers were washed at 37°C to remove the loosely adherent cells on top of the endothelial monolayer. The cocultures on membranes were fixed, mounted and subendothelial monocytes were enumerated under 625× magnification. In pilot experiments culture supernatants were screened for chemotactic activity using the monocytic cell lines Mono Mac 6 (32) or THP-1 which resulted in values in complete agreement with those obtained using human peripheral blood monocytes (data not shown).

Monocyte adhesion assay. The cocultures were grown in gelatin coated 96-well microtiter plates and were treated with native LDL (250–350 mg/ml) in M199 media containing 1% pooled human serum in the absence or presence of various test compounds for 24 h. The culture supernatants were subsequently transferred to untreated cocultures and were incubated for 4 h. A 50 μl monocyte suspension in DME providing 2.5×10^5 cells/cm^2 was then added per well. Extreme care was taken to avoid drying out of the endothelial cells during the changes and throughout the assay. The loosely adherent cells were washed off with DME, the cultures fixed with 1% glutaraldehyde in PBS and the monocytes enumerated under 200× magnification. Alternatively monocytes that were prelabeled with carboxyfluorescine were used and the adherent monocytes were enumerated using a Cytofluor 2300 (Millipore, Bedford, MA).

Enzyme supplementation of acute phase HDL. Purified paraoxonase or PAF-AH was added at a final concentration of 2×10^{-2} U /ml to 1.0 mg/ml of the acute phase HDL. After 1 h of incubation at 37°C under argon with gentle mixing, HDL was reisolated using 100,000 mol wt cut off Centricons.

Enzyme activity assays. PAF-AH activity was determined by two different methods described previously (20, 33). In the method described by Steinbrecher and Pritchard (31) 10 nmol of the substrate 1-palmitoyl 2-[6-(7-nitrobenzoxadiazoyl)-amino]caproyl phosphatidylcholine (C_6NBD PC) was incubated with HDL in 1 ml PBS. The reaction was terminated by vortexing with methanol/chloroform. The fluorescence of the aqueous layer was measured at excitation 470 nm and emission 533 nm. The mass of the substrate hydrolyzed was calculated following the method described (33). When higher sensitivity was required, 2-[acetyl-^{3}H]PAF was used as substrate (20). The [^{3}H]acetate

generated during the hydrolysis by PAF-AH is separated from labeled substrate and quantitated. In a typical assay sample aliquots of 10 μl were mixed with 40 μl of 0.1 mM [acetyl-^{3}H]PAF, incubated for 30 min at 37°C. This was followed by addition of 50 μl of acetic acid and 1.5 μl of sodium acetate solution. Each reaction mixture was then passed through a C^{18} gel cartridge and the filtrates were collected in scintillation vials. Each reaction tube was then washed with sodium acetate and the wash was also passed through the cartridge and combined with the original effluent and the radioactivity was determined in a scintillation counter. Enzymatic activity was expressed in mmoles/min after appropriate corrections (20). There was complete agreement between the values obtained using the two assay methods.

Paraoxonase activity was determined using paraoxon as the substrate and measuring the increase in the absorbance at 412 nm due to formation of 4-nitrophenol. Enzyme activity was measured in 50 mM Tris/HCl buffer at pH 7.4 and 8.0, and in 50 mM glycine/NaOH at pH 10.5. The sample to be tested was added to start the reaction and the increase in the absorbance at 412 nm was recorded (24). The amount of generated 4-nitrophenol was calculated from the molar extinction coefficients at pH 7.4, 8.0, and 10.5, which were 12,800, 17,100, and 18,290 M^{-1} cm^{-1}, respectively. Blanks contained substrate without the lipoprotein sample or purified enzyme. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per min under the above assay conditions. The advantage of this assay is that a microtiter plate reader can be readily used for a large number of samples. When a higher degree of sensitivity was required, however, the enzyme activity was measured in an arylesterase assay (24). The cuvette contained 1 mM phenylacetate in 20 mM Tris/HCl pH 8.0. The reaction was initiated by the addition of the enzyme solution or the lipoprotein samples and the increase in the absorbance at 270 nm was recorded over a 90-s period. Blanks were included to correct for the spontaneous hydrolysis of phenylacetate. Enzymatic activity was calculated from the molar extinction coefficient 1310 M^{-1} cm^{-1}. A unit of arylesterase activity is defined as 1 μmol phenylacetate hydrolyzed per min under the above assay conditions (24).

Western blot analysis

To determine the presence of ceruloplasmin in rabbit and human lipoproteins, equal amounts of lipoprotein (50–100 μg) were electrophoresed in 7% SDS PAGE gels and applied to Hybond™ ECL Nitrocellulose membrane (Amersham) in Tris-buffered saline. Chemiluminescent detection of proteins was carried out using the Amersham ECL Western blotting kit (Amersham) according to the manufacturers suggested protocol. The primary antibody used was goat anti-human ceruloplasmin (Sigma Chemical Co.) which also showed high cross-reactivity with 132-kD rabbit lipoprotein ceruloplasmin. The secondary antibody used was horseradish peroxidase-conjugated anti-goat IgG (Amersham). The blots were exposed to Hybond™ ECL film according to the manufacturers suggested protocol and analyzed.

Quantitation of gene expression

Total RNA was extracted from the coculture cells according to the technique of Chomczyski and Sacchi (34). Northern blot analysis was used to quantitate the mRNA levels of monocyte chemotactic protein-1 (MCP-1) (35). For each sample, 15 μg of RNA were electrophoresed on formaldehyde/1% agarose gels and transferred to 20× SSC equilibrated Hybond ECL™ nitrocellulose membrane. Membranes were hybridized after UV cross-linking, and washed at a high stringency (65°C, 0.1× SSC). The MCP-1 probe had a sequence of 5′ CGG ATG TTG GGT TTG CTT GTC CAG GTG GTC CAT GG 3′. The blots were also hybridized using a cDNA probe for α-tubulin to normalize the quantities of RNA loaded into the gel lanes.

Other procedures

Lipid hydroperoxide formation was measured using the method of Auerbach et al (36). The protein content of cells and lipoproteins was measured using a microtiter plate assay (37) based on the method of Lowry et al. (38). SDS-PAGE was performed according to the procedure of Laemmli (39). Serum amyloid A levels were determined as previously described (5). MCP-1 levels were determined using an ELISA reported previously (23). Cytotoxicity was kindly evaluated by Dr. Theodore Sarafian of the Department of Pathology at UCLA using propidium iodine as a fluorescent probe which was added to cell cultures at a 50 μM final concentration. Fluorescence at 536 nm excitation and 590 nm emission (f_1) was determined on a Cytofluor 2300. Digitonin at 200 μM was subsequently added and fluorescence was determined (f_2). The ratio f_1/f_2 was used to determine the level of cytotoxicity. Statistical analyses were carried out first using Model I ANOVA to determine if differences existed among the group means, followed by a Paired Student's t distribution to identify the significantly different means.

Results

Lack of protection by acute phase HDL against LDL oxidation. As demonstrated in Fig. 1 *A*, normal HDL (C-HDL), obtained before cardiac surgery, completely inhibited the LDL-induced increase in monocyte transmigration. In contrast, acute phase HDL (AP-HDL), obtained from the same patients 2–3 d after surgery, not only did not prevent the LDL-induced increase in monocyte transmigration, but amplified it by up to 1.8-fold ($P < 0.01$). Incubation of AP-HDL alone with the modifying cocultures did not affect the number of monocytes in the subendothelial space of the target cocultures. While normal HDL was capable of inhibiting the LDL-induced increase in lipid hydroperoxide levels in cocultures by 82%, AP-HDL was significantly less effective ($P < 0.01$, Fig. 1 *B*).

AP-HDL acts via MCP-1 stimulation. Our laboratory had previously shown in this coculture system that LDL-induced monocyte transmigration (MTM) was inhibited > 90% by antibody to MCP-1 (17, 40). As shown in Fig. 1 *C*, the LDL-induced increase in MCP-1 levels was almost completely abolished by the presence of normal HDL. Presence of AP-HDL, however, did not reduce but significantly elevated ($P < 0.008$) the LDL-induced increase in MCP-1 levels. As the Northern blot analysis in Fig. 2 shows, cells incubated in the presence of LDL showed an increase in MCP-1 expression that was further enhanced by incubation of LDL with AP-HDL. This is in contrast to that seen in cells coincubated with LDL and normal HDL which suppressed LDL-stimulated MCP-1 expression.

AP-HDL express ceruloplasmin. Since ceruloplasmin is an acute phase reactant and was reported to stimulate LDL oxidation in vitro, we determined if ceruloplasmin was present in AP-HDL. Using Western blot analysis, (Fig. 3) it was found that AP-HDL from patients 2–3 d after cardiac surgery expressed ceruloplasmin whereas HDL from healthy subjects did not. HDL was also isolated from rabbits before and 24, 48, and 72 h after injection with croton oil. As can be seen, although no ceruloplasmin could be detected in the HDL from rabbits before croton oil injection, by 48 h, ceruloplasmin had appeared and remained for at least 72 h.

To determine if ceruloplasmin distributed to acute phase lipoproteins other than HDL, and to reduce the possibility of ultracentrifugal artifacts, FPLC was used to fractionate plasma from rabbits at the peak of the acute phase (27) (Fig. 4). The Western Blot analysis shows that ceruloplasmin could be detected only in HDL and not VLDL nor LDL from acute phase plasma.

Ehrenwald et al. (11) noted that the ability of ceruloplasmin to increase the copper-induced oxidation of LDL was dependent upon intact nondegraded protein of 132 kD. In the experiment

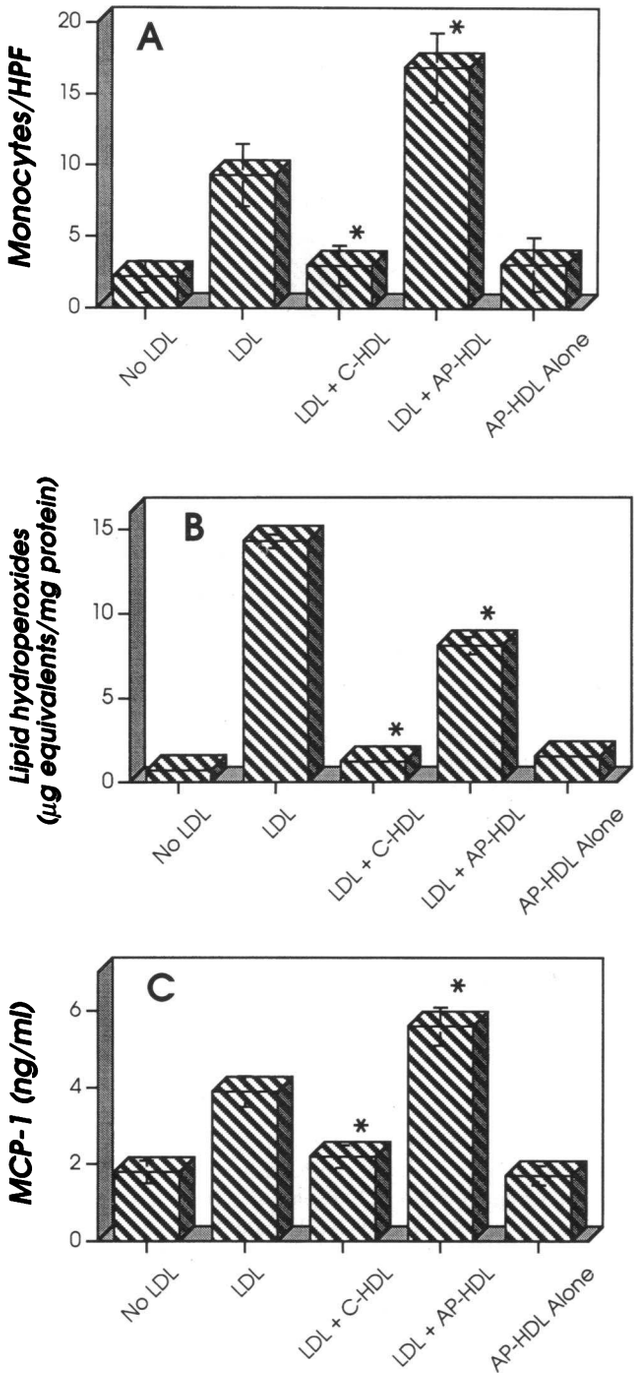

Figure 1. (*A*) Protective effect of HDL. Cocultures of human aortic EC and SMC were treated with freshly isolated LDL at 350 μg/ml (LDL). To some wells the LDL was added after the addition of HDL (500 μg/ml) that was obtained from five individuals prior to surgery (*+C-HDL*), or HDL obtained during the acute phase response after the surgery (*+AP-HDL*). After 24 h of incubation, conditioned medium was transferred to target cocultures. The medium was removed from the target cocultures after 24 h of incubation and a suspension of DiI-labeled monocytes was added at 2.5×10^5 cells/cm^2 to the endothelial side of the cocultures. The cultures were returned to the incubator and maintained for 60 min. The medium was then removed, cultures were washed and were fixed with 10% neutral buffered formalin for 24 h. The cocultures were then mounted and subendothelial monocytes were enumerated under 625× total magnification. Values shown are mean±SD of the number of monocytes in 25 fields in five cocultures for each treatment in four independent experiments. (*) Statistically significant difference from "LDL" at $P < 0.01$. (*B*) Lipid hydroperoxides. LDL at 350 μg/ml was incubated with artery wall cell cocultures for 8–16 h and lipid hydroperoxide levels were determined in the supernatants as described in Methods. Cocultures in two group of wells received C-HDL or AP-HDL before the addition of LDL. Other wells received AP-HDL alone. Values are mean±SD of triplicate determinations of lipid hydroperoxides (linoleic acid equivalent) in three independent experiments. (*) Statistically significant difference from "LDL" at $P < 0.01$. (*C*) MCP-1 levels. Cocultures were incubated with LDL at 350 μg/ml for 16 h. The medium was then transferred to target cocultures and was incubated for 6 h to stimulate MCP-1 induction. The medium was subsequently removed and the cocultures were washed with M-199 medium at 37°C. The cocultures were then incubated in serum free medium for 12 h followed by determination of MCP-1 protein in the supernatants using an ELISA referred to in Methods. The values are mean±SD of quadruple measurements per treatment in three independent experiments. (*) Statistically significant difference from "LDL" at $P < 0.01$.

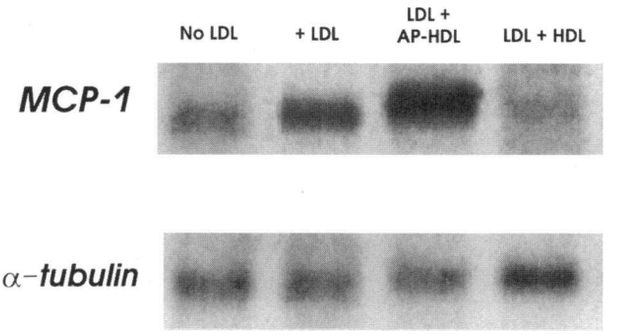

Figure 2. Northern blot analysis of coculture cells treated with AP-HDL. Coculture cells were treated as in Fig. 1 and total RNA was prepared for Northern blot analysis as described in Methods. Expression of α-tubulin mRNA is shown for normalization of the quantities of RNA loaded in gel lanes. This figure is representative of three independent experiments.

shown in Fig. 5 we tested the effects of purified ceruloplasmin on LDL modification in cocultures. Whereas ceruloplasmin alone had no effect on MTM, 100 μg/ml of ceruloplasmin coincubated with LDL enhanced the LDL-induced response.

We then tested if in vitro enrichment of HDL with purified ceruloplasmin would affect the ability of HDL to protect LDL from modification in the coculture system. In the experiment shown in Fig. 6, HDL was incubated in media alone or with 100 μg/ml of ceruloplasmin and then ultracentrifugally re-isolated. In contrast to the inhibitory effect of C-HDL on LDL modification and monocyte transmigration, ceruloplasmin-treatment of HDL (CP-HDL) stimulated the effect. Consistent with these results, CP-HDL further enhanced LDL-induced expression of MCP-1 mRNA, whereas control HDL reduced expression (Fig. 6 *B*).

Enzyme supplementation of the acute phase HDL. We next determined if the HDL associated enzymes, paraoxonase and PAF-AH were altered in an APR. Unlike normal HDL obtained before surgery, paraoxonase activity in AP-HDL was reduced 76% ($P < 0.01$, Table I). In addition while normal HDL hydrolyzed PAF efficiently, AP-HDL showed an 8-fold lower hy-

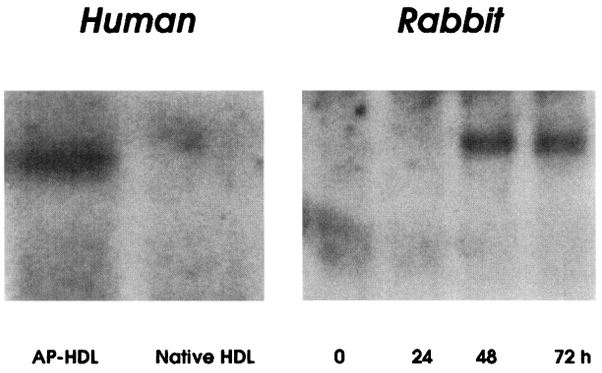

Figure 3. Western blot analysis of ceruloplasmin in humans and rabbits. Western Blot Analysis was conducted as described in Methods. Human HDL (d = 1.063–1.21 g/ml) were isolated from normal subjects and from the same subjects after cardiac surgery. Rabbit HDL were isolated from animals before (0), and 24, 48, and 72 h after intramuscular injection of 1% vol/vol croton oil.

drolytic activity toward PAF ($P < 0.01$, Table I). Incubation of AP-HDL with purified human paraoxonase or with purified human PAF-AH followed by reisolation of AP-HDL increased the paraoxonase activity 2.8-fold and the PAF-AH activity 5.1-fold (Table I).

Elevated levels of SAA and reduced levels of apo A-I. Fig. 7 *A* shows that human AP-HDL contained high levels of SAA but levels of apo A-I were reduced 58%. Similarly, HDL that had been highly enriched with purified human SAA in vitro (designated as SAA-HDL) had lost 87% of its apo A-I content,

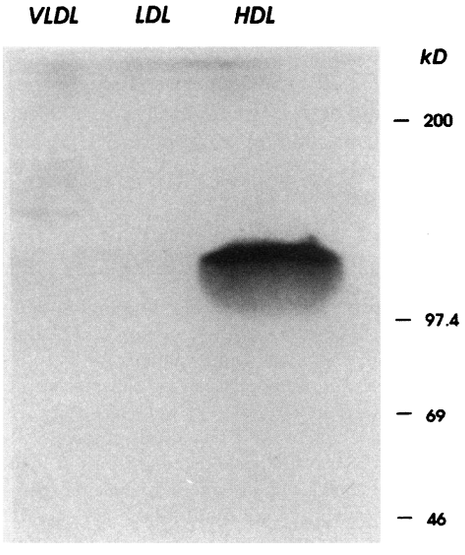

Figure 4. Western blot analysis of ceruloplasmin FPLC fractions from acute phase rabbits. FPLC fractions were isolated from plasma samples of acute phase rabbits as described in Methods. The peak fractions representing VLDL, LDL and HDL were then subjected to Western blot analysis as described in Methods. Molecular weight markers are shown on the right. The band exhibits an apparent molecular weight of 132 kD.

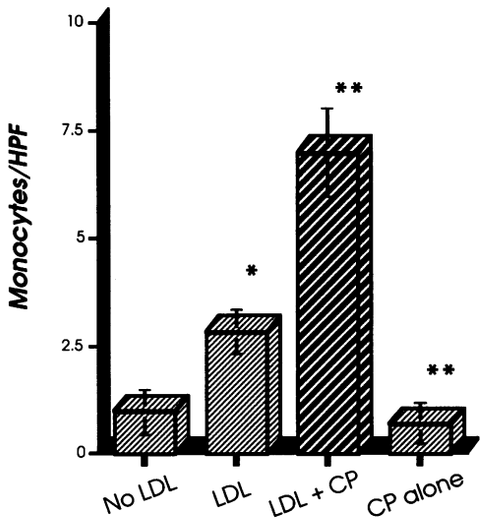

Figure 5. Bar graph showing the effect of ceruloplasmin on monocyte transmigration. Cocultures were incubated in medium containing either no additional LDL (*No LDL*), with 250 μg/ml LDL either alone (*LDL*) or coincubated with 100 μg/ml purified ceruloplasmin (*LDL + CP*), or incubated with purified ceruloplasmin alone (*CP*) for 16 h. Conditioned medium was then transferred to target cocultures and was further incubated for 6 h. The medium was then removed and a suspension of DiI-labeled monocytes was added at 2.5×10^5 cells/cm^2 to the endothelial side of the cocultures. The cultures were returned to the incubator and maintained for 45–90 min. The medium was then removed, cultures were washed and were fixed with 10% neutral buffered formalin for 24 h. The cocultures were then mounted and subendothelial monocytes were enumerated under 625 × total magnification. The values shown are the mean±SD in 15 fields from triplicate cocultures. Asterisks represent a statistically significant difference ($P < 0.05$) from No LDL (*) or from LDL (**).

91% of its paraoxonase activity, and 88% of its PAF-AH activity (data not shown). Incubation of the AP-HDL (Fig. 7 *B*) or SAA-HDL (Fig. 7 *C*) with pure human paraoxonase or with 2×10^{-2} U/ml PAF-AH rendered AP-HDL protective against LDL modification in that it inhibited the LDL-induced monocyte transmigration (Fig. 7, *B* and *C*). Inclusion of purified paraoxonase or purified PAF-AH without HDL also completely abolished the modification of LDL and the subsequent induction of monocyte transmigration (Fig. 7 *B*). Paraoxonase and PAF-AH supplementation of acute phase HDL had the same effects on LDL-induced adhesion of monocytes to endothelial monolayers as it did on transmigration (data not shown).

Whereas paraoxonase and PAF-AH in native HDL conferred protection when HDL was included together with LDL in the coculture from the start of the incubation, addition of the purified enzymes to cocultures that had been exposed to LDL previously modified by a first set of cocultures did not affect the number of monocytes in the subendothelial space of the cocultures (data not shown). This indicated that paraoxonase and PAF-AH did not block the ability of the cells to produce MCP-1 upon stimulation by mildly oxidized LDL (MM-LDL). If, however, LDL was incubated with paraoxonase or PAF-AH in a test tube at 37°C for 4–6 h with gentle rocking and LDL was reisolated and incubated with cocultures, the enzyme-treated MM-LDL lost its ability to induce MCP-1 and monocyte migration while the sham-treated MM-LDL was still active

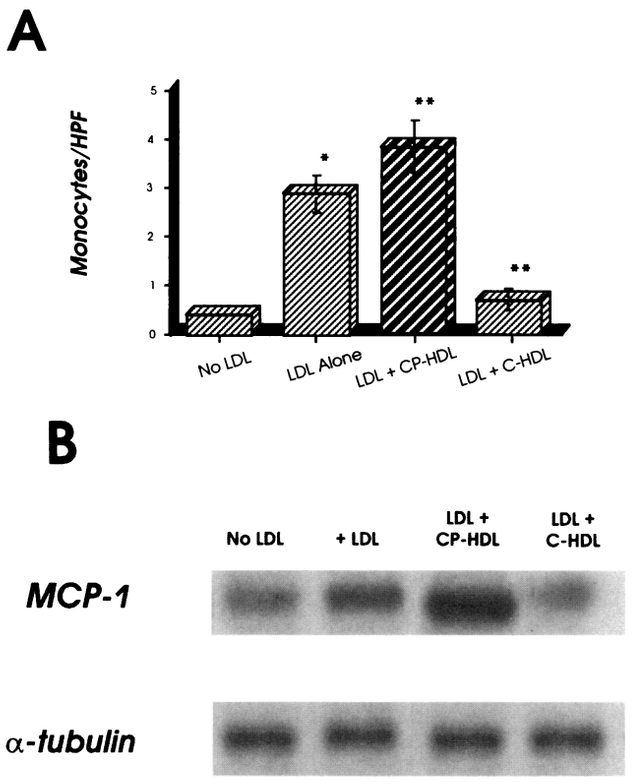

Figure 6. (*A*) Bar graph showing the effect of ceruloplasmin-enriched HDL on monocyte transmigration. Cocultures were incubated in medium containing either no additional LDL or with 250 μg/ml LDL alone or in combination with either 500 μg/ml HDL that had been incubated with either purified ceruloplasmin (*CP-HDL*) or incubated with PBS alone for 2 h and then ultracentrifugally reisolated (*C-HDL*). Conditioned medium was then transferred to target cocultures and was further incubated for 6 h. The medium was then removed and a suspension of DiI-labeled monocytes was added at 2.5×10^5 cells/cm^2 to the endothelial side of the cocultures. The cultures were returned to the incubator and maintained for 45–90 min. The medium was then removed, cultures were washed and were fixed with 10% neutral buffered formalin for 24 h. The cocultures were then mounted and subendothelial monocytes were enumerated under 625 × total magnification. The values shown are the mean±SD in 15 fields from triplicate cocultures. Asterisks represent a statistically significant difference ($P < 0.05$) from No LDL (*) or from LDL (**). (*B*) Northern blot analysis of coculture cells treated with AP-HDL. Coculture cells were treated as in A and total RNA was prepared for Northern blot analysis as described in Methods. Expression of α-tubulin mRNA is shown for normalization of the quantities of RNA loaded in gel lanes. This figure is representative of three independent experiments.

(data not shown). The presence of paraoxonase or PAF-AH in control cocultures containing recombinant human MCP-1 did not affect the number of monocytes migrating into the subendothelial space (data not shown). This indicated that the HDL associated enzymes did not have any detectable effect on the interaction of MCP-1 with monocytes and their subsequent transmigration. Furthermore, there was no cytotoxicity observed as a result of the addition of AP-HDL, SAA-HDL, or the purified enzymes to the cocultures (data not shown). These results are consistent with enzymatic destruction of the biologically active lipids in MM-LDL by PAF-AH and paraoxonase associated with HDL.

Table I. Enzyme Supplementation of HDL

	Paraoxonase/arylesterase activity U/mg protein	PAF acetylhydrolase activity nmoles/min per mg protein
Pre-op. HDL*	1.7±0.3[§]	6.1±0.91[‖]
Post-op. AP-HDL[‡]	0.4±0.03[§]	0.8±0.22[‖]
Following enzyme supplementation		
AP-HDL/paraoxonase	1.1±0.29[¶]	0.92±0.31
AP-HDL/PAF acetylhydrolase	0.55±0.11	3.9±0.56[¶]

* Pre-op. HDL indicates HDL isolated from plasma of individuals prior to cardiac surgery. [‡] Post-op. AP-HDL indicates the HDL isolated during the acute phase 2–3 d after the surgery. Human paraoxonase and PAF acetylhydrolase used in the supplementation were isolated as described in Methods. Supplementation was carried out by gentle mixing under argon at 37°C followed by reisolation of HDL. The data are mean±SD of values obtained from five separate experiments using HDL from the same five individuals prior and subsequent to surgery. [§] Indicates a significant difference from Pre-op. HDL values at $P < 0.01$. [‖] Indicates a significant difference from Pre-op. HDL values at $P < 0.001$. [¶] Indicates a significant difference from AP-HDL values at $P < 0.01$.

Changes in SAA and HDL apo A-I in the rabbit model of acute phase. Fig. 8 *A* demonstrates that the injection of croton oil in the rabbits resulted in a marked increase in serum SAA levels to a maximum on day three of 60.6-fold the level present before the induction of the acute phase, with levels showing a decline on day four. As demonstrated in Fig. 8 *B*, parallel to the elevations in serum concentrations of SAA, increases in SAA and concomitant decreases in apo A-I levels were observed in HDL isolated on days one to three of the APR. There was a significant reduction of SAA and a moderate increase in apo A-I in HDL on day four of the APR. There were no detectable differences in the HDL obtained on days zero to four from rabbits injected with saline alone (data not shown).

Changes in HDL enzymes and the protective effect. Paraoxonase and PAF-AH activities showed precipitous declines in HDL obtained from these rabbits between days zero and three (Fig. 9, *A* and *B*, respectively). The loss of enzyme activity was paralleled by a disappearance of HDL's protective effect against LDL oxidation and the resulting monocyte transmigration (Fig. 9 *C*). There appeared to be some recovery of enzymatic activity and HDL's protective effect on day 4 of the acute phase. Thus the levels of paraoxonase and PAF-AH activity in HDL paralleled HDL's ability to prevent LDL modification by the artery wall cells.

Discussion

Mild modification (oxidation) of LDL in serum-containing multilayer cocultures of human aortic wall cells induces increased monocyte adhesion to and transmigration through the endothelial cell layer in the cocultures (17, 23). The cells of the artery wall therefore, may be capable of creating a microenvironment that excludes aqueous antioxidants and thus permits the formation of mildly oxidized LDL (MM-LDL). In earlier studies we found that the ability of the cells to generate MM-

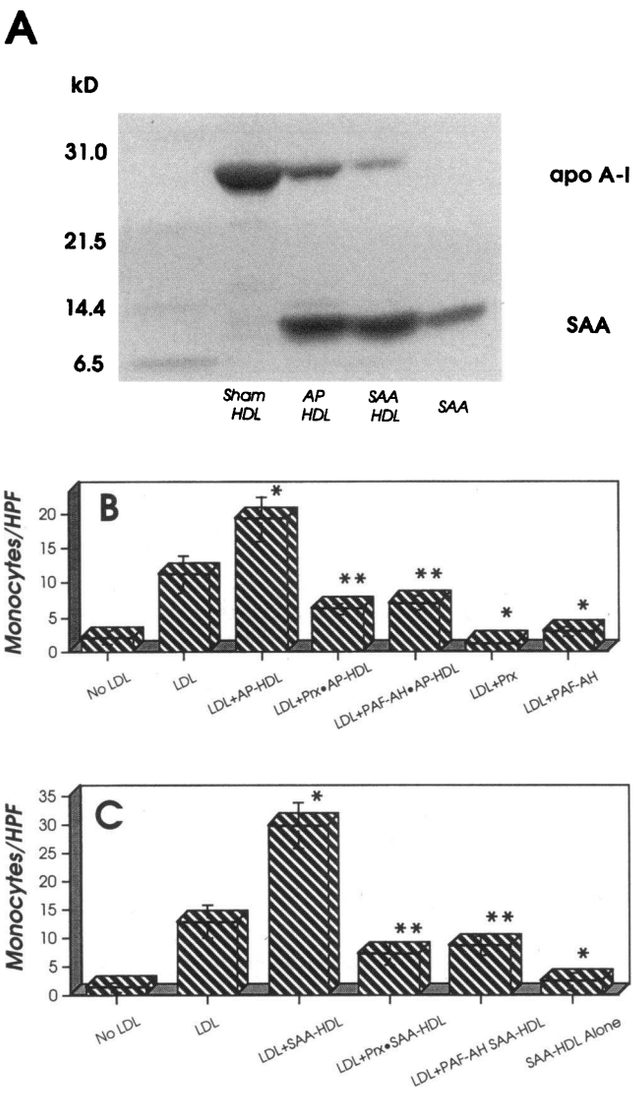

Figure 7. (*A*) SDS -PAGE of AP-HDL. Human HDL obtained before and after acute phase response was analyzed for apo A-I and SAA content. To the native HDL was added buffer (*Sham HDL*) or purified human SAA (*SAA-HDL*) and HDL was subsequently reisolated. Electrophoresis was carried out as stated in Methods. (*B*) Effect of purified enzymes and restoration of protective effect to AP-HDL. Cocultures were incubated with LDL at 350 μg/ml. To a group of wells was added AP-HDL at 500 μg/ml in addition to LDL. To other groups of wells was added together with LDL, AP-HDL that had been previously incubated with purified paraoxonase at 2.0 μg/ml (1×10^{-2} U/μg) (*Prx·AP-HDL*) or PAF-AH at 2×10^{-2} U/ml (*PAF-AH·AP-HDL*) and had been subsequently reisolated. Additional wells were incubated with purified paraoxonase (+*Prx*) or PAF-AH (+*PAF-AH*) at 2×10^{-2} U/ml and LDL was subsequently added. A monocyte migration assay was conducted at the end of the incubation time as described for Fig. 1. Asterisks represent a statistically significant difference ($P < 0.05$) from LDL (*) or from LDL + AP-HDL (**). (*C*) Effect of purified enzymes and restoration of protective effect to SAA-HDL. Cocultures were incubated with LDL at 350 μg/ml. To a group of wells was added SAA-HDL at 500 μg/ml in addition to LDL. To another group of wells was added LDL and SAA-HDL that had been previously incubated with 2×10^{-2} U/ml of purified paraoxonase or PAF-AH and had been subsequently reisolated (*Prx·SAA-HDL* and *PAF-AH·SAA-HDL*, respectively). Some wells were incubated with 500 μg/ml of SAA-HDL alone. Incubations and monocyte migration assay was carried out as for Fig. 1 *A*. The values are mean±SD of number of monocytes in 12 fields from 4 wells in each treatment in four independent experiments. Asterisks represent a statistically significant difference ($P < 0.05$) from LDL (*) or from LDL + SAA-HDL (**).

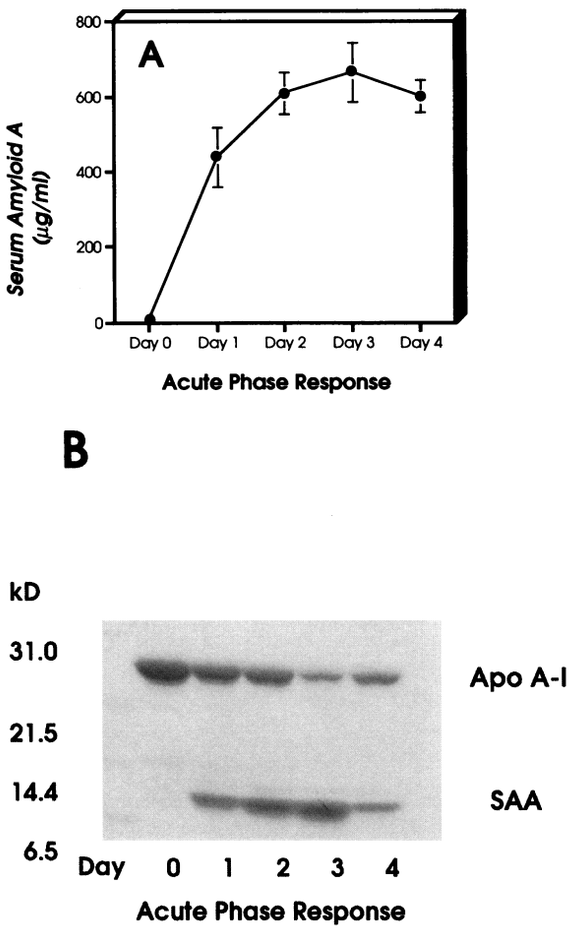

Figure 8. SAA and apo A-I during the acute phase response in rabbits. New Zealand white rabbits were injected with croton oil and blood samples were collected on days zero to four of the acute phase response. (*A*) SAA levels were determined on pooled serum samples from five rabbits using a polyclonal antibody raised against and monospecific for rabbit SAA, and following the procedure referred to in Methods. (*B*) HDL was isolated from the pooled rabbit serum samples obtained on consecutive days during the acute phase response. Shown are the representative results observed following SDS-PAGE and Coomassie Blue staining.

LDL from native LDL was markedly inhibited by pretreatment of the coculture with antioxidants such as probucol, α-tocopherol, or β-carotene (23). This finding is consistent with the hypothesis that the transfer of cellular membrane lipid peroxides to LDL is an essential first step in initiating LDL lipid oxidation as suggested by Witztum and Steinberg (41) and by Chisolm (42). Numerous epidemiological studies have associated HDL with an inverse risk for coronary artery disease. This "protective" effect of HDL may be due in part to inhibition of the oxidative modification of LDL (17) or by promoting cholesterol efflux from peripheral cells (43). Our results would suggest that under certain conditions, such as an APR, the normally

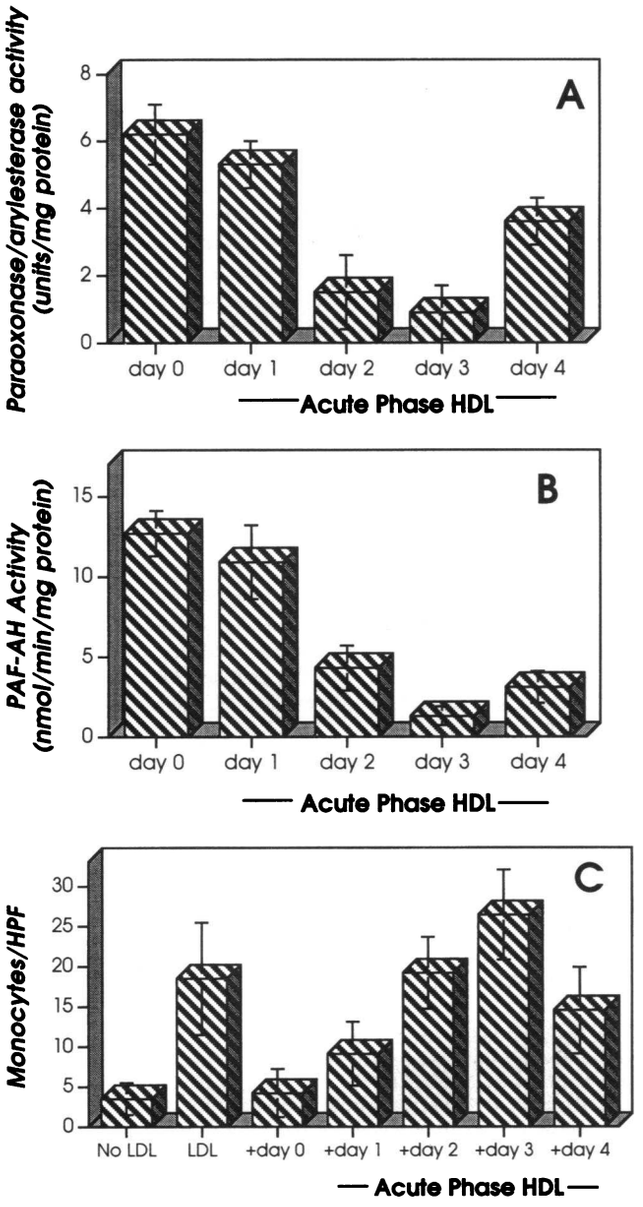

Figure 9. Enzyme activity and protective effect of acute phase HDL. HDL from rabbits before and following injection with croton oil was analyzed for paraoxonase/arylesterase (*A*), and PAF-AH (*B*) as described in Methods. In the case of arylesterase, activity is expressed as mmoles of phenyl acetate hydrolyzed and for PAF-AH activity, nmoles of PAF hydrolyzed. (*C*) Protective effect of HDL obtained before and following the acute phase response was determined by the degree of prevention of LDL-induced monocyte transmigration as described in Fig. 1 *A*. Values shown represent mean±SD of number of migrated monocytes in 15 fields for triplicate cocultures in each treatment. This figure is representative of three independent experiments.

antioxidant nature of HDL is altered. We found that in contrast to native HDL which inhibited LDL modification and monocyte adhesion to endothelial cells in an arterial coculture system, AP-HDL enhanced monocyte adhesion. It is well known that during the APR, lipoprotein composition is altered dramatically from non-acute phase conditions (2, 44). As shown here, HDL exhibits a marked increase in SAA protein with a concomitant loss of apo A-I (2). Apo A-I-containing HDL particles appear to contain the majority of lecithin cholesteryl acyl transferase (LCAT), responsible for the esterification of plasma cholesterol (45), and are the HDL ligands responsible for the interaction of HDL with cells. The displacement of apo A-I by SAA during the acute phase might be expected to inhibit LCAT activity in HDL suppressing cholesterol efflux from peripheral cells and retargeting HDL and its lipids to alternative sights. Moreover, one can envision that under the conditions of an APR a markedly different interplay among plasma components would occur. VLDL would become enriched in apo B, triglyceride, and SAA, but be depleted of apo E (44). These alterations would favor an increased plasma residence time and a shift away from hepatic removal (primarily mediated by apo E) toward uptake by peripheral tissues (via the apo B, E receptor). In the present study, we have shown that AP-HDL possesses less paraoxonase and PAF-AH activities, enzymes able to catalyze the hydrolysis of the biologically active lipid in MM-LDL (46). Taken together these modifications in plasma lipoproteins would provide an environment that promotes host defense, providing more lipid for fuel and tissue remodeling as well as promoting lipid oxidation to combat bacterial infection during an acute illness.

Ceruloplasmin is an acute phase reactant that has been reported to stimulate LDL oxidation in vitro (11, 12, 47). We observed a marked elevation of ceruloplasmin in AP-HDL but not normal HDL. That this response was specific for AP-HDL was demonstrated by the absence of detectable ceruloplasmin in VLDL and LDL isolated from acute phase rabbits. In addition, purified ceruloplasmin, although having had no effect on monocyte adhesion itself, stimulated LDL-induced monocyte adhesion. Moreover, purified ceruloplasmin incorporated into native HDL rendered HDL no longer protective of LDL modification. Ehrenwald and his coworkers (11) have provided evidence that the pro-oxidant activity of ceruloplasmin requires intact 132-kD protein and that much of the antioxidant activity of ceruloplasmin may be due to varying amounts of proteolytic degradation products. The preparation used in our studies was predominantly the 132-kD form (85%). One could postulate that during an APR, an increase in newly synthesized ceruloplasmin would promote pro-oxidant activity in HDL toward lipoprotein or membrane lipids, switching HDL from an "anti-inflammatory" to a "pro-inflammatory" particle. As the APR subsides, proteolytic degradation of ceruloplasmin (48, 49) would result in the loss of pro-oxidant activity and an eventual return of HDL to a more antioxidant particle.

An important early event in atherogenesis is an increased recruitment of monocytes into the arterial subendothelium (50). Both endothelial cells and smooth muscle cells in culture constitutively produce a chemotactic factor, MCP-1, that acts on monocytes but not neutrophils (51). This laboratory had previously shown in a coculture system that LDL-induced monocyte transmigration was inhibited > 90% by antibody to MCP-1 (17, 40). In the present study we found that coincubation of coculture cells in the presence of LDL with AP-HDL resulted in an enhanced production of MCP-1. This would support the hypothesis that local modification of LDL in the arterial wall, and the subsequent monocyte infiltration may increase during an APR at inflammatory sites (fatty streaks).

The oxidative modification of lipoproteins, particularly LDL, may be causative in atherosclerotic lesion development. Although transition metals such as iron and copper serve important roles in biology, both iron and copper have been shown

to cause oxidative damage in biological systems in vitro (52). The evidence that high serum levels of copper and ceruloplasmin are associated with coronary heart disease (10) are consistent with ceruloplasmin acting as a pro-oxidant for LDL. In contrast to the inhibitory effect of normal HDL on LDL modification, enhancement of LDL modification by AP-HDL may be due in part to its ceruloplasmin content providing a source of transition metals for oxidative reactions. These observations would support the scenario that a local modification of LDL in the arterial wall, and a subsequent monocyte infiltration may be due in part to an increase in ceruloplasmin at inflammatory sites.

HDL is known to prevent LDL modification (13–17), and enzyme systems associated with HDL have been shown to play a role in HDL's ability to protect LDL from oxidation (18–22). Our group has demonstrated that active phospholipid species responsible for inducing monocyte adhesion to endothelial cells are destroyed after PAF-AH treatment (53) and that paraoxonase as part of HDL or in the purified form inhibited or abolished the bioactivity of MM-LDL (46). In the present study, the protective effect of HDL was evaluated using normal HDL, AP-HDL, and HDL after displacement by SAA and subsequent to restoration of its paraoxonase and PAF-AH activities (Table I). The observation that enrichment of normal HDL with purified human SAA abolishes the protective effect of HDL (Fig. 7 *C*) suggests that during the APR the incorporation of SAA into HDL could result in changes in the HDL particle that leads to the loss of its protective capacity against LDL oxidation. The AP-HDL not only did not inhibit the LDL oxidation and the resulting monocyte migration but in fact amplified it.

In human serum most if not all of the paraoxonase activity is associated with HDL (18, 54). Paraoxonase is present in a distinct HDL subspecies containing apo A-I and clusterin or apo J. Paraoxonase co-elutes with apo A-I. Thus immunoabsorption with anti-apo A-I removed 90% of paraoxonase from plasma, whereas anti-apo A-II eliminated only 10% of paraoxonase (55). It is likely that enzymes associated with apo A-I such as paraoxonase are responsible for the antioxidant effects of HDL and not apo A-I itself. We previously reported that incubation of cocultures with purified human apo A-I in a concentration range of 50–500 μg/ml did not prevent LDL-induced monocyte migration in the human artery wall cell cocultures (17).

Clinically, serum paraoxonase activity has been shown to be significantly lower in both familial hypercholesterolemia and insulin dependent diabetes mellitus (55). Additionally, in streptozotocin-diabetic rats, a progressive decrease in serum paraoxonase activity down to 36% of controls was observed (57). The presence of paraoxonase during copper-induced oxidation was reported to reduce the amount of lipoperoxides in the LDL particle (18). It was suggested that paraoxonase may act by hydrolyzing the lipid hydroperoxides produced during oxidation to nonreactive alcohols and carboxylic acids and thus prevent reactive aldehyde formation (22).

In our study, incubation of AP-HDL with purified human serum paraoxonase or PAF-AH followed by removal of unbound enzyme rendered AP-HDL protective against LDL modification. These results would suggest that the in vitro protective effect of HDL against LDL oxidation by human aortic wall cells significantly depends on the paraoxonase and PAF-AH activities of HDL. Moreover, these studies suggest that the acute phase reaction in both animals and humans is associated with a loss of these HDL associated enzyme activities and a gain in ceruloplasmin, thus accounting for the loss of HDL's ability to protect LDL from oxidation by artery wall cells. Liuzzo and colleagues have reported that the concentration of the sensitive indicators of inflammation, C-reactive protein and SAA, exceeded the 90th percentile of normal distribution in 65% of patients with unstable angina and 76% of those with acute myocardial infarction (57). The authors demonstrated that the elevation of C-reactive protein and SAA predicted a poor outcome and may reflect an important inflammatory component in the pathogenesis of unstable angina. Marked reduction of paraoxonase and PAF-AH and a gain in ceruloplasmin in HDL during the acute phase with a consequent loss of the protective effect of AP-HDL against LDL oxidation coupled with the resulting monocyte/endothelial interaction observed in this study may be contributing factors to the inflammatory reaction in such patients.

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