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A human monoclonal antibody F_{ab} reactive to oxidized LDL and carbamylated LDL recognizes human and mouse atherosclerotic lesions

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This study was undertaken to produce a F_{ab} fragment of a human monoclonal antibody reactive to oxidized and carbamylated low-density lipoprotein (oxLDL and cLDL) using phage display technology. An analysis of DNA sequences of this F_{ab}, termed plaque 15,16-46 F_{ab}, revealed that the rearranged V_H was highly mutated. Complementarity-determining regions of the V_H showed a very high R/S ratio and contained many positively charged amino acids. In direct binding and competitive ELISA, the F_{ab} reacted strongly with both MDA-LDL and Cu-oxLDL forms of oxLDL, and also showed high affinity for cLDL. Immunofluorescence and immunohistochemical analyses showed that this F_{ab} positively stained atherosclerotic aortic plaques in ApoE$^{-/-}$ mice as well as those in patients with atherosclerosis. The F_{ab} also showed positive staining in placental decidua from patients with preeclampsia. It is suggested that the plaque 15,16-46 F_{ab} against oxLDL and cLDL might possibly be applicable for developing a diagnostic reagent for both human and rodent animal research to detect and characterize atherosclerotic disease progression in atherosclerotic lesions as well as exploring the pathogenesis of atherogenic diseases such as preeclampsia.

Keywords: human monoclonal antibody; Fab fragment; oxidized LDL; carbamylated LDL; atherosclerosis

Introduction

Atherosclerosis is characterized by chronic inflammation of the arterial intima (Hansson 2005). Oxidative modification of low-density lipoprotein (LDL) occurs early in the natural progression of atherosclerotic plaques (Jeon et al. 2007; Goncalves et al. 2009; Lewis et al. 2009). Oxidized LDL (oxLDL) accumulates within the intramural extracellular matrix of the coronary and carotid vasculature, where it binds to macrophages (Feng et al. 2010). Oxidative modification of LDL produces reactive aldehydes, phospholipids, and lipid peroxidation products such as malondialdehyde-modified LDL (MDA-LDL), a representative form of oxLDL in which oxidation occurs mainly among the free lysine amino groups of apolipoprotein B. OxLDL levels in vivo, both in plasma and in atheromatous plaques, are strongly associated with coronary artery disease progression, the vulnerability of atherosclerotic lesions to rupture, and the severity of the clinical presentation (Nishi et al. 2002; Faviou et al. 2005; Hiki et al. 2009). Scavenger receptors on macrophages interact with oxLDL, leading to the formation of macrophage foam cells and fatty streaks, and the progression of atherosclerotic plaques (Jeon et al. 2007; Osto et al. 2008). Atherosclerotic lesions have been shown to contain materials with properties similar to those of oxLDL, particularly with respect to their physical and immunochemical properties (Itabe et al. 1994). Carbamylated LDL (cLDL) is another modified LDL that is related to the pathogenesis of atherosclerosis through vascular cell injury, proliferation, dysfunction, and increased expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Kraus and Kraus 2001; Apostolov et al. 2007; Asci et al. 2008). Urea-mediated or myeloperoxidase-catalyzed carbamylation contributes to the production of cLDL. Plasma levels of cLDL may predict the risk of coronary artery disease, future myocardial infarction, and stroke (Ok et al. 2005; Wang et al. 2007).

In humans with atherosclerosis and in preclinical animal models of disease (e.g., hypercholesterolemic rabbits and apoE$^{-/-}$ or LDL receptor-null mice), the titers of autoantibodies to oxLDL are increased; in mice models, autoantibody titer is correlated with the extent of atherosclerosis (Palinski et al. 1994, 1996;

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Sherer et al. 2001). The role of anti-oxLDL antibodies in the pathogenesis of atherosclerosis is not yet clearly defined, although possibilities for the potential use of anti-oxLDL autoantibodies as therapeutic agents have been reported (Palinski et al. 1996; Hörkkö et al. 1999; Shaw et al. 2001; Schiopu et al. 2004; Torzewski et al. 2004; Jeon et al. 2007; Goncalves et al. 2009). Further research efforts should improve prognostic and diagnostic strategies based on probes of pathogenic determinants of atherosclerosis. However, only a few antibodies to oxLDL or other epitopes are known to detect antigens in atherosclerotic lesions. Mouse antibodies against oxLDL, MDA-conjugated lysine, and/or 4-hydroxynonenal-conjugated lysine have been shown to positively stain atheroma of LDL receptor-null mice and/or Watanabe heritable hyperlipidemia rabbits (Harberland et al. 1988; Palinski et al. 1996; Shaw et al. 2001; Torzewski et al. 2004), and a human antibodies against apoB-100 peptide and oxLDL have been shown to immunostain atherosclerotic lesions in mice, rabbits, and humans (Shaw et al. 2001; Schiopu et al. 2004; Goncalves et al. 2009).

Preeclampsia is a characteristic hypertensive disorder of human pregnancy. Although the pathogenesis of this condition has not yet been clearly established, there is evidence for a significant contribution of atherogenic and pro-inflammatory mechanisms. Preeclampsia shares major similarities with atherosclerosis, including the presence of oxLDL accumulation and atheromatous plaques in the lesion, the involvement of common risk factors (e.g., oxidative stress and endothelial dysfunction) in the pathophysiology (Belo et al. 2008), and higher titers of anti-oxLDL autoantibodies (Tulppala et al. 1995; Fialová et al. 2002; Jain et al. 2004).

The genetic structures of only a few anti-oxLDL monoclonal antibodies have been analyzed in mice (Palinski et al. 1996; Choi et al. 1998; Shaw et al. 2000) and humans (Shaw et al. 2001; Schiopu et al. 2004; Jeon et al. 2007). The development of human monoclonal antibodies against epitopes relevant to atherogenic diseases, such as oxLDL and cLDL, is valuable, not only for clarifying the genetic structures and potential role(s) of these antibodies in the diseases, but also for exploring their possible use as diagnostic probes. In this study, we produced a F_{ab} fragment of a human monoclonal antibody specific to oxLDL and cLDL using human atheromatous plaques from two atherosclerosis patients as source material for the construction of a phage-display antibody library. We analyzed the primary structures of the heavy and light chain variable regions (V_H and V_L), binding specificities, and biological activity of this F_{ab} fragment, termed plaque 15,16-46 F_{ab}. In particular, we assessed the ability of this F_{ab} fragment to recognize antigen(s) located in atherosclerotic plaques in the aortas of patients with atherosclerosis, in the placental deciduas of patients with preeclampsia, and in aortas of ApoE$^{-/-}$ mice.

Materials and methods

Human and animal subjects

Written informed consent forms were obtained from atherosclerosis and preeclampsia patients who had visited Ajou University Hospital, S. Korea. The consent forms and study plans were approved by the Institutional Review Board of the Medical Center of Ajou University. The procedures used in human and mouse studies were in accordance with the institutional guidelines of the Medical Center of Ajou University and the School of Medicine at Washington University, St. Louis, USA.

Human combinatorial IgG library construction and phage display

Human combinatorial IgG library construction and phage display procedures were performed as described previously (Jeon et al. 2007), with additional procedures for obtaining RNA from plaques. Total RNA was obtained from atherosclerotic aortic plaques isolated from two atherosclerosis patients during thoracic surgery. The plaques were preserved in RNAlater® (Ambion, USA) at -20°C until use. The plaque tissues were minced with scissors and pulverized using a tissue crusher (Tissue-Tearor®, Biospec Product, USA) in TRIzol reagent (Invitrogen, New Zealand). During the phage display procedure, the monoclonal antibody clone was selected by comparing the reactivity of the phage supernatant to MDA-LDL and native LDL.

Modification of LDL and HDL

Native LDL and high-density lipoprotein (HDL) were purchased from Chemicon International (USA). LDL and HDL were MDA-modified using the method of Fogelman et al. (Fogelman et al. 1980; Choi et al. 1998), oxidized with $CuSO_4$ to produce Cu-oxLDL using the method of Kakutani et al. (2000), acetylated using the method of Basu et al. (1976), and carbamylated using the methods described by Ok et al. (2005). All modifications were confirmed by the different electrophoretic mobilities of the modified lipoproteins (compared to native LDL) on agarose gels (Asci et al. 2008).

Sequencing of F_{ab} V region genes

Phagemid DNA of clones that reacted positively to MDA-LDL in phage enzyme-linked immunosorbent assays (ELISAs) were sequenced in an automatic sequencer (CoreBio Technology, Korea) using the sequencing primers 5′-ACC TAT TGC CTA CGG CAG CCG-3′ (for V_H) and 5′-AAG ACA GCT ATC GCG ATT GCA G-3′ (for V_L). Sequence data were compiled and analyzed using the Basic Local Alignment Search Tool (BLAST) and DNAPLOT (V BASE).

RT-PCR of AID mRNA transcripts

Activation-induced cytidine deaminase (AID) mRNA transcripts were amplified by reverse transcription-polymerase chain reaction (RT-PCR) from cDNA generated from peripheral blood mononuclear cells (PBMCs) and plaque samples obtained from atherosclerosis patients. PCR was performed according to previous protocols (Basu et al. 1976) using the primers 5′-GAG GCA AGA AGA CAC TCT GG-3′ (AID1P), 5′-GTG ACA TTC CTG GAA GTT GC-3′ (AID2P), 5′-TAG ACC CTG GCC GCT GCT ACC-3′ (AID3P), and 5′-CAA AAG GAT GCG CCG AAG CTG TCT GGA G-3′ (AID4P).

Preparation, purification, and immunoblotting of soluble F_{ab} fragment

Preparation, purification, and immunoblotting of soluble F_{ab} fragment were performed as described previously (Jeon et al. 2007).

Analysis of F_{ab} binding by ELISA

In the direct binding ELISA, 100 μl of buffer containing 1 μg antigen was placed in each well of a 96-well microtiter plate for antigen coating and incubated overnight at 4°C. For oxidized lipoproteins, PBS buffer with 20 μM butylated hydroxytoluene (BHT) and 0.27 mM ethylenediaminetetraacetic acid (EDTA) was used. For carbamylated and acetylated lipoproteins, PBS buffer with 0.01% EDTA was used. After washing three times with PBS, the wells were blocked with PBS containing 5% BSA for 2 h at room temperature (RT). Following three washes with PBS containing 0.05% Tween (PBST), the F_{ab} fragment was allowed to react for 2 h at RT. Alkaline phosphatase-conjugated goat anti-human IgG antibody (diluted 1:5000 in PBS/1% BSA) was then added and incubated for 2 h at RT. The wells were then washed twice with PBST, and once with TBS containing 0.05% Tween. The remaining alkaline phosphatase activity was determined spectrophotometrically at 405 nm with an ELISA reader (model EL_X808, DI Biotech, Korea) using *p*-nitrophenyl phosphate as a substrate. In the competitive ELISA, before F_{ab} was incubated with the coated MDA-LDL (30 μg/ml), various concentrations (0, 1, 5, 25 μg/ml) of inhibitors were incubated with F_{ab} for 1 h in the BSA-blocked wells of ELISA plates. After transferring the pre-incubated samples to MDA-LDL coated wells, procedures used were same as those for direct binding ELISA.

Immunofluorescence (IF), immunohistochemical (IHC), and FACS analyses

Damaged parts of tissues from human placental deciduas (obtained at Ajou University Hospital) were isolated for freezing them for IF analysis and single cell separation for FACS analyses at the same time. For IF analyses, frozen tissue sections from the placental deciduas and atherosclerotic aortic plaques (BioChain, USA) were reacted with 0.01 g/L F_{ab} fragment for 2 h at RT, washed to remove unbound ligand, then exposed to 0.02 g/L fluorescein isothiocyanate (FITC)-labeled anti-human IgG (Vector Labs, USA) for 1 h at RT. The staining reaction was analyzed by routine immunofluorescence microscopy using an Olympus fluorescence microscope (model BX-50). For IHC analyses, frozen tissue sections from $ApoE^{-/-}$ mouse aorta bifurcations were reacted with 0.01 g/L F_{ab} fragment, stained with Vectastain ABC kit (Vector Labs, USA), and microscopically imaged with a fluorescence microscope (model BX-61, Olympus, USA).

For single cell separation for identification of macrophages in adjacent areas of placental tissues removed for freezing for IF analyses, samples of placental decidua were washed thoroughly with PBS and minced in a Petri dish. The minced tissue was treated with collagenase and incubated at 37°C in a CO_2 incubator for 3 h. Digested tissue samples were then passed through a macrofiltration mesh (mesh opening, 53–88 μm; Spectrum Labs), and recovered single cells were centrifuged at $300 \times g$ for 10 min at 4°C. The isolated single-cell suspension was admixed into greater than 10 volumes of red blood cell (RBC) lysis buffer (155 mM NH_4Cl, 9.9 mM $KHCO_3$, 99 nM EDTA disodium salt) for 2 h to lyse RBCs, and then re-centrifuged at $300 \times g$ at 4°C. The procedure was repeated until RBCs were removed completely. The recovered single-cell suspension was washed twice with PBS containing 2% fetal bovine serum (FBS). Non-homogeneous single-cell suspensions were dispersed by passing once more through a nylon filter (mesh opening, 53 μm; Spectrum Labs). Single-cell suspensions (1×10^6 cells/tube) were incubated with anti-CD68 mouse antibody (primary antibody) for 15 min, washed

with PBS/2% FBS, and collected by centrifugation at $1000 \times g$ for 10 min (twice), and then incubated with FITC-conjugated anti-mouse IgG (secondary antibody) for 15 min. After washing twice with PBS/2% FBS, the stained cells were analyzed by fluorescence-activated cell sorting (FACS) using a FACSVantage system (BD Biosciences, USA).

Results

Analysis of the genetic structure of the variable regions

The monoclonal anti-oxLDL antibody F_{ab} clone, termed plaque 15,16-46 F_{ab}, was selected by comparing the reactivity of F_{ab} phage libraries to MDA-LDL and native LDL coated on the wells of a 96-well plate. The ELISA signal from MDA-LDL increased over the course of three rounds of panning, whereas the signal from the negative control (BSA) remained low throughout the procedure (data not shown). To analyze the detailed molecular structure of the selected F_{ab} fragment, we determined the V_H and V_L cDNA sequences. The amino acid sequences of the V_H and V_L regions and their analyses are presented in Figure 1 and Table 1, respectively. A comparison of the gene segments utilized by the F_{ab} fragment with their germline counterparts revealed that the V_H and V_L regions utilized V_H3-48 and V_k3-20 genes, respectively. The D region of V_H used the D3-16 gene segment, and the J regions of V_H and V_L utilized J_H4 and J_k1 gene segments, respectively. The complementarity-determining region 3 (CDR3) of V_H and V_L were four and ten amino acids in length, respectively.

The V_H of plaque 15,16-46 F_{ab} was mutated extensively both in CDR and framework regions (FRs) (Figure 1A and Table 1B). The similarity between the V_H sequence and germline gene counterpart was 87.8%, and that of V_L was 96.8%, suggesting the presence of somatic hypermutations. An amino acid-level analysis of the mutational characteristics of V_H and V_L showed that six of the 22 amino acid mutations in CDRs and FRs of V_H involved substitutions to positively charged amino acids (arginine, lysine, or histidine); four of these were changes to arginine. In the CDRs and FRs of V_L, three of the five mutations involved a change to a basic amino acid, one of which was a change to arginine. The ratio of replacement (R) to silent (S) mutations in CDRs of V_H was remarkably high (14/0) compared to that of the framework regions (FRs) of V_H (8/5), the CDRs of V_L (4/0), and the FRs of V_L (1/3) (Table 1B). The R/S ratios of the CDRs of V_H and V_L, which were greater than 2.9, also suggested hypermutation and antigenic selection during the development of B cells. Further evidence of somatic hypermutation was obtained by analyzing the local expression of mRNA transcripts encoding AID in the atherosclerotic plaques from which plaque 15,16-46 F_{ab} was produced (Figure 2). The correct AID RT-PCR products ($\sim$335 base pairs) were observed in both plaque and PBMC samples from the same patients.

Binding selectivity of soluble F_{ab}

To characterize the anti-oxLDL monoclonal antibody F_{ab} fragment at the protein level, we purified soluble F_{ab} from the culture supernatant and periplasmic

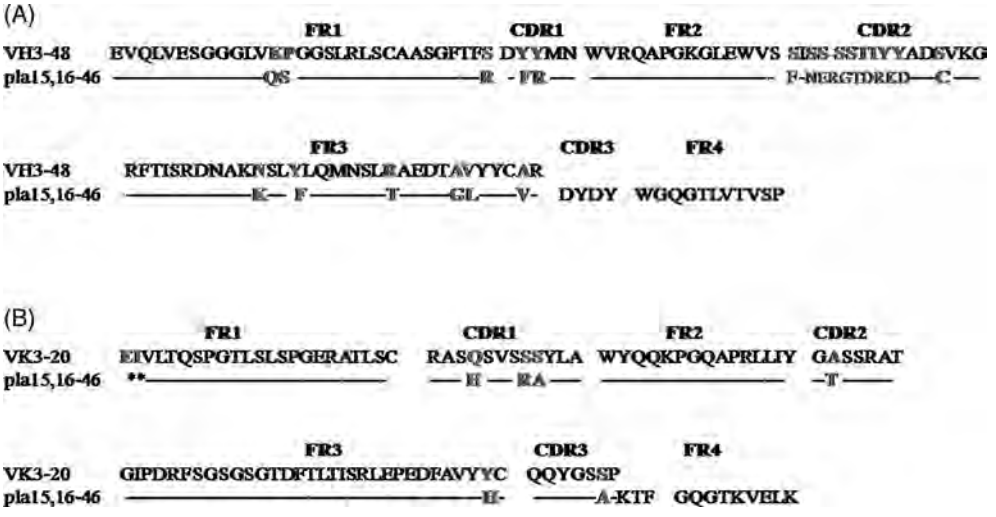

Figure 1. Amino acid sequence comparison of V_H and V_L genes utilized by plaque 15,16-46 F_{ab}. GenBank accession numbers of the V_H and V_L are AY957524 and AY957525, respectively. In each alignment, the top sequence represents the germline gene that displays the highest degree of similarity to the expressed genes. Identical residues are indicated by dashes. The residues that correspond to mutations are denoted in red.

Table 1. Gene usages, similarity to germline sequences, and mutational analysis of V_H and V_L sequences of plaque 15,16-46 F_{ab}

A. Characteristics of V genes used by plaque 15,16-46 F_{ab}.

	V gene	D_H segment	J_H segment	CDR3 length (aa)	DNA similarity to germline (%)
V_H	V_H3-48	D3-16	J_H4	4	87.8
V_L	V_k3-20	-	J_K1	10	96.8

B. Mutational analysis of V_H and V_L sequences.

	Number of mutations										R/S ratio	
	FR1		CDR1		FR2		CDR2		FR3		CDRs	FRs
	R	S	R	S	R	S	R	S	R	S		
V_H	2	0	3	0	0	0	11	0	6	5	14/0	8/5
V_L	0	2	3	0	0	0	1	0	1	1	4/0	1/3

extract after the induction of protein expression. Purified soluble F_{ab} fragments were separated by SDS-PAGE under non-reducing conditions and analyzed by immunoblotting. Purified plaque 15,16-46 F_{ab} specifically reacted with an anti-human IgG (F_{ab}-specific) antibody, exhibiting an apparent molecular weight of $\sim$45 kDa (Figure 3).

The binding properties of plaque 15,16-46 F_{ab} were assessed by ELISA (Figure 4). In direct binding ELISA (Figure 4A), plaque 15,16-46 F_{ab} reacted strongly with both MDA-LDL and Cu-oxLDL forms of oxLDL, and also showed high affinity for cLDL, although this F_{ab} was originally isolated on the basis of its binding to MDA-LDL. No significant reactivity against MDA-HDL, Cu-oxHDL, cHDL, acetylated LDL, acetylated HDL, native LDL, or native HDL was detected. Binding of plaque 15,16-46 F_{ab} to MDA-LDL, Cu-oxLDL, and cLDL was also shown in competitive ELISA (Figure 4B). Binding of plaque 15,16-46 F_{ab} to MDA-LDL was dose-dependently inhibited by pre-incubation of the F_{ab} with various concentrations of MDA-LDL, Cu-LDL, or cLDL. In the case of native LDL or native HDL, the inhibition was not significant. Plaque 15,16-46 F_{ab} also exhibited no affinity for the unrelated antigens, calf thymus double-stranded DNA and histones.

Recognition of atherosclerotic lesions by soluble F_{ab}

The ability of plaque 15,16-46 F_{ab} to recognize atherosclerotic lesions was tested using IF and IHC. Frozen tissue sections of aortas from an atherosclerosis patient were stained with the F_{ab} and analyzed by IF. Whereas negative control without plaque 15,16-46 F_{ab} did not show clear staining of the atherosclerotic plaques (Figure 5A-1), positive staining was shown at both $\times$40 (Figure 5A-2) and $\times$200 (Figure 5A-3) magnifications.

Atherosclerotic lesions from aortas of ApoE$^{-/-}$ mice in which lesion-formation was induced by a high-fat diet were analyzed by IHC using plaque 15,16-46 F_{ab}. Foam cells in atherosclerotic plaques were specifically recognized by plaque 15,16-46 F_{ab}, resulting in purple-colored staining (Figure 5B). Although the staining was not very bright, staining intensity was clearly greater at higher concentrations of plaque 15,16-46 F_{ab} (Figure 5B-3) than at lower concentrations (Figure 5B-2), and both high and low concentrations stained more intensely than the negative control

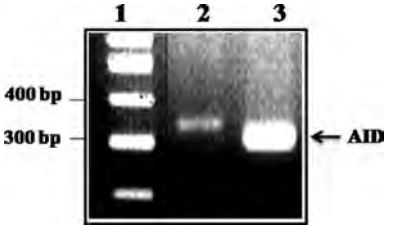

Figure 2. Amplification of AID mRNA transcripts from plaques and PBMCs of patients with atherosclerosis. RT-PCR products were electrophoresed on 1.5% agarose gels. Lane 1, 100-base-pair DNA ladder marker; lane 2, patient plaques; lane 3, patient PBMCs.

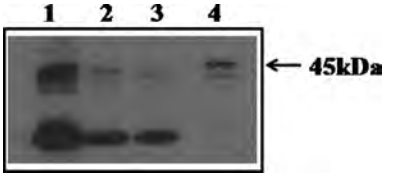

Figure 3. Immunoblotting of purified plaque 15,16-46 F_{ab}. Samples from total bacterial pellet (lane 1), total soluble extract (lane 2), flow-through of soluble extract during application to the column (lane 3), and eluted F_{ab} fragment (lane 4) were separated by SDS-PAGE under non-reducing conditions and then analyzed by immunoblotting with a monoclonal anti-human IgG (F_{ab}-specific).

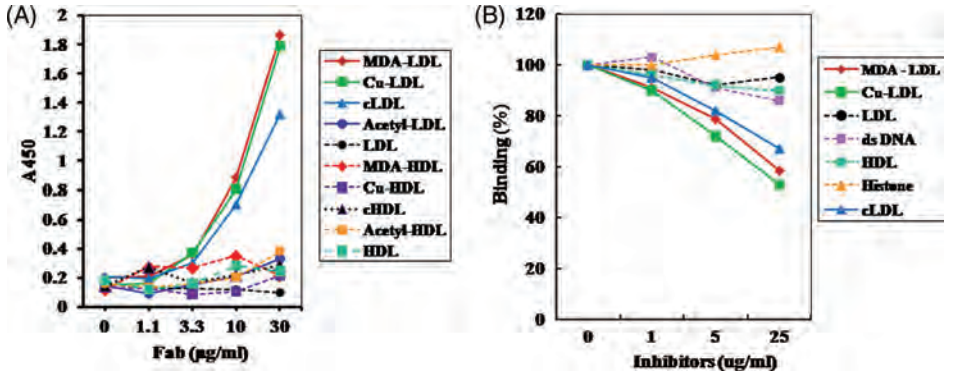

Figure 4. Analysis of plaque 15,16-46 F_{ab} binding to different forms of modified LDLs and HDLs by direct binding (A) or competitive ELISA (B). Antigens were coated on the wells of microtiter plates. After washing and blocking the wells, F_{ab} pre-incubated with inhibitors (for competitive ELISA) or non-incubated F_{ab} (for direct binding ELISA) was allowed to react with coated antigen(s) and then AP-conjugated goat anti-human IgG antibody was added. AP activity on a substrate was determined spectrophotometrically.

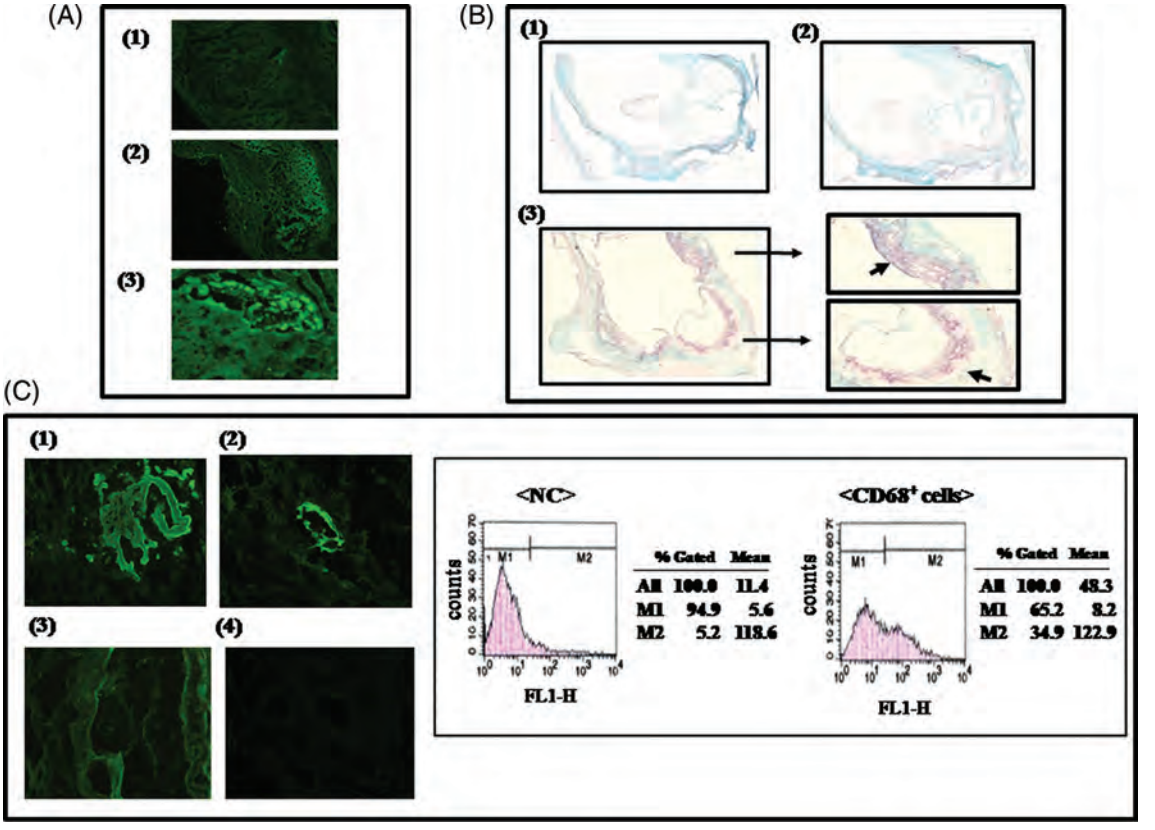

Figure 5. Recognition of atherosclerotic lesions by plaque 15,16-46 F_{ab}. Specific binding to aortic lesions of frozen sections from an atherosclerosis patient (A) and paraffin sections from ApoE$^{-/-}$ mice (B) were analyzed by IF and IHC, respectively. Arterial lesions of placental deciduas from preeclampsia patients was analyzed by IF (C). The fluorescent images were collected at magnifications of $\times 40$ (A1 and A2), $\times 200$ (A3, C1, and C2) or $\times 400$ (C3 and C4). Negative controls (A1, B1, and C4) were performed without plaque 15,16-46 F_{ab} (i.e., secondary antibody only). The boxes in C present a FACS analysis of macrophages using single cells isolated from placental deciduas. Cells stained only with the secondary antibody were used as a negative control (NC). In IHC (B), the colorized images were collected at a magnification of $\times 40$. Thicker boxes represent enlarged, higher-magnification ($\times 100$) images of different areas in B3. Arrows in the thicker boxes indicate atherosclerotic plaques.

(Figure 5B-1). The thicker boxes corresponding to higher-magnification images of two different locations from Figure 5B-3 clearly revealed the presence of atherosclerotic plaques.

One mechanism that might induce preeclampsia is atherogenesis, and atheromatous plaques were indeed found in damaged vascular lesions. We therefore analyzed thin sections of human placental deciduas from a preeclampsia patient by IF using plaque 15,16-46 F_{ab} (Figure 5C). The F_{ab} fragment specifically recognized the damaged atheroma plaques of placental tissue. Several different IF-positive locations were observed at 200 × (Figure 5C-1, -2) and 400 × (Figure 5C-3) magnifications. Interestingly, a cross section of the atherosclerotic blood vessel stained with plaque 15,16-46 F_{ab} clearly revealed a circular shape (Figure 5C-2). The presence of macrophages around the damaged vascular lesion of the tissue was analyzed by flow cytometry using single cells isolated freshly from a portion of the tissues, parts of which were used for the thin-section IF study. As shown in boxes in Figure 5C, the flow cytometric analysis of single cells stained with mouse anti-human CD68 IgG followed by FITC-conjugated anti-mouse IgG revealed that the percentage of CD68-positive cells (M2 in the histogram) was increased (34.9%), compared to the negative control (5.2%). The FACS data provide indirect evidence for the presence of macrophages around the lesion that was also recognized by plaque 15,16-45 F_{ab} in IF.

Discussion

In this study plaque 15,16-46 F_{ab}, an antibody fragment against oxLDL with cross-reactivity to cLDL, was produced from human atherosclerotic plaques. The V_H of plaque 15,16-46 F_{ab} utilized V_H3, which is one of the gene families used frequently by anti-oxLDL antibodies (Jeon et al. 2007). An analysis of the molecular structure of this F_{ab} fragment suggested the occurrence of somatic hypermutations in germinal centers, which are commonly produced during antigenic selection in vivo. Specifically, the variable regions, especially V_H, were highly mutated from germline sequences, and the R/S ratios of CDRs of V_H and V_L were greater than 2.9, which is indicative of antigen-driven selective pressure during the accumulation of mutations. The occurrence of somatic hypermutations was confirmed by analyzing the local expression of mRNA transcripts encoding AID, which is accepted as evidence of somatic hypermutation and class-switch recombination (Coker et al. 2003), in the atherosclerotic plaque from which plaque 15,16-46 F_{ab} was isolated. Only a few studies have reported analyses of the genetic structures and characteristics of human anti-oxLDL antibodies. Somatic hypermutations, however, have been reported in several antibodies, including seven monoclonal antibodies reported previously by our group (Shaw et al. 2001; Jeon et al. 2007). Plaque 15,16-46 F_{ab} can be added to the list of anti-oxLDL antibodies having somatic hypermutations in their variable regions.

Anti-oxLDL antibodies that have many positively charged amino acids in their CDRs, especially in the V_H region, tend to have higher affinities for oxLDL (unpublished data). Plaque 15,16-46 F_{ab} also contained a large number of mutations to positively charged amino acids in the V_H and V_L segments. Among these mutations, arginine was dominant in the V_H of plaque 15,16-46 F_{ab}. Charge-charge interactions between positively charged amino acids in the V_H of plaque 15,16-46 F_{ab} and negative charges of oxLDL might be involved in epitope(s) recognition by the F_{ab} fragment and contribute to the selection of a high-affinity antibody. The present results suggest that somatic hypermutations and an antigen-driven selection process occur in the atherosclerotic plaques during the selection of pathogenic anti-oxLDL-producing B cells.

Several mouse and human antibodies against MDA-LDL are known to block uptake of oxLDL by human and/or mouse macrophages (Hörkkö et al. 1999; Shaw et al. 2001; Torzewski et al. 2004; Jeon et al. 2007), and to reduce the extent of atherosclerosis in mice (Schiopu et al. 2004), suggesting the potential therapeutic application of these antibodies. Combinations of specific antibodies with aggressive chronic medical management, such as statin therapy, could have a role in acute passivation of plaque progression (Apostolov et al. 2009). Increased levels of oxLDL in vivo strongly suggest plaque destabilization and vulnerability as well as coronary artery disease progression (Tsimikas et al. 2003). The levels of cLDL in plasma could also be a biomarker of coronary artery disease, future myocardial infarction and stroke, or uremia (Wang et al. 2007). Therefore, quantification of oxLDL or cLDL, for example using modification-specific, radio-labeled antibodies, might have an important role in biomarker-based identification of disease risk. In the current study, plaque 15,16-46 F_{ab} was analyzed in vitro to explore its potential use in diagnostic applications. Plaque 15,16-46 F_{ab} could distinguish oxidized forms of LDL and cLDL from other forms of LDL and HDL in ELISAs, including native LDL and HDL. IF and IHC analyses showed that plaque 15,16-46 F_{ab} immunostained atherosclerotic plaques, suggesting that this F_{ab} could potentially be applied to detect oxLDL or cLDL in plaques or plasma as an identifier of coronary artery disease progression. Plaque 15,16-46 F_{ab} reacted with atherosclerotic plaques from both humans and mice. There have been

several monoclonal anti-oxLDL antibodies reactive to plaques, some are cross-reactive to human and rabbit atherosclerotic lesions (Itabe et al. 1994; Palinski et al. 1996; Shaw et al. 2001), another to human and mouse lesions similarly to plaque 15,16-46 F_{ab} (Schiopu et al. 2004). Because of its cross-species reactivity between human and mouse, plaque 15,16-46 F_{ab} could be tested in well-defined transgenic mouse models, and the results of such diagnostic studies could be directly translated to a clinical setting. Interestingly, plaque 15,16-46 F_{ab} also immunostained placental tissues from a patient with preeclampsia, a condition whose pathogenesis is not clearly established but which has features in common with atherosclerosis. Although the present study provides evidence for the occurrence of atherosclerotic damage in decidua, further studies (e.g., using impaired arteries from diverse stages of preeclampsia) are required to clearly determine the significance of atherosclerosis in the pathogenesis of the disease.

In conclusion, plaque 15,16-46 F_{ab} is a human-derived F_{ab} fragment specific for oxLDL and cLDL that is cross-reactive with mouse and human biomarkers, but has no cross-reactivity with native lipoproteins. It might be thus poised for potential use as a diagnostic reagent for both human and rodent animal research to detect and characterize atherosclerotic disease progression in plasma or atherosclerotic lesions. A human IgG antibody immunostained atherosclerotic plaques of both apoE$^{-/-}$ mice and human (Schiopu et al. 2004; Goncalves et al. 2009). A previous report described a human monoclonal antibody F_{ab} fragment that immunostained atheromatous plaques in humans and animal models (Shaw et al. 2001). Thus, our investigation is the second account of a human anti-oxLDL monoclonal antibody F_{ab} that could immunostain atherosclerotic lesions of both humans and animals. Notable additional characteristics of plaque 15,16-46 F_{ab} include its cross-reactivity to cLDL and ability to bind placental deciduas from patients with preeclampsia. Further studies on the role(s) of modified LDLs in atherosclerosis and preeclampsia using plaque 15,16-46 F_{ab} are now in progress.

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