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Research Article

Terminal complement protein complexes C5b-9 have been found in human atherosclerotic lesions. Insertion of C5b-9 in the endothelial cell membrane alters permeability, induces membrane vesiculation, and activates secretion. We hypothesized that complement might also alter interactions of the endothelial surface with lipoproteins, particularly high density lipoprotein (HDL), which is reported to inhibit C5b-9-induced hemolysis. We now demonstrate that exposure to C5b-9 increases (by 2- to 50-fold) specific binding of HDL and its apolipoproteins (apo) A-I and A-II to endothelial cells. Binding to cells exposed to antibody, C5b67, and C5b-8 was virtually unchanged. Enhanced binding was also dependent on the number of C5b-9 complexes deposited on the cells. Other agonists that activate endothelial secretion did not augment binding. Calcium was required for full exposure of new binding sites by C5b-9. The C5b-9-induced increase in binding was independent of the increase observed after cholesterol loading. In addition, apo A-I and A-II appear to compete for the same binding sites on untreated and C5b-9-treated cells. In contrast to the data reported for red cells, we were unable to detect significant inhibition of C5b-9-mediated endothelial membrane permeabilization by HDL (up to 1 mg/ml) or by apo A-I (up to 100 micrograms/ml). These data demonstrate that the C5b-9 proteins enhance endothelial binding of HDL and its apoproteins, suggesting that intravascular complement activation may alter cholesterol […]

The Terminal Complement Proteins C5b-9 Augment Binding of High Density Lipoprotein and Its Apolipoproteins A-I and A-II to Human Endothelial Cells

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Abstract

Terminal complement protein complexes C5b-9 have been found in human atherosclerotic lesions. Insertion of C5b-9 in the endothelial cell membrane alters permeability, induces membrane vesiculation, and activates secretion. We hypothesized that complement might also alter interactions of the endothelial surface with lipoproteins, particularly high density lipoprotein (HDL), which is reported to inhibit C5b-9–induced hemolysis. We now demonstrate that exposure to C5b-9 increases (by 2- to 50-fold) specific binding of HDL and its apolipoproteins (apo) A-I and A-II to endothelial cells. Binding to cells exposed to antibody, C5b67, and C5b-8 was virtually unchanged. Enhanced binding was also dependent on the number of C5b-9 complexes deposited on the cells. Other agonists that activate endothelial secretion did not augment binding. Calcium was required for full exposure of new binding sites by C5b-9. The C5b-9–induced increase in binding was independent of the increase observed after cholesterol loading. In addition, apo A-I and A-II appear to compete for the same binding sites on untreated and C5b-9–treated cells. In contrast to the data reported for red cells, we were unable to detect significant inhibition of C5b-9–mediated endothelial membrane permeabilization by HDL (up to 1 mg/ml) or by apo A-I (up to 100 μg/ml). These data demonstrate that the C5b-9 proteins enhance endothelial binding of HDL and its apoproteins, suggesting that intravascular complement activation may alter cholesterol homeostasis in the vessel wall. (*J. Clin. Invest.* 1991. 88:1833–1840.) Key words: apolipoprotein A-I • apolipoprotein A-II • atherosclerosis • cholesterol • inflammation

Introduction

The endothelial cell surface expresses high-affinity binding sites for circulating lipoproteins which are believed to be important in regulating cholesterol homestasis of the vessel wall. Among these are low density lipoprotein (LDL) receptors, which mediate internalization of the LDL particle and uptake of cholesterol. High density lipoprotein (HDL; 1, 2) and apolipoproteins (apo) A-I and A-IV (3) have also been shown to bind to endothelial cells. However, membrane binding of HDL does not appear to result in significant internalization of the HDL particle (1). The nature of the interaction of HDL with cell surfaces, and its functional significance, remain poorly understood. Both HDL and free apo A-I and A-II have been demonstrated to serve as acceptors for removal of cholesterol from several types of cells in culture (4–7), and HDL and apo A-I and A-IV binding is increased significantly after cholesterol loading of some cells (8, 9). However, it is not known whether HDL binds to a membrane protein receptor, or directly with membrane lipid. An HDL-binding protein, which increases in activity after cholesterol loading, has been reported in fibroblasts and bovine aortic endothelial cells (10). However, given the high affinity of apo A-I and A-II for phospholipid (11–13), direct binding of these apoproteins to membrane lipid remains an important possibility.

Membrane insertion of the terminal complement proteins C5b-9 has been shown to disrupt membrane phospholipid organization, creating patches of nonbilayer phospholipid and facilitating transbilayer movement of phospholipid (14–16). Assembly of C5b-9 complexes has been shown to perturb the endothelial surface by inducing shedding of membrane microvesicles and fusion of secretory granules with the plasma membrane (17, 18). The membrane vesicles express binding sites for factor Va and thus provide catalytic surface for assembly of the prothrombinase enzyme complex. It has been suggested that factor Va–binding sites induced by C5b-9 in platelet and endothelial membranes may result from C5b-9–induced surface expression of acidic phospholipids, which are normally a component of the inner leaflet of the cell membrane bilayer (18).

Recently, C5b-9 complexes have been identified in human atherosclerotic plaques (19, 20). Moreover, endothelial C5b-9 deposition has been demonstrated before lesion development in the hypercholesterolemic rabbit model of atherosclerosis, suggesting an interrelationship between complement activation and atherogenesis (21). The specific role of the terminal complement proteins in this process remains unknown. The known interaction of the C5b-9 proteins with plasma lipoproteins, including the inhibitory effects of HDL and its apoproteins on C5b-9–mediated hemolysis (22, 23), raises the possibility that C5b-9 deposition on the endothelial surface alters the interaction of HDL with the vascular wall.

As a first step towards elucidating the effects of complement activation on endothelial interaction with plasma lipoproteins, we have examined the effects of the C5b-9 complex on the binding of HDL and its major apolipoproteins apo A-I and A-II to the endothelial cell surface. These experiments demonstrate that complement activation results in a significant increase in the specific binding of both HDL and its free apolipoproteins to human endothelial cells, and that the exposure of these membrane binding sites requires incorporation of C9 into the C5b-9 complex.

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Methods

Cell culture. Endothelial cells were harvested from human umbilical vein and cultured as previously described (17). First-passage cells grown in 1-cm^2 wells in 48-well plates were used in all experiments described here. Before HDL binding experiments, cells were grown for 24 h in media containing 10% lipoprotein-deficient human serum (LPDS)[1] and 50 μg/ml 25-hydroxycholesterol, rather than 20% fetal bovine serum. For some apolipoprotein binding experiments, cells were preloaded with cholesterol by incubating for 24 h in medium containing 10–30 μg/ml 25-hydroxycholesterol as indicated in the figure legends.

Purification of human complement proteins. Human C8 and C9 were purified as previously reported (24). Human serum deficient in complement component C8 was prepared by absorption of normal serum against rabbit antibody to C8 coupled to agarose (17). For some experiments, LPDS (prepared as described below using Liposorb, Calbiochem-Behring Corp., San Diego, CA) was used to prepare serum which was both C8- and lipoprotein-deficient.

Preparation of polyclonal rabbit anti-endothelial cell antibody. Plasma membranes partially purified from cultured human endothelial cells were used to immunize rabbits (17). IgG was prepared by absorption to protein A-agarose (17).

Purification and iodination of HDL. HDL was purified from human plasma anticoagulated with EDTA by sequential flotation ultracentrifugation (*d* 1.063–1.21) (25). HDL was iodinated using IODOGEN (Pierce Chemical Co., Rockford, IL). Free iodine was removed by gel filtration followed by extensive dialysis against phosphate-buffered saline (PBS, 0.15 M NaCl, 9 mM Na_2HPO_4, 1.6 mM KH_2PO_4) containing 2% KI, followed by dialysis against PBS and activated charcoal, to reduce the amount of ^{125}I associated with lipid. The specific activity was 860 cpm/ng; > 98% of the radioactivity was precipitable with trichloroacetic acid.

Purification of apo A-I and A-II. Apo A-I and A-II for preliminary experiments were obtained from Sigma Chemical Co., St. Louis, MO. The apolipoproteins were later purified from HDL (prepared by sequential flotation ultracentrifugation of human plasma). HDL was dialyzed into 5 mM ammonium bicarbonate, 1 mM EDTA, pH 8.0, and lyophilized. Apo HDL was prepared by two extractions with diethyl ether, and five extractions with ethanol/ether (3:1) as described by Osborne (26), dried under nitrogen, and dissolved in 3 M guanidine HCl, 20 mM Tris, pH 8.0. The HDL apolipoproteins were chromatographed on Sephacryl S200 (2.6 × 85 cm) yielding an elution pattern similar to that previously reported with Sephadex G200 (27). Apo A-I and A-II peaks were further purified by reverse-phase HPLC (SynChropak RP-P C18, 250 × 21.2 mm; SynChrom, Inc., Lafayette, IN) using a gradient of acetonitrile (in water containing 0.1% trifluoroacetic acid) from 25% to 58% at 1.0%/min, 20°C, flow rate 8 ml/min (28). Apolipoproteins were lyophilized and stored at −70°C. Before use, the apolipoproteins were dissolved in 3 M guanidine, 0.02 M Tris, pH 8.0, and dialyzed into PBS. Apo A-I and A-II were iodinated using immobilized lactoperoxidase (Enzymobeads, Bio-Rad Laboratories, Richmond, CA) to specific activities of 500–2,660 and 1,700–4,900 cpm/ng, respectively. After iodination, purity was assessed by SDS-PAGE followed by autoradiography; in the absence of disulfide bond reduction, apo A-I and A-II yielded single bands of molecular mass 28 and 17 kD, respectively.

Preparation of LPDS. LPDS was prepared by ultracentrifugation of human serum at density 1.23 (adjusted by the addition of solid KBr); the infranate was dialyzed against HBSS to remove KBr. Alternatively, human serum was absorbed with Liposorb according to the manufacturer's instructions. LPDS was heat-inactivated at 56°C for 30 min before use on cultured cells.

Exposure of endothelial cells to activated complement. The buffer solution used for all endothelial cell experiments consisted of HBSS containing 10 mM Hepes and 1% bovine serum albumin (HHBSS-BSA). Cultured endothelial cells were exposed to various stages of complement activation by sequential incubation at 37°C with antiendothelial IgG (4 mg/ml) for 15 min, 25% C8-deficient serum for 10 min, and human C8 and C9 for 10 min at the concentrations indicated in the figure legends. In some experiments, the above buffer with 0.1 mM EGTA and no calcium was used for the C8 and C9 incubations, as detailed in the figure legends.

Lipoprotein binding experiments. After two washes to remove complement components, cell monolayers were incubated with ^{125}I-HDL for 2 h at 37°C. Cells were then washed six times rapidly with HHBSS-BSA at 4°C to remove unbound radioactivity, solublized in 4% SDS, and counted in a γ counter (Beckman Instruments, Inc., Fullerton, CA). Nonspecific binding was determined by parallel incubations in the presence of 50-fold excess of unlabeled HDL, and was always ~ 50% of total binding. Specific binding, defined as total bound radioactivity minus nonspecific, is reported in the figures. Binding experiments using apo A-I and A-II were performed identically except that duration of binding was 10–40 min as indicated in the figure legends, and nonspecific binding was determined in the presence of a 15-fold excess of unlabeled apolipoprotein. At the concentrations used in these experiments, nonspecific binding for apo A-I was ~ 45% of total in untreated cells and decreased to ~ 30% in C5b-9–treated cells; for apo A-II, nonspecific binding was ~ 45% of total in control cells and only 20% of total in C5b-9–treated cells.

Quantitation of endothelial membrane permeabilization. Membrane permeabilization by C5b-9 was monitored using 2-deoxy-D-[^3H]glucose ([^3H]DOG) release (29). Cells were loaded by incubation with [^3H]DOG 10 μCi/ml (2.75 μCi per well) for 24 h. After washing to remove extracellular radioactivity, C5b-9 was deposited as described above. Media were removed after 10 min, pooled with two washes, and subjected to scintillation counting. Results are expressed as percent reduction by potential lipoprotein inhibitors of C5b-9–induced [^3H]-DOG release (counts per minute released from C5b-9–treated cells minus counts per minute released from untreated cells).

Results

HDL and apolipoprotein binding to C5b-9–treated endothelial cells. HDL are small, spherical or discoid particles containing about one half protein and one half lipid by weight. The protein components include apo A-I (M_R 28,000; 60–70%), apo A-II (M_R 17,000; 20–30%), and small amounts of apo C and E (30). The role of these apolipoproteins in the interaction of HDL with cell surfaces is not known, but these apolipoproteins contain extensive regions of amphipathic α-helix which have high affinity for phospholipids. Apo A-I dissociates readily from HDL, and "free" apo A-I is found in both plasma and the interstitial space (30). As illustrated in Fig. 1, binding of immunoglobulin or complement activation through C8, does not alter the binding of either of the HDL apolipoproteins (A-I or A-II) to the endothelial surface. By contrast, addition of C9 (to membrane C5b-8) was found to increase binding of both apo A-I and A-II, suggesting a specific effect of the assembled C5b-9 complex on the exposure of membrane binding sites for these apolipoproteins. Apolipoprotein binding to C5b-9–treated cells increased from 2- to 50-fold above binding to untreated cells. This variability was in part due to the large variability in specific binding observed for untreated cells. In addition to a requirement for C9, the exposure of specific membrane binding sites for apo A-I and A-II was also found to increase as a function of the number of cell surface C5b-9 complexes, generated by the addition of limiting amounts of C8 to preformed membrane C5b67 (Fig. 2). Increased binding to human endothelial cells after exposure to C5b-9 was also observed for purified HDL particles (Fig. 3). As was the case for the apolipoproteins, HDL binding was virtually unchanged in cells exposed to

1. *Abbreviations used in this paper:* [^3H]DOG, 2-deoxy-D-[^3H]glucose; LPDS, lipoprotein-deficient human serum.

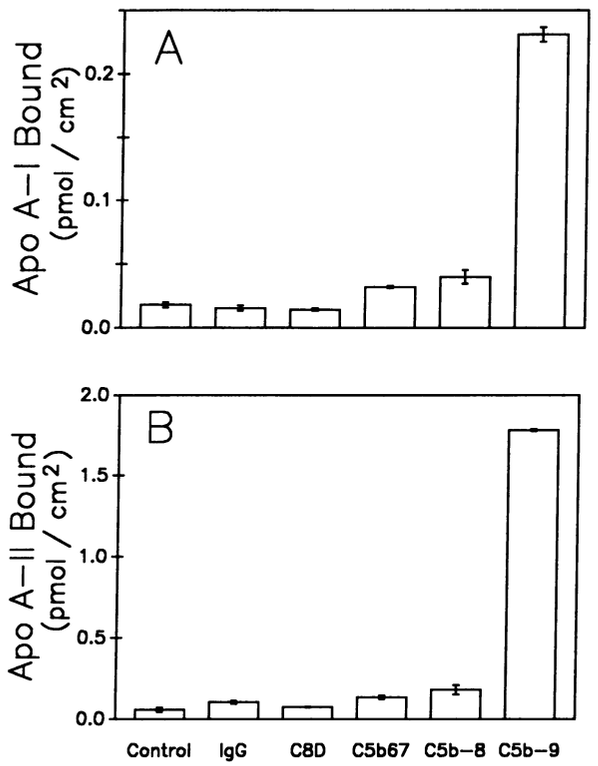

Figure 1. Effects of complement deposition on binding of apo A-I and A-II to human endothelial cells. Complement was activated, as described in Methods, by sequential exposure of cells to antiendothelial IgG, C8-deficient serum, and human C8 (0.2 μg per well) and C9 (1 μg per well). After complement activation, cells were incubated with either ^{125}I-apo A-I (10 μg/ml, *A*), or ^{125}I-apo A-II (10 μg/ml, *B*) at 37°C for 30 min. Effects of exposure to antibody only (*IgG*), C8-deficient serum only (*C8D*), antibody plus C8-deficient serum to assemble *C5b67*, C5b67 plus C8 (*C5b-8*), and C5b-8 plus C9 (*C5b-9*) are shown. Data are specific apolipoprotein binding (mean±range, n = 2), and are representative of four experiments performed. Note the difference in scale for *A* and *B*.

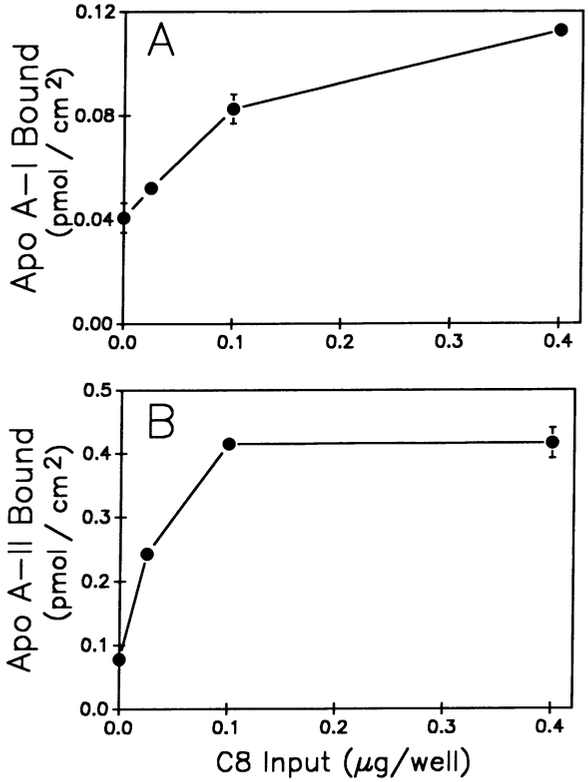

Figure 2. Effect of number of membrane C5b-9 complexes on binding of apo A-I and A-II. Complement was activated by sequential exposure of cells to antiendothelial IgG, C8-deficient serum, and human C8 and C9. Cells were then incubated with either ^{125}I-apo A-I (10 μg/ml, *A*) or ^{125}I-apo A-II (10 μg/ml, *B*) at 37°C for 20 min. Membrane deposition of C5b-9 complexes was controlled by limiting C8 input, as indicated on the abscissa. C9 input was 2 μg per well (16 μg/ml). Specific binding data shown are the mean of duplicate determinations; error bars indicate range. Of note, error bars are frequently obscured by symbols. Data are representative of three experiments performed. Note the different scales for apo A-I and A-II.

antibody and complement activation through C8, but increased with addition of C9 to the membrane C5b-8 complex, suggesting a specific requirement for the membrane-inserted C5b-9 complex. The similarity of the results obtained with HDL and with apo A-I and A-II (cf. Figs. 1–3) suggests that the binding of HDL to the C5b-9–induced membrane sites is mediated by the apolipoprotein components of these particles.

Relationship of exposure of apolipoprotein binding sites to endothelial storage granule secretion. In addition to increasing cell surface binding of HDL and its apolipoproteins (above), C5b-9 has been shown to induce secretion of endothelial storage granules (17), raising the possibility that the expression of these new binding sites is related to the fusion of Weibel-Palade bodies with the plasma membrane. Several other agonists are also known to activate endothelial cell secretion, including histamine, thrombin, the phorbol ester phorbol 12-myristate 13-acetate (PMA), and the calcium ionophore A23187 (31). By contrast to C5b-9, when endothelial cells were exposed to these agonists at concentrations that activate maximal von Willebrand factor secretion, no increase in specific binding of apo A-II was observed (Fig. 4). Similar results were obtained for binding of apo A-I (data not shown). These results suggest that neither permeabilization of the plasma membrane to calcium (as occurs after A23187), release of intracellular calcium stores (as induced by histamine and thrombin), nor activation of protein kinase C (by PMA) is sufficient to expose the plasma membrane binding sites for the HDL apolipoproteins. These data also suggest that the increase in binding of the HDL apolipoproteins observed in C5b-9–treated endothelial cells is unrelated to induced secretion of endothelial storage granules. Consistent with this interpretation, the C5b-9–induced binding sites for apo A-I and A-II were observed to persist on the endothelial surface during prolonged incubation at 37°C (Fig. 5). This contrasts the transient cell surface exposure and rapid reinternalization of the granule membrane-derived protein, GMP-140, that is observed in association with endothelial secretion induced under these conditions (17).

Effect of plasma membrane cholesterol loading on apolipoprotein binding. It has been reported that the binding of HDL and apo A-I and A-IV to bovine endothelial cells increases markedly when the cells are preincubated with cholesterol, either in the form of LDL, acetylated LDL, or 25-hydroxycholesterol (3, 8, 9). The increased binding does not follow accumulation of esterified cholesterol but requires an increase in free cholesterol in the membrane (32). In our preliminary experiments, we confirmed this effect of cholesterol on HDL and apo

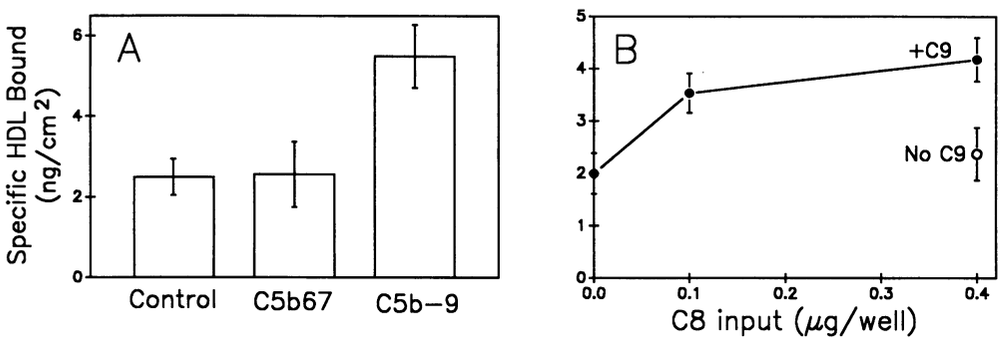

Figure 3. Increased HDL binding to human endothelial cells exposed to C5b-9. (*A*) Endothelial cells were exposed to buffer only (*Control*), antiendothelial IgG followed by C8-deficient human serum to deposit *C5b67*, or IgG, C8-deficient serum, and then human C8 (0.2 μg per well, 1.6 μg/ml) and C9 (2 μg per well, 16 μg/ml) to deposit *C5b-9*. After washing to remove complement components, cells were incubated for 2 h with ^{125}I-HDL (30 μg/ml) with and without a 50-fold molar excess of unlabeled HDL. Specific binding is shown, and is representative of two experiments so performed. Data are mean±SD, n = 3. (*B*) Endothelial cells were exposed to antiendothelial IgG and C8-deficient serum to deposit C5b67. The number of C5b-9 complexes deposited was controlled by adding human C8 in the amounts indicated on the abscissa, in the presence of human C9 at 2 μg per well (•) or 0 μg per well (○). Data shown are mean±SD, n = 3.

A-I binding to human endothelial cells, and found a similar increase in apo A-II binding. In order to determine whether cholesterol loading and C5b-9 deposition might synergistically alter HDL apolipoprotein binding, we examined the effect of cholesterol on C5b-9–induced exposure of these endothelial binding sites (Fig. 6). As illustrated by these data, the effects of cholesterol and C5b-9 are approximately additive, suggesting that plasma membrane cholesterol content does not influence the magnitude of C5b-9–induced binding of these apoproteins. To be noted, distinct morphologic changes were observed in the human endothelial cells incubated with 10 μg/ml 25-hydroxycholesterol, even though concentrations of 40–100 μg/ml have been commonly used to enhance HDL and apo A-I binding (3, 8). Moreover, C5b-9 assembly was poorly tolerated by the cholesterol-loaded cells, necessitating a decrease in C8 concentration (0.05 μg per well) to avoid lysis and detachment of the cells used in these experiments.

Requirement for calcium. C5b-9 induces the secretion endothelial vWF through a pathway that requires extracellular Ca^{2+}, and can be abolished by EGTA (17). In order to determine whether calcium influx is required for the C5b-9–induced generation of new binding sites for apo A-I and A-II, we compared binding to C5b67-bearing cells when C8, C9, and ^{125}I-apolipoprotein were added in the presence of calcium or EGTA. As

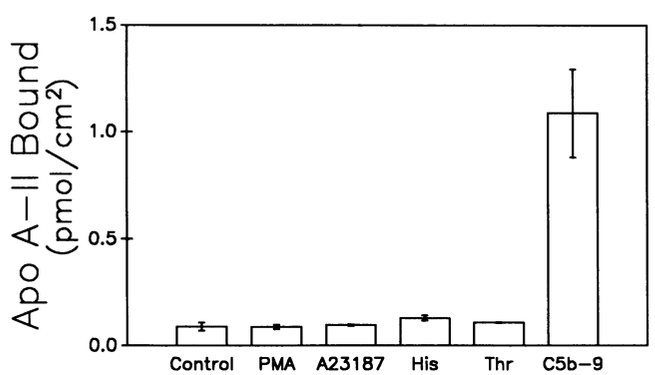

Figure 4. Comparison of C5b-9 to other activators of endothelial secretion. Endothelial cells were washed, and treated sequentially with antiendothelial IgG and C8-deficient serum (C5b-9–treated cells) or buffer only (control and other agonists). After washing, ^{125}I-apo A-II (10 μg/ml with and without 15-fold excess of unlabeled apo A-II) was added, followed by agonists at the following concentrations: *PMA*, 10^{-7} M; *A23187*, 10^{-5} M; Histamine (*His*), 10^{-4} M; thrombin (*Thr*) 1 U/ml; C8 (0.2 μg per well) and C9 (1 μg per well) (*C5b-9*). Specific binding was measured after 30 min at 37°C. Von Willebrand factor secretion into the supernatant was assayed and was greater than five times control for all agonists. Data shown are mean±range, n = 2.

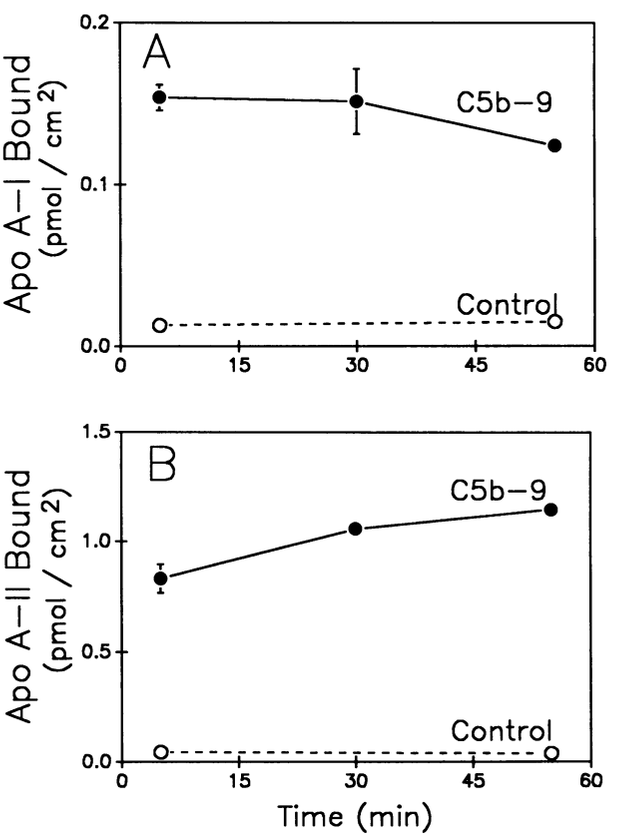

Figure 5. Stability of the C5b-9–induced apolipoprotein binding sites. Endothelial cells were incubated with antiendothelial IgG, C8-deficient human serum, and then C8 (0.2 μg per well) plus C9 (1 μg per well) (*C5b-9*) or buffer only (*Control*) for 8 min. Cells were then washed to terminate complement deposition and remained in buffer until addition of ^{125}I-apo A-I (*A*) or ^{125}I-apo A-II (*B*) at 0, 25, and 50 min after completion of C5b-9 assembly. The ^{125}I-apoproteins were used at 10 μg/ml in the presence and absence of 15-fold excess of unlabeled apoprotein. Duration of binding was 10 min, and data are shown at the midpoint of the binding interval (mean±range, n = 2). Note the different scales for *A* and *B*.

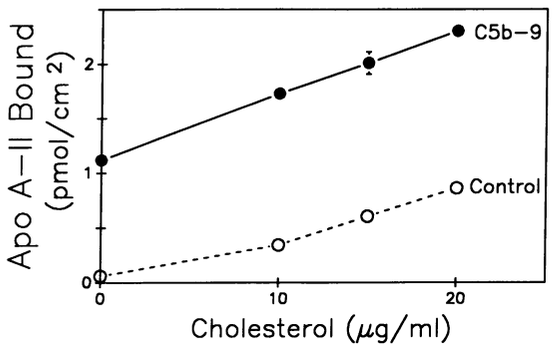

Figure 6. Effect of cholesterol loading on the C5b-9–induced response. Endothelial cells were preincubated for 24 h with culture medium containing 25-hydroxycholesterol at the concentration given on the abscissa. Cells were then washed five times and treated sequentially with antiendothelial IgG, C8-deficient serum, and C8 (0.05 μg per well, 0.4 μg/ml) and C9 (1 μg per well, 8 μg/ml). Control cells were incubated with buffer only at each stage. After two washes to remove C8 and C9, cells were incubated for 30 min with ^{125}I-apo A-II (10 μg/ml) for 30 min at 37°C. Data shown are specific binding (mean±range, n = 2), and are representative of two experiments performed.

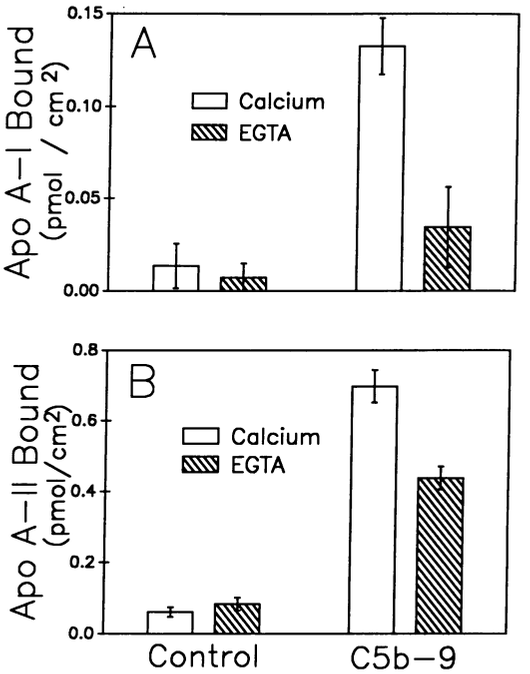

Figure 7. Requirement for calcium. C5b67 was assembled by incubating endothelial cells with antibody and C8-deficient serum. Cells were then washed twice in buffer containing either 2 mM calcium (*open bars*) or 0.1 mM EGTA (*hatched bars*), followed by addition of this same buffer without (*Control*) or with C8 and C9 (0.2 and 2 μg per well respectively, *C5b-9*); ^{125}I-apo A-I (20 μg/ml, *A*) or ^{125}I-apo A-II (10 μg/ml, *B*) was also included. Binding proceeded for 20 min at 37°C. Data shown are specific binding, mean±SD, n = 3, and are representative of three similar experiments. Note the different scales for apo A-I and A-II.

shown in Fig. 7 *A*, the C5b-9–induced increase in apo A-I binding was reduced ~ 80% by EGTA. In general, a smaller effect of EGTA on the C5b-9–induced binding of apo A-II was observed (Fig. 7 *B*). These data suggest that extracellular calcium, or the influx of calcium into the cytosol, contributes to full expression of the C5b-9–induced apolipoprotein binding sites, particularly in the case of apo A-I. By contrast, calcium does not appear to influence the interaction of these apolipoproteins with the untreated endothelial cell.

The calcium requirement for the C5b-9–induced apolipoprotein binding demonstrated in Fig. 7 may be due to the calcium influx required for cell activation by C5b-9 or to an effect of calcium on the interaction of the apolipoprotein with the newly expressed binding site. In order to examine these effects, we performed C5b-9 assembly from C5b67 sites, and apo A-I binding in separate stages; the effects of EGTA on (*a*) cell "activation" by C5b-9, and (*b*) apo A-I binding could then be observed. As demonstrated by the data in Table I, calcium appears to be important at the time of complement deposition on the membrane. Once the complement pore has been formed in the presence of calcium, apolipoprotein binding is unaffected by removal of calcium. These data suggest a calcium dependence of expression of the binding site and do not support a calcium dependence of ligand interaction with the site.

Effect of C5b-9 on internalization of apolipoprotein. Our binding studies were performed at 37°C, and may potentially reflect both surface bound and internalized ligand. In order to determine whether the increases in apo A-I and A-II binding to C5b-9 (vs. untreated) cells (demonstrated in Fig. 1, 4, 5, and 7) reflected an internalization of the plasma membrane-bound apolipoprotein, the cells were subjected to a tryptic digest, to distinguish surface-bound from internalized ligand. In the C5b-9–treated cells incubated with ligand at 37°C, the percentage of cell surface-bound apo A-I remained essentially constant with time (67% trypsin-releasable at 10 min vs. 69% at 40 min, Table II). These data suggest that the 2- to 30-fold increases in specific apo A-I binding is not accounted for by increased membrane internalization in the C5b-9–treated cells.

Competition between apo A-I and A-II for the binding sites on C5b-9–treated endothelial cells. Apo A-I and A-II exhibit significant structural homology and both contain extensive regions of amphipathic helix which are believed to be important in the phospholipid binding and lipid transport function of these proteins (33). Because both apolipoproteins exhibited similar enhancement in binding to C5b-9–treated endothelial cells, we wished to determine whether this binding occurred at

Table I. Calcium Requirement for Expression of C5b-9–induced Apo A-I Binding Sites: Effects of EGTA on Cell Activation by C5b-9 and on Apo A-I Binding

C5b-9 assembly from C5b67 sites*	Apo A-I binding‡	Apo A-I bound§
		pmol/cm²
Ca	Ca	0.159±0.006
Ca	EGTA	0.151±0.020
EGTA	EGTA	0.061±0.006

* C8 (0.2 μg per well) and C9 (1 μg per well) in either calcium 2 mM or EGTA 0.1 mM were added to cells bearing preformed C5b67 sites and incubated for 10 min at 37°C to assemble C5b-9. Cells were then washed twice with the calcium- or EGTA-containing buffer to be used for apoprotein binding. ‡ Binding of ^{125}I-apo A-I was performed for 15 min at 37°C in the presence of calcium 2 mM or EGTA 0.1 mM, after termination of C5b-9 assembly. § Results are mean±range, n = 2.

Table II. Accessibility to Trypsin of Apo A-I Bound to C5b-9–treated Endothelial Cells

Duration of binding	Apo A-I bound: Total	Apo A-I bound: Trypsin-releasable	Percentage of trypsin releasable
min	*pmol/cm²*		
10	0.255±0.021	0.172±0.006	67
40	0.495±0.023	0.340±0.028	69

Endothelial cells were treated with antiendothelial IgG and C8-deficient serum, and then incubated with C8 (0.2 μg per well, 1.6 μg/ml), C9 (1 μg per well, 8 μg/ml) for 10 min to deposit C5b-9. After washing twice, ^{125}I-apo A-I (10 μg/ml, with and without a 15-fold excess of unlabeled apo A-I) was added and incubated for 10 or 40 min. Cells were then transferred to ice and rapidly washed six times. In some wells, cells were solubilized in SDS to yield specific apo A-I bound. In other wells, cells were incubated with trypsin (1 mg/ml) at 4°C for 75 min; trypsinization was terminated and wells were washed with soybean trypsin inhibitor (2 mg/ml). After microcentrifugation, supernatants were counted to yield the trypsin-releasable fraction. Data shown are mean±range, n = 2.

the same membrane site. As shown in Fig. 8 *A*, apo A-II effectively competed for the ^{125}I-apo A-I binding sites exposed in C5b-9–treated cells. Likewise, apo A-I effectively competed for the ^{125}I-apo A-II binding sites (Fig. 8 *B*), although in all experiments, apo A-II was observed to compete binding of the radiolabeled apolipoproteins (apo A-I or apo A-II) more effectively than apo A-I. Similar results were obtained for control cells (data not shown). To be noted, apo A-II has been reported to associate with lipid more rapidly than apo A-I, and to displace apo A-I from HDL particles (34).

Effect of HDL and apo A-I and A-II on the C5b-9 pore. As noted above, it has been reported that apo A-I and A-II inhibit C5b-9–induced lysis of human erythrocytes, suggesting that these apoproteins inhibit assembly of the C5b-9 pore (22, 23). In order to determine whether the HDL particle or its apolipoproteins inhibits C5b-9 assembly on the endothelial surface, we examined the effect of HDL and apo A-I on C5b-9–induced permeabilization of the plasma membrane. In these experiments, the inhibition of C5b-9–induced release of [^{3}H]DOG by HDL and apolipoprotein was measured. Under the conditions of our experiments, neither HDL (at concentrations up to 1 mg/ml) nor apo A-I (at concentrations up to 100 μg/ml) were found to inhibit significantly C5b-9 pore formation: percent inhibition by apo A-I 100 μg/ml, 10±16% (NS); in the presence of HDL 1 mg protein/ml, there was no inhibition, but rather a small increase in [^{3}H]DOG release (21±8%, NS). By contrast, it has been reported that HDL (at 30 μg/ml) and apo A-I (at 1.5–9.5 μg/ml) causes 50% reduction in C5b-9–induced lysis of human erythrocytes (22).

Discussion

These experiments establish that membrane assembly of the C5b-9 complex can lead to a significant increase in the binding of HDL and apo HDL to the endothelial cell surface, suggesting a possible mechanism by which cholesterol metabolism of the vessel wall might be altered by intravascular complement activation. To our knowledge, this response to C5b-9 is the first

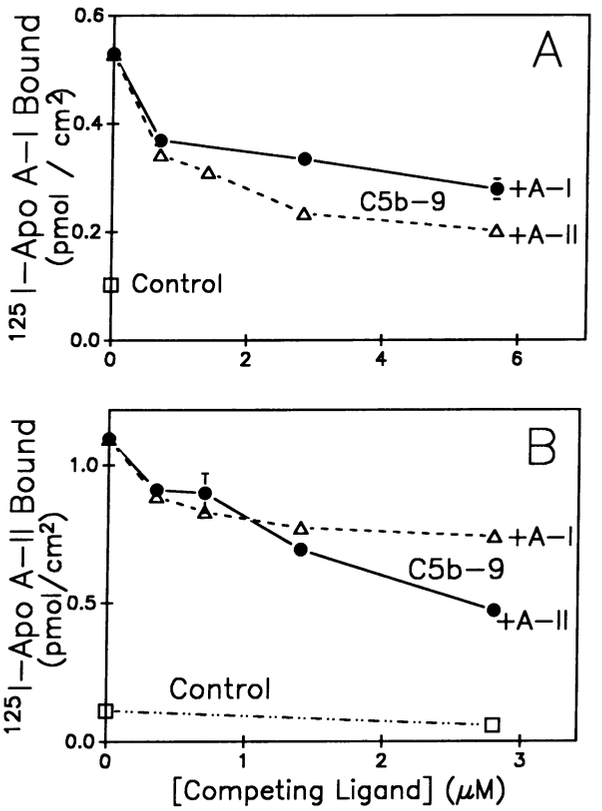

Figure 8. Apo A-I and A-II compete for a common binding site on C5b-9–treated endothelial cells. (*A*) ^{125}I-apo A-I (20 μg/ml, 0.7 μM) binding to C5b-9–treated cells is shown in the presence of 0- to 8-fold excess of unlabeled apo A-I and A-II. (*B*) ^{125}I-apo A-II (6 μg/ml, 0.35 μM) binding to C5b-9–treated cells is shown in the presence of 0- to 8-fold excess of unlabelled apo A-I and A-II. Binding to control cells is shown for comparison. Data shown are mean±range, n = 2. Note the different scales for *A* and *B*.

evidence for a direct effect of an inflammatory mediator on lipoprotein binding to the endothelial surface.

It is of interest to note that the only reported mechanism for increasing the binding of HDL (and its apolipoproteins) to endothelial cells is through increased concentration of free cholesterol in the plasma membrane. Since increasing plasma membrane cholesterol concentration and membrane insertion of the C5b-9 proteins are both known to alter the arrangement of membrane phospholipid, it is possible that the increased binding of HDL and HDL apolipoproteins observed under these conditions reflects formation of lipid domains that favor intercalation of these amphipathic proteins into the plasma membrane. Alternatively, C5b-9 may expose a protein receptor for apo A-I and A-II on the endothelial surface, perhaps by inducing translocation of such receptors to the plasma membrane from an intracellular pool or by inducing a conformational change in preexisting receptors. Of note, an HDL-binding protein with affinity for apo A-I and A-II has been described in fibroblasts and bovine aortic endothelial cells (10), although evidence against a protein receptor for HDL has also been presented (35).

A review of the known effects of the C5b-9 complex on endothelium suggests several mechanisms by which these proteins might generate new binding sites for HDL. First, C5b-9 stimulates release of secretory granules and surface expression

of protein(s) derived from the granule membrane (17). However, fusion of the secretory granules with the plasma membrane cannot be fully responsible for the observed increase in HDL binding, because other agonists which stimulate secretion did not increase binding (Fig. 4). C5b-9 also induces endothelial membrane vesiculation and expression of new binding sites for factor Va, an effect that has been related to the surface exposure of phosphatidylserine and other negatively charged phospholipid under these conditions (18). Although an affinity of apo A-I or A-II for acidic phospholipids might underlie the observed increase in binding to C5b-9–treated endothelial cells, it is of note that most of the membrane binding sites for factor Va (and presumably, most of the exposed acidic phospholipid) are vesiculated from the endothelial surface, and are unlikely to account for the binding of HDL to the cell monolayers that we report here. As HDL and its apoproteins have been reported to inhibit C5b-9–induced hemolysis, direct binding of these lipoproteins to components of the C5b-9 complex can also be considered (22, 23). As noted above, with endothelial cells we were not able to demonstrate an inhibitory effect of apo A-I or HDL on C5b-9 pore formation, and were unable to inhibit apo A-II binding to C5b-9–treated cells with a polyclonal antibody to C9 (data not shown). Nevertheless, the possibility that these lipoproteins bind directly to the membrane-bound C5b-9 proteins cannot be excluded.

The function of HDL is not fully understood, and the physiologic and pathophysiologic consequences of enhanced HDL binding to vascular endothelium remains to be determined. HDL and apo A-I have received attention because epidemiologic data suggests an inverse relationship between their plasma levels and the risk of cardiovascular disease in industrialized countries (36, 37). Whether HDL has a direct protective effect against atherogenesis or is a marker for the efficiency of other metabolic processes (e.g., clearance of triglyceride-rich lipoproteins) is controversial (for discussion, see Tall [30]). HDL are believed to play a role in transport of cholesterol from peripheral cells to the liver (30), and thus enhanced binding to endothelial cells might result in increased cholesterol efflux. Alternatively, since apo A-I is a potent activator of lecithin:cholesterol acyl transferase, the possibility exists that the C5b-9–induced apo A-I binding to the endothelial plasma membrane might result in the accumulation of cholesterol ester in the cell. Furthermore, HDL are heterogeneous, and it is now recognized that a significant population of apo A-I–containing lipoproteins exists outside the HDL density range (38, 39). The effect on cholesterol homeostasis or other cellular functions may depend upon the subclass of apo A-I–containing lipoprotein that is actually bound to the plasma membrane: for example, there is evidence in vitro that binding of HDL containing apo A-I but not A-II, Lp (A-I without A-II), may activate cholesterol efflux, whereas Lp (A-I with A-II) binds but fails to activate this process (5). Thus, the C5b-9–induced increased HDL binding may serve to activate or inhibit cholesterol efflux, if a particular subclass is preferentially bound.

In addition to its potential role in reverse cholesterol transport, apo A-I has recently been shown to circulate in association with a complement inhibitor (clusterin, complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, apolipoprotein J; 40–42). By augmenting apo A-I binding, C5b-9 deposition could increase the local concentration of an inhibitor which prevents further C5b-9 assembly on the cell surface, perhaps limiting injury. Since HDL and apo A-I/phospholipid complexes have been shown to bind prostacylin (43, 44), an inhibitor of platelet aggregation that is produced by endothelium in response to C5b-9 and other stimuli (45), the possibility also exists that bound HDL may alter the antithrombotic properties of the endothelial surface.

The interrelationship between complement activation and atherogenesis remains unresolved. Detection of C5b-9 complexes in atherosclerotic plaques (19, 20) and the association of anti–HLA antibodies with the development of accelerated coronary artery disease in transplanted hearts (46) suggests that the humoral immune response may play a role in the initiation or progression of the atherosclerotic lesion. Our finding that the C5b-9 proteins induce binding of HDL to the endothelial surface suggest one mechanism by which intravascular complement activation might alter cholesterol homeostasis, and contribute to the development of atherosclerosis.

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