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Appearance of cross linked proteins in human atheroma and rat pre-fibrotic liver detected by a new monoclonal antibody

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Abstract

A new monoclonal antibody against malondialdehyde (MDA)-treated low density lipoprotein (LDL) was raised using homogenate of human atheroma as immunogen. This antibody, DLH2, was obtained by selecting the clones which did not react to native LDL but did react to copper-induced oxidized LDL (OxLDL). DLH2 showed a greater reactivity to MDA-LDL than to OxLDL. When LDL was treated with various aldehyde containing reagents, treatment of LDL with glutaraldehyde or MDA greatly increased the reactivity to the antibody, while LDL treated with 2,4-hexadienal or 4-hydroxynonenal was not reactive. Among many proteins tested, high density lipoprotein, bovine serum albumin and hemoglobin showed significant reactivity to DLH2 after they were treated with MDA or glutaraldehyde. When low density and high density lipoproteins treated with MDA were subjected to immunoblot analysis, newly formed products larger than the original apolipoproteins were detected with the antibody, suggesting that this antibody recognizes aggregated proteins with divalent short chain cross linkers. The antigenic materials were shown by immunohistochemical analysis to be present in foamy macrophages in human atheromatous lesions. DLH2 antigen did not colocalize either with apolipoprotein B. Furthermore, we found a massive accumulation of the antigenic material in Kupffer cells in the liver of rats treated with alcohol and carbonyl iron, a model of hepatic fibrosis due to oxidative stress. These results suggest the presence of cross linked proteins in damaged tissues. © 1998 Elsevier Science B.V.

Keywords: Monoclonal antibody; Cross linked protein; MDA-LDL; Atherosclerosis; Fibrosis; Macrophage

Abbreviations: Ab, antibody; mAb, monoclonal antibody; LDL, low density lipoprotein; HDL, high density lipoprotein; OxLDL, oxidized low density lipoprotein; AcLDL, acetylated low density lipoprotein; MDA, malondialdehyde; MDA-LDL, malondialdehyde treated low density lipoprotein; GA, glutaraldehyde; GA-LDL, glutaraldehyde treated low density lipoprotein; HNE, 4-hydroxynonenal; apoB, apolipoprotein B; BSA, bovine serum albumin; ALP, alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay

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1. Introduction

Lipid peroxidation has been thought to be involved in a number of diseases and pathological conditions [1,2]. A number of studies have shown increased levels of lipid peroxidation products in lesions and sera in a variety of pathological conditions including atherosclerosis and alcoholic liver disease [2–8]. Lipid peroxidation products containing aldehyde group, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are known to react to proteins [9–11]. Modification of proteins could be an important factor in the pathogenesis of various diseases through inactivation of enzymes and deformation of structural proteins. For example, accumulation of MDA-modified collagens was demonstrated in nephrotic glomeruli [12].

It is well known that long term intake of alcohol leads to hepatic cell damage and finally to liver cirrhosis. Hepatic fibrogenesis is a complicated process, which has not been fully clarified yet, but induction of cytochrome *P*-450 and lipid peroxidation appeared to be involved in [5,13].

Oxidative modification of low density lipoprotein (LDL) has been extensively studied, since it is thought to be involved in early development of atherosclerosis [2,10]. Modified LDL such as MDA-treated LDL (MDA-LDL) was shown to be taken up by macrophages through scavenger receptors to form foam cells [9,10,14,15], which are typical features observed in atherosclerotic lesions [16]. Presence of anti-MDA-LDL autoantibodies in patients of cardiovascular diseases also suggested the pathological implication of MDA-LDL [17].

Several investigators have suggested the presence of MDA-LDL-like products in atherosclerotic aortas of rabbits [18,19] and other tissues [5,10] using monoclonal antibodies against MDA-lysine adducts. MDA-LDL should be one good model of OxLDL which is a mixture of heterogenously modified lipoprotein particles. However, little has been studied on the structural changes of these modified LDLs as well as correlation between MDA-LDL and OxLDL in vivo. Monoclonal antibodies (mAbs) against certain modified proteins is a useful tool for characterizing their structures and metabolism in vivo. We have obtained a series of mAbs which react with copper-induced OxLDL, using homogenate of human atheroma as immunogen [20]. One of these mAbs, DLH3, was shown to recognize oxidized phosphatidylcholines containing aldehyde groups [21]. In the present study we focused on another mAb in the series which reacted strongly with cross linked proteins. We found the presence of cross linked proteins in damaged tissues especially in foam cells in human atheroma, and in Kupffer cells in pre-fibrotic rat liver.

2. Materials and methods

2.1. Preparation and modification of lipoproteins

LDL and other lipoproteins were separated from human plasma by sodium bromide stepwise density gradient centrifugation [22]. All sodium bromide stock solutions contained 0.25 mM EDTA. The fractions with a density of 1.019–1.063 g/cm^3 and 1.063–1.15 g/cm^3 were pooled as LDL and high density lipoprotein (HDL), respectively. MDA-LDL was prepared by the method of Fogelman et al. [13]. To 1 ml of LDL solution (0.5 mg/ml) in 50 mM phosphate buffer, pH 6.5, was added 5 μl of 1 M MDA reagent (prepared from malondialdehyde bis(dimethyl acetal) (Aldrich, Milwaukee, WI) by acidic hydrolysis just before use), and the mixture was incubated for 3 h at 37°C. The reaction was stopped by extensive dialysis of the reaction mixture against phosphate-buffered saline (PBS) containing 0.25 mM EDTA at 4°C. Modification of LDL with glutaraldehyde (GA), 2,4-hexadienal, and HNE (a kind gift from Dr. Esterbauer of University of Graz, Austria) were carried out using the same procedure as for MDA-LDL. Modification of other proteins with MDA and GA was also carried out using the same method as for MDA-LDL. Prior to oxidation, LDL was passed through a 10DG desalting column (Bio-Rad, Richmond, VA, USA) to remove the EDTA and the LDL (0.2 mg/ml) was oxidized by incubation with 5 μM $CuSO_4$ at 37°C for 3 h. The OxLDL prepared under the condition showed an increase in relative mobility (1.8 + 0.2) on agarose electrophoresis and production of 56.7 + 9.7 nmol of thiobarbituric acid reactive substances (TBARS) per mg protein. The reaction was stopped by addition of EDTA (final concentration 0.25 mM). Glycated LDL was prepared according to the procedure of Bucala et al. [23].

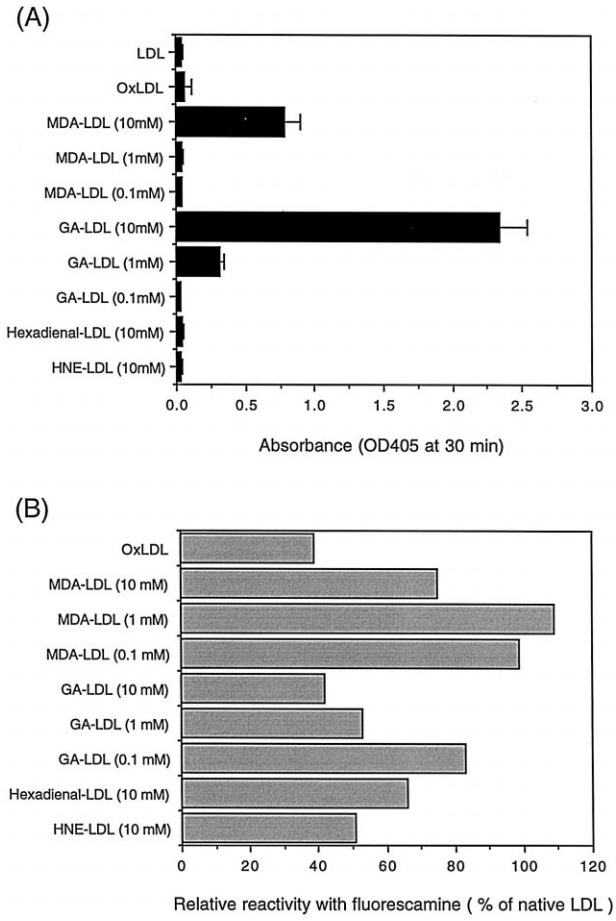


Fig. 1. The reactivity of mAbs DLH2 to modified LDLs. (A) 1 μg each of native LDL and various modified LDL were coated as antigen onto microtiter wells. The ELISA was carried out with DLH2 (1:2000 dilution, closed bar) as described under the Section 2. Values indicated are mean ± standard deviation of three different experiments. (B) The free amino group was determined using a fluorescent dye fluorescamine. The values represent relative amino group concentrations of modified LDL preparations against the original native LDL.

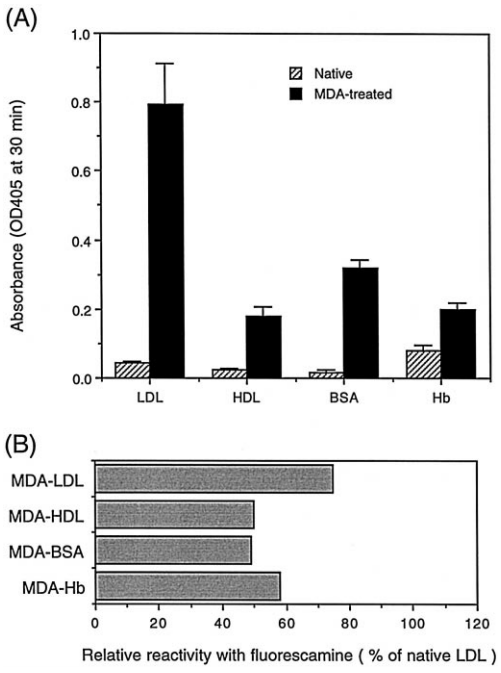
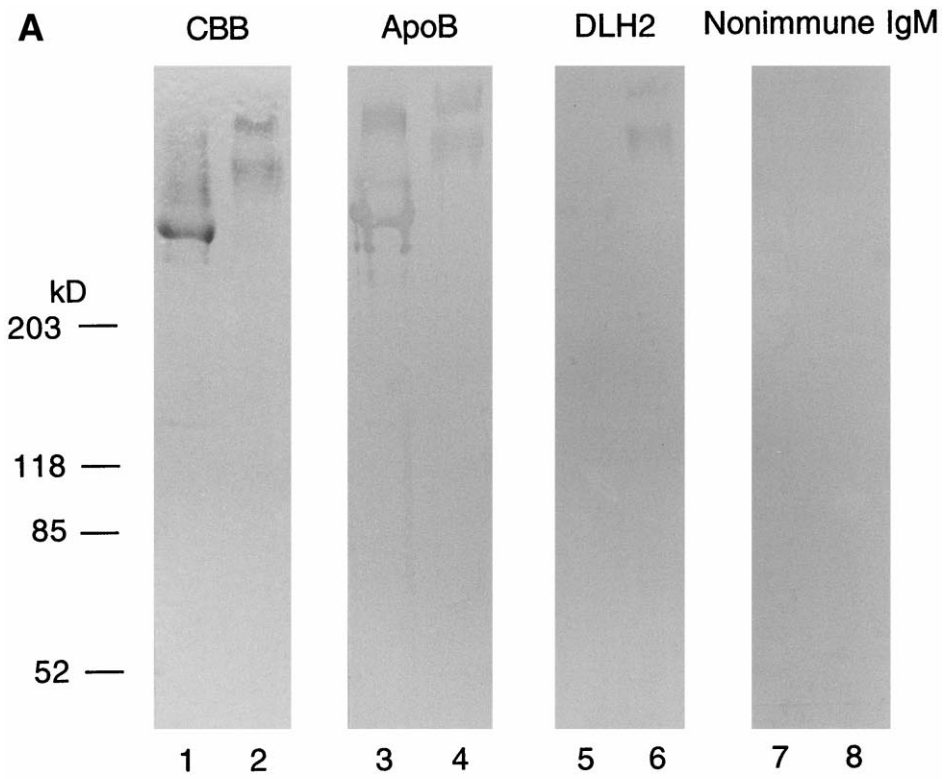


Fig. 2. The reactivity of DLH2 to MDA-modified proteins. (A) Various proteins were treated with 10 mM MDA. 1 μg of MDA-treated (closed bar) and untreated (hatched bar) samples were coated as antigen onto the microtiter wells. The ELISA was carried out with DLH2 (1:2000 dilution). Values indicated are mean ± standard deviation of three different experiments. (B) The free amino group was determined using a fluorescent dye fluorescamine. The values represent relative amino group concentrations of modified LDL preparations against the original native LDL. Hb; bovine hemoglobin.

2.2. Preparation of mAb

The anti-OxLDL mAb DLH2 was prepared as described previously [20]. Briefly, female BALB/c mice were immunized three times with homogenates of human atheromatous plaques of aortas (obtained from autopsy cases, kind gifts from Dr. Numano of Tokyo Medical and Dental University, Tokyo, Japan) over a period of two months. Spleen cells from the immunized mice were fused with P3/U1 murine myeloma cells. Culture supernatants of the hybridoma were screened by enzyme-linked immunosorbent assay (ELISA) using OxLDL and native LDL as antigen (1 μg protein/well). The isotype of DLH2

Fig. 3. Immunoblot analyses of MDA-LDL and MDA-HDL with DLH2. (A) LDL and MDA-LDL (10 μg for lanes 1–6, 20 μg for lane 7, and 30 μg for lane 8) were analyzed by non-reducing SDS-PAGE on a 3–10% gradient gel. Gel was stained with Coomassie brilliant blue (lanes 1 and 2). Nitrocellulose membranes were treated with anti-human apoB Ab (lanes 3 and 4), DLH2 (lanes 5 and 6) or non-immune IgM (lanes 7 and 8), and positive bands were visualized by BCIP-NBT method. Lanes 1, 3, 5 and 7; native LDL, lanes 2, 4, 6 and 8; MDA-HDL. (B) HDL and MDA-HDL (20 μg) were analyzed by non-reducing SDS-PAGE on a 4–15% gradient gel. Gel was stained with Coomassie brilliant blue (lanes 1 and 2). Immunoblot analysis with DLH2 (lanes 3 and 4) or nonimmune IgM (lanes 5 and 6) was visualized using [^{125}I]- protein A. Lanes 1, 3, and 5; native HDL, lanes 2, 4 and 6; MDA-HDL.


A
CBB
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Nonimmune IgM
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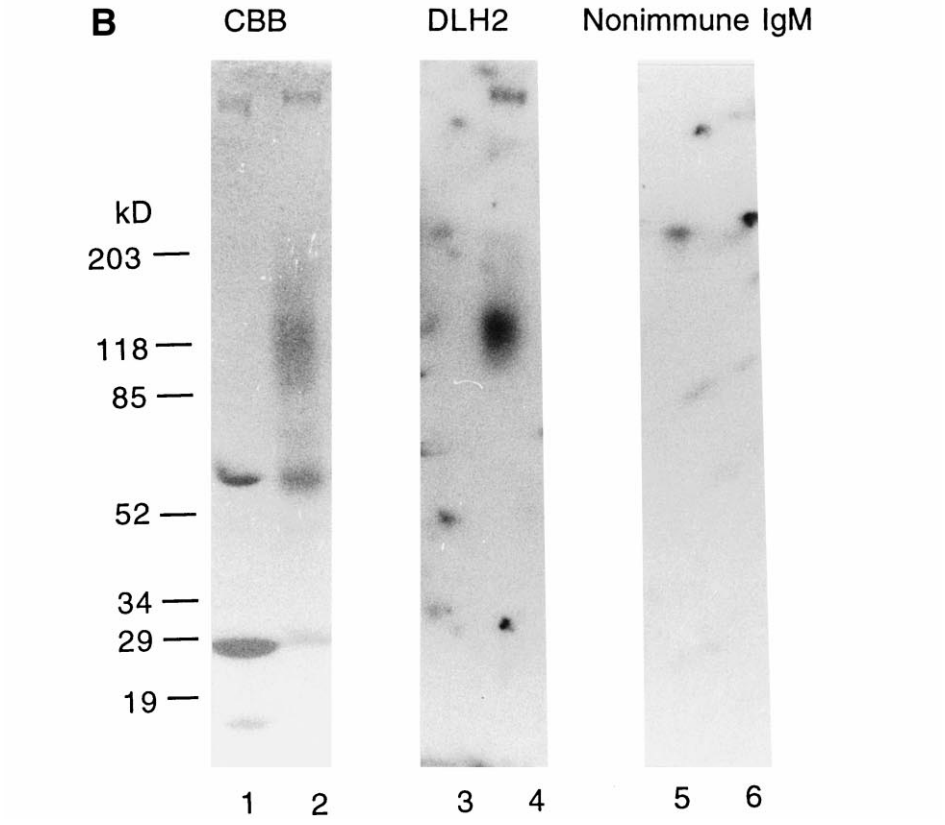
B
CBB
DLH2
Nonimmune IgM
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was IgM with κ-light chain determined using Mouse typer kit (Bio-Rad). Ascites fluids were obtained by injecting hybridomas into mice previously primed with pristane. The mAb was partially purified from the ascites fluid by ammonium sulfate precipitation followed by gel filtration column chromatography on Toyopearl HW-65 (Tosoh, Tokyo, Japan).

2.3. ELISA

Antigens suspended in PBS (1 μg/100 μl) were placed in wells of a 96-well microtiter plate and incubated for 18 h at 4°C. After removing the antigen fluid, the wells were blocked with Tris-buffered saline (TBS) containing 2% skim milk (TBS-skim milk) for 2 h at room temperature. After washing the wells three times with TBS containing 0.05% Tween 20, DLH2 (1:2000 diluted in PBS) was reacted for 2 h at room temperature, followed by an alkaline phosphatase (ALP) conjugated goat anti-mouse Ig(G + M) antibody (Ab) (Tago, Burlingame, CA, U.S.A.) (1:5000 diluted in TBS-skim milk) for 2 h at room temperature. Finally, the remaining ALP activity was determined using *p*-nitrophenylphosphate as substrate. The results were read spectrophotometrically as optical density on an ELISA plate reader (Bio-Rad, model 550) with the filter at 405 nm.

2.4. Immunoblot analysis

LDL (10 μg) and HDL (20 μg) treated with or without MDA were run through 4–15% gradient polyacrylamide gel containing 0.1% SDS. After electrotransfer, the nitrocellulose membranes were incubated with either 30 μg/ml DLH2 or murine IgM, or sheep Ab to human apoB (Binding Site, 1/1000). After incubating with second Abs (rabbit anti-mouse Ig(G + M) Ab, ALP-conjugated goat anti-mouse Ig(G + M) Ab, or ALP-conjugated donkey anti-sheep IgG Ab), the positive bands were visualized either by incubating with coloring reagent containing 5-bromo-4-chloroindoryl phosphate (BCIP) and nitro blue tetrazolium (NBT) according to the method of Blake et al. [24] or by incubating with 0.1 μCi/ml of [^{125}I]-labeled protein A for 30 min followed by an autoradiogram taken by the method of Small et al. [25].

2.5. Induction of liver fibrosis in rats

Male Wistar rats (140–150 g) were pair-fed *ad libitum* for 12 weeks with a nutritionally adequate liquid high fat diet containing ethanol (36% of the total caloric intake) or isocaloric carbohydrate with or without dietary carbonyl iron (0.5% w/v) [26]. The intake volumes the rats pair-fed isocalorically were 70–150 ml/day. Dietary carbonyl iron (FERRONYL) was purchased from ISP Chemicals, Tokyo, Japan. At the end of the experiments, animals were sacrificed and immunohistochemical analysis of the livers was carried out. All animals were humanely treated according to NIH guide for the care and use of laboratory animals.

2.6. Immunohistochemical analysis

Serial sections of human abdominal aortic tissue and rat liver were provided for indirect immunohistochemical staining. Briefly, acetone-treated (10 min) thin sections were washed in PBS with agitation, and then incubated with PBS containing 5% bovine serum albumin (BSA) for 10 min. The first Abs used were DLH2, HAM56 (Dako) which recognizes human macrophages, anti-human apoB (Chemicon), and Mar1 (Seikagaku, Japan) which recognizes rat macrophages and Kupffer cells [27]. The sections were incubated with the diluted first Abs overnight at 4°C. Negative control experiments were carried out either by using non-immune murine IgM (Zymed Lab) or omitting first Ab. After agitation-washing with PBS, the sections were incubated with ALP-conjugated second Abs against mouse or sheep immunoglobulins (Dako) for 30 min followed by incubation with ALP-conjugated anti-ALP Ab. Sections were then washed with PBS and stained with 0.2 M Tris–HCl buffer (pH 8.2) containing 0.01% new fucshin (Merck), 0.01% $NaNO_2$, 10 mg naphtol AS-BI phosphate (Sigma), 0.1 ml *N*,*N*-dimethyl formamide (Wako, Osaka) and 10 mM levamisol (Sigma). Sections were then fixed with 10% formalin, and counter-stained with hematoxylin.

2.7. Other analytical methods

Protein concentration was measured by the bicinchoninic acid (BCA) method [28]. Free amino groups were measured using fluorescamine as a probe [29].

3. Results

3.1. Reactivity of DLH2 to chemically modified LDLs

The reactivity of mAb DLH2, which was raised using homogenate of human atheroma as immunogen, to variously modified and untreated LDLs was tested. In the ELISA assay, 1 μg each of these samples was precoated onto plastic ELISA plates to test the reactivity with the mAb. DLH2 reacted to MDA-LDL and GA-LDL effectively, while native LDL was inert (Fig. 1A). It reacted little to OxLDL under this experimental condition, although a significant reactivity was observed at a higher concentration of DLH2 (1/500 dilution). The reactivity of DLH2 to OxLDL did not change by prolonged incubation of LDL with $CuSO_4$ up to 24 h (data not shown). When LDL was treated with lower concentrations of MDA or GA, the antigenicity of GA-LDL or MDA-LDL reduced significantly and it was almost diminished by reducing the concentration of the reagents added to LDL down to 0.1 mM. The loss of free amino group was minimal in LDLs treated with 0.1 mM of MDA or GA (Fig. 1B). The mAb, however, did not react to LDL treated with HNE or 2,4-hexadienal, even when 10 mM of the reagents were used. The HNE- and 2,4-hexadienal-treated LDL preparations lost more amino groups than MDA-LDL. Chemical modification of LDL did not affect the amounts adsorbed onto the plastic surface of microtiter wells in this ELISA assay (data not shown). In a separate experiment, DLH2 showed no reactivity to glycated LDL (remaining free amino group 51%) (data not shown). These results suggest that modification of LDL by divalent reagents could be antigenic, but the modification of LDL with aldehyde reagents is not enough.

3.2. Reactivity of DLH2 to cross linked proteins

The specificity of the mAb against apoB moiety of modified LDL was investigated by treatment of various proteins with MDA. As shown in Fig. 2, HDL, BSA and bovine hemoglobin as well as LDL were positive after the treatment. MDA-LDL showed the strongest reactivity, although the loss of free amino groups in the MDA-treated LDL proteins was slightly less than the others. Other proteins tested, such as lysozyme, ovalbumin, and collagen were not reactive with DLH2 by the ELISA assay when they were treated with MDA (data not shown). Poor solubility (modified collagen and ovalbumin), or poor reactivity to the aldehyde cross linkers (lysozyme) might cause the low reactivity. It seems likely that DLH2 is not specific for apoB but requires certain physicochemical properties of proteins to be modified.

To further investigate the reactivity of DLH2 to cross linked proteins, immunoblot analyses of MDA-LDL and MDA-HDL were carried out (Fig. 3). Treatment of LDL with MDA reduced its electromobility suggesting the formation of cross linked apoB proteins (Fig. 3B), lanes 2 and 4). DLH2 detected the MDA-LDL (10 μg) whilst non-immune murine IgM failed to detect MDA-LDL even though 30 μg of antigen were used (Fig. 3A, lanes 6 and 8). After treatment of HDL with MDA, a smeared band ranging from 90–140 kDa appeared which corresponds to cross linked proteins (Fig. 3B, lane 2). The smeared band was positive when the transfer membrane was treated with DLH2 (Fig. 3B, lane 4). This HDL preparation was not highly purified so that it still contained small amount of albumin and apoB. However, it is noteworthy that the apolipoprotein AI and albumin remained at the original sizes in the MDA-HDL preparation were not reactive to DLH2, although they were possibly modified by MDA. A small band at 500 kDa was also detected by DLH2. Since the 500 kDa band was slightly larger than apoB present in the HDL preparation, it may be apoB cross linked with smaller proteins. A weak band at 240 kDa was observed on the native HDL, but this band was also detected with non-immune IgM (Fig. 3B, lanes 3 and 5). Proteolytic treatment of MDA-LDL or GA-LDL with trypsin or V8 protease abolished their reactivity to DLH2 (data not shown). These results suggest that DLH2 recognizes cross linked proteins, but it is not specific for modified apoB.

3.3. Distribution of DLH2 antigen in human atheromatous lesions

Surgical specimens of aorta were freshly obtained from patients (67 year old, female, and 60 year old, male) receiving an aneurysm resection of abdominal aorta. Serial thin sections of the aortic tissue were subjected to immunohistochemical analysis to determine the localization of DLH2 antigens in athero-

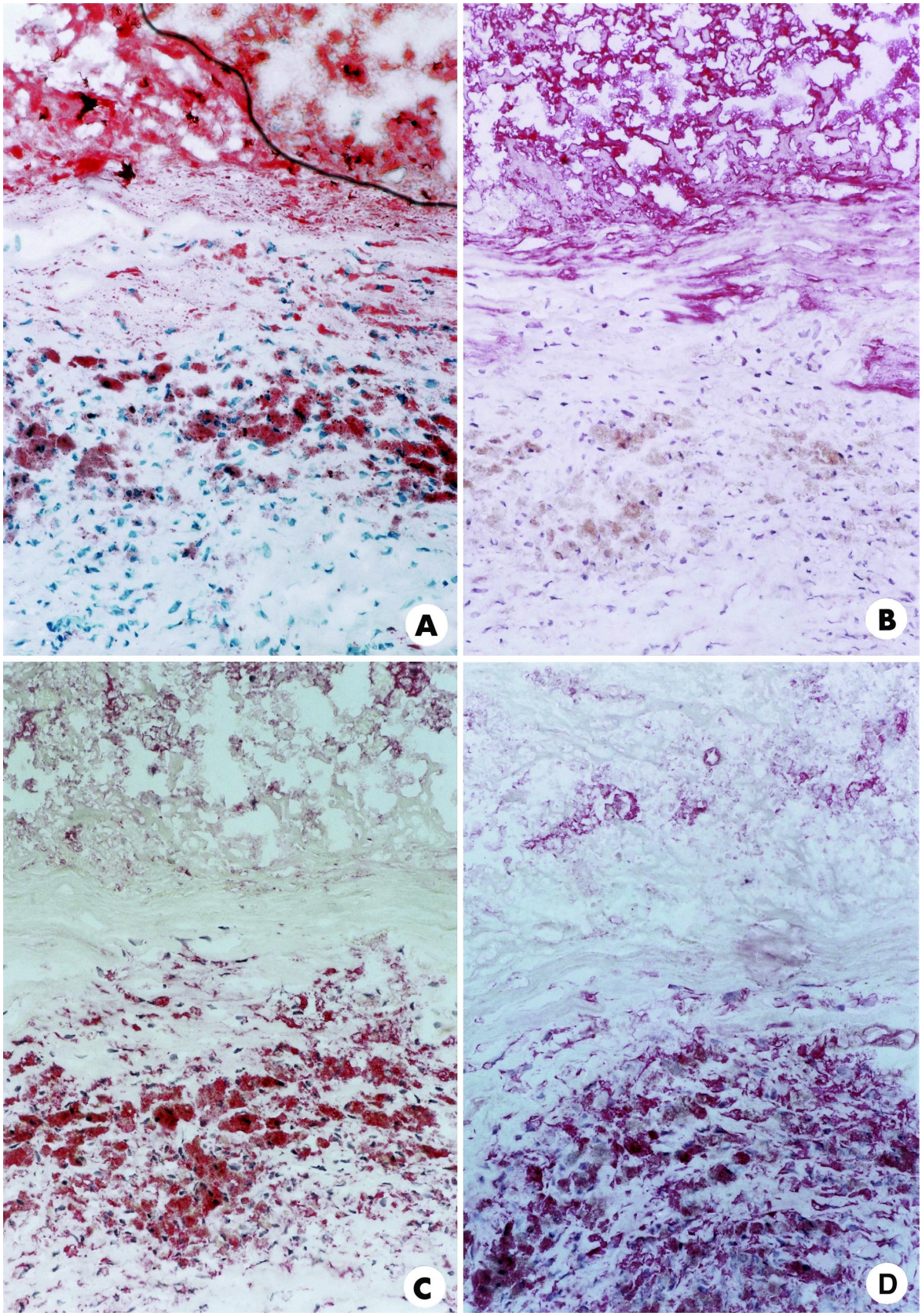

Fig. 4.

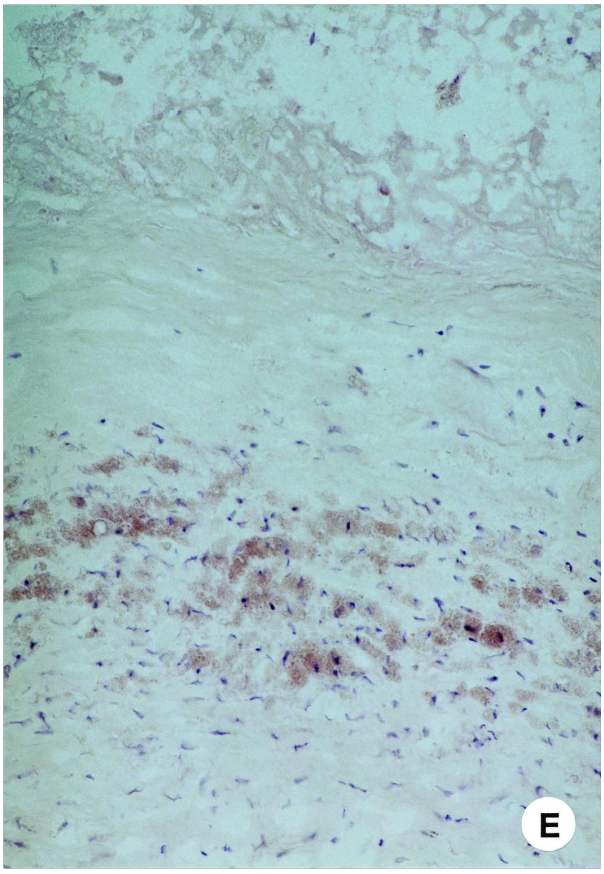

Fig. 4. Immunohistochemical detection of DLH2 antigen in human atherosclerotic lesion in a patient with abdominal aneurysm (67 year old, female). Serial sections of atheromatous lesion in the aorta were stained with Oil Red-O (A), anti-human apoB (B), HAM56 (C), DLH2 (D), or without first Ab (E) (magnification: ×50). Large amounts of Oil Red-O positive lipids and apoB were deposited in necrotic core (upper part). Many infiltrating macrophages were observed at the edge of the core. DLH2 antigen was primarily detected in the foamy macrophages, while apoB was detected diffusely in collagenous matrix but not in the cells.

sclerotic lesions. DLH2 was found in necrotic core (Fig. 4D), where HAM56 positive materials colocalized (Fig. 4C). While apoB (Fig. 4B) and neutral lipids (Fig. 4A) were diffusely accumulated in necrotic lesion. DLH2 antigen was found intracellularly in neutral lipid-laden foam cells (Fig. 4A, C and D). ApoB, however, was found to be deposited in collagenous matrix but not in the cells (Fig. 4B). Fig. 5 shows a vaso vasorum in atheromatous lesion. This small artery shows mild fibromascular intimal thickening. Although no lipid accumulation was detected in this artery (Fig. 5A), both DLH2 antigen and apoB were clearly observed in the endothelial layer (Fig. 5B and C). No staining was observed when either the first Ab was omitted (Fig. 4E and Fig. 5D or non-immune murine IgM was used (data not shown). The staining was abolished by addition of MDA-LDL to the DLH2 antigen solution incubating with the histochemical specimens, whilst either native LDL or OxLDL did not reduce the staining (data not shown). These results indicate that the presence of DLH2 antigen(s) in human atherosclerotic lesions. Furthermore it is interesting to note that the localization of DLH2 does not correspond to that of apoB.

3.4. Appearance of the antigen in Kupffer cells in pre-hepatic fibrosis

It has been known that lipid peroxidation is involved in progressive liver injury from fatty liver to cirrhosis. We used an established animal model in which hepatic fibrosis is induced by feeding a high fat diet containing alcohol and carbonyl iron [22]. The liver of rats fed alcohol together with iron in the present study, showed a pre-fibrosis state which is characterized by normohepatic tissue with mild bile retention. DLH2 antigen clearly accumulated in an enlarged cellular cytoplasm (Fig. 6B). The DLH2 positive cells, which located near the portal areas of the liver in rats treated with alcohol and iron, were identified as Kupffer cells by Mar1 antibody (data not shown). DLH2 antigen in the rats treated with alcohol alone had a scattered distribution in Kupffer cells and the size of the positive cells was markedly smaller than that in the rats treated with alcohol and iron (Fig. 6A). Almost no cell was stained in the liver of control rats. When an anti-human apoB Ab was used for reference, apoB seemed to be localized along the sinusoid, but not in the Kupffer cells where DLH2 antigen was accumulated (data not shown).

4. Discussion

The new mAb DLH2 obtained in this study has appeared to react strongly to cross linked proteins.

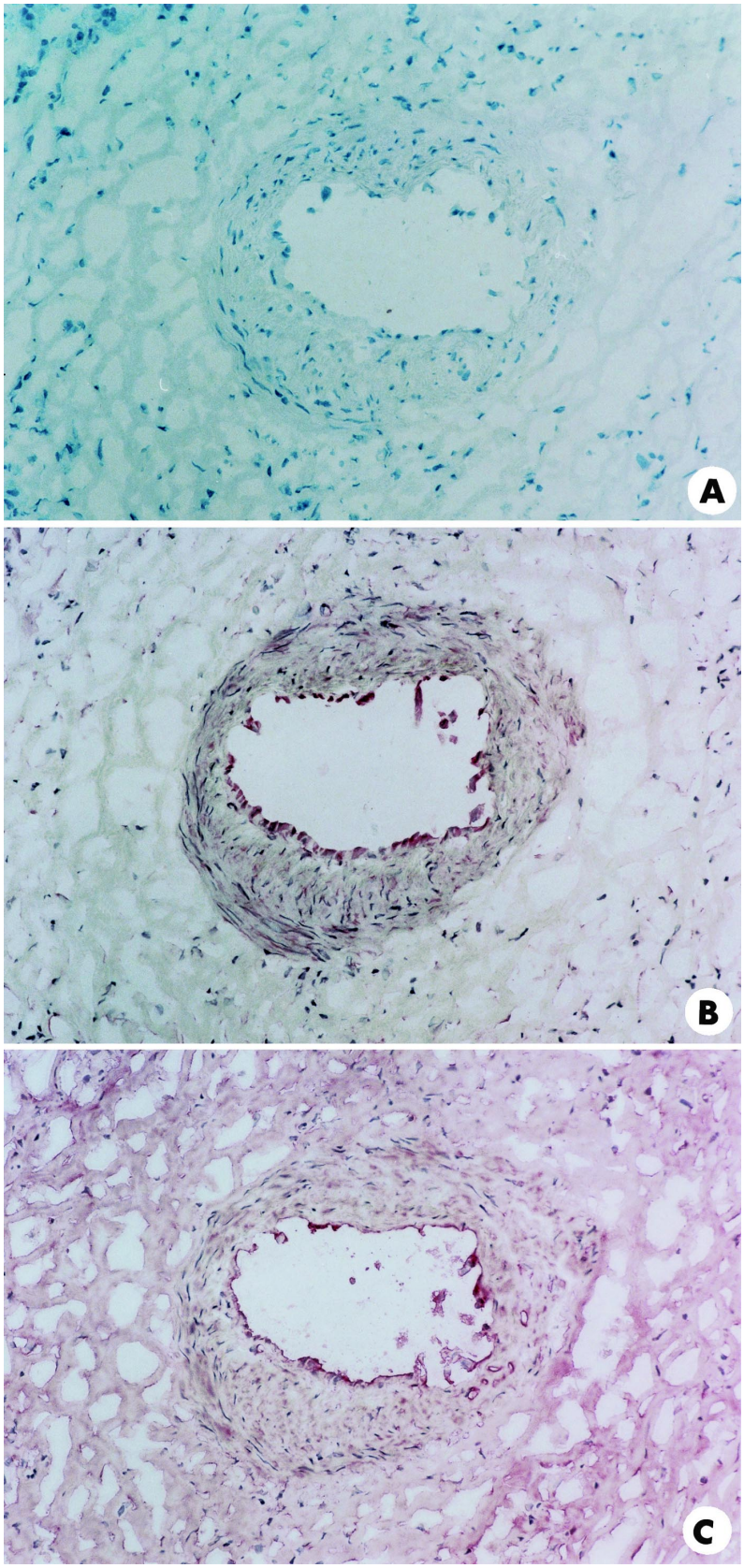

Fig. 5.

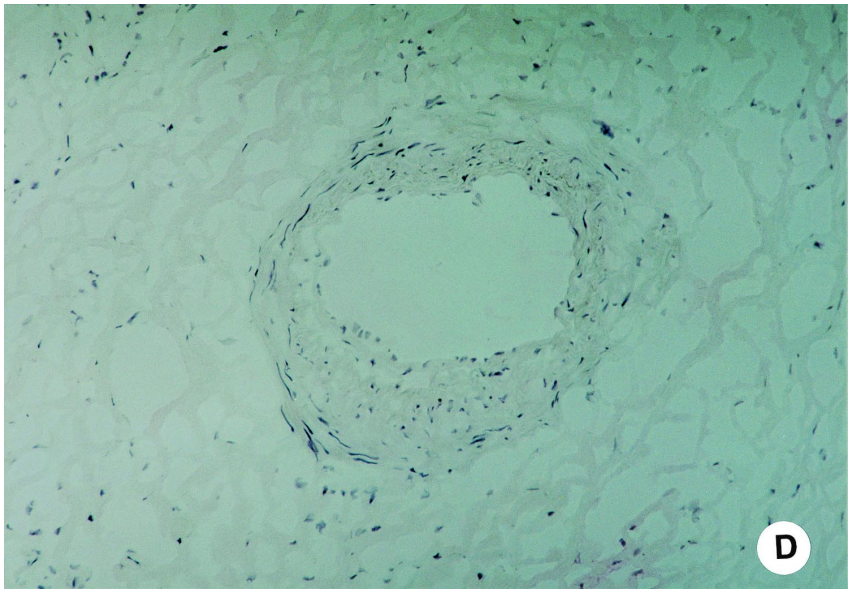

Fig. 5. Immunohistochemical detection of DLH2 antigen in a small artery with mild intimal thickening in a patient with abdominal aneurysm (60 year old, male). Serial sections of the artery were stained with Oil Red-O (A), DLH2 (B), anti-human apoB (C), or without first Ab (D) (magnification: ×50). Although no lipid was deposited in the artery, DLH2 antigen was detected in endothelial layer, where apoB was also deposited.

Firstly, DLH2 was highly reactive to MDA-LDL and GA-LDL while HNE-LDL and 2,4-hexadienal-LDL failed. Secondly, cross linked products formed in MDA-LDL and MDA-HDL preparations were detected by immunoblot analysis. A unique feature of this antibody is to recognize cross linked proteins, not merely MDA adducts. However, this mAb is not specific for apoB protein, therefore certain protein structure or peptide motif is likely to be an additional important factor in antigen recognition.

Using this mAb we demonstrated the presence of cross linked proteins in human atheroma. The distribution of DLH2 antigen corresponded well to macrophage-derived foam cells. It should be noted that the distribution of DLH2 antigen did not correspond to that of apoB protein in atheromatous lesions and in fibrotic liver. One possibility is that the antigen(s) in the lesions could be derived from certain materials other than LDL. Accumulation of MDA-modified collagen in the glomeruli in rats with nephrotic syndrome was reported [12]. Another possibility is that lysosomal degradation of modified LDL in macrophages decreases the immunoreactivity of apoB moiety of the modified LDL. Lysosomal degradation of [^{125}I]-labeled OxLDL has been shown to be less efficient than that of native LDL, and the degradation of modified LDLs was suggested to depend on cross linking and types of reagents [30,31].

Several other groups have obtained anti-MDA-lysine mAbs [18,19]. Antigens detected by the anti-MDA-lysine mAbs showed cell associated as well as extracellular distributions in atherosclerotic lesions of WHHL rabbit and balloon-injured rabbit aortas [18,19]. Palinski et al. [32] reported that antigens detected by anti-MDA-lysine and anti-HNE antibodies were observed in macrophages in early lesions, but they were also present in extracellular spaces in advanced lesions of atheromatous aorta in apolipoprotein E knockout mice. These results together with ours suggest that the possible importance of protein modification with MDA in atherosclerosis, and that a part of MDA-modified proteins previously detected in pathological conditions may be cross linked proteins. There are some difference in the antigen distributions detected by these antibodies in atherosclerotic lesions. DLH2 is likely to have different determinants of antigen recognition from the anti-MDA-lysine mAbs, since DLH2 is not specific for MDA adducts

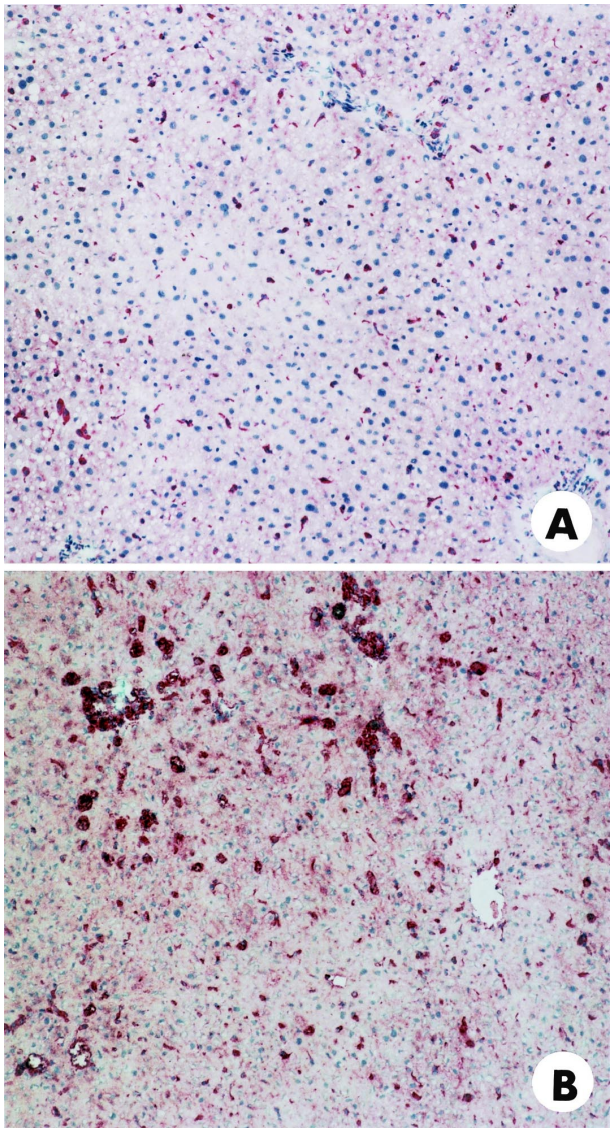

Fig. 6. Immunohistochemical detection of DLH2 antigens in rats treated with alcohol and carbonyl iron. The liver in rats fed with alcohol alone (A) or alcohol and carbonyl iron (B) was stained with DLH2 (magnification: ×25). DLH2 antigen was markedly accumulated in an enlarged cellular cytoplasm of Kupffer cells in rats fed with alcohol and iron, while rats treated with alcohol alone accumulated DLH2 antigen to a less extent.

but is reactive to modified proteins cross linked by divalent aldehydes including MDA.

Our present study also demonstrated that there is massive accumulation of DLH2 antigen in Kupffer cells of rats treated with alcohol and iron. Involvement of lipid peroxidation in this alcoholic liver disease model has been shown by increased levels of thiobarbituric acid reactive substances and HNE [5,13]. Early hepatic damage induced by alcohol and iron may induce oxidative modification of various components in addition to LDL. The possibility that the proteins are modified in extra hepatic tissues cannot be ruled out, since DLH2 antigen did not accumulate significantly in liver parenchyme, including hepatocytes. Oxidatively modified LDL is effectively incorporated in Kupffer cells presumably to remove it from blood stream, and Kupffer cells have been shown to incorporate modified LDLs when injected intravenously [33]. Cross linked proteins may also be effectively incorporated by Kupffer cells. Polyclonal antisera raised against MDA-LDL and HNE-LDL, however, stained liver parenchyme of rats treated with alcohol and iron in our previous study [5]. These results suggest the possibility that structurally different modified proteins could have metabolic differences as well.

DLH2 antigen in human atheromatous aorta was localized to foam cells. The apparent distribution of DLH2 antigen in pre-fibrotic rat liver seemed similar to that of aorta, since it was found primarily in macrophages or Kupffer cells. On the other hand, DLH2 antigen was also detected in endothelial layer of small artery which did not have atherosclerosis. The antigenic materials may localize differently in early stages of lesion formation and in advanced necrotic lesions. Macrophage-like cells in a variety of tissues including Kupffer cells express trimeric scavenger receptor, SR-AI and II [34], which may be responsible for the accumulation of DLH2 antigen in atheromatous aorta and in prefibrotic liver. However, there are more than one, possibly several different types of receptors for modified LDL on macrophages [33–37]. Although all of those are capable of incorporating OxLDL and various modified proteins, they may have different specificity for cross linked proteins and different ability to metabolize them.

In conclusion, using a unique mAb we found cell-associated appearance of cross linked proteins in damaged tissues in which lipid peroxidation is thought to be involved. More work is needed to understand how the DLH2 antigen accumulates in injured tissues. In future studies we intend to further investigate the metabolism and pathological involvement of oxidative modification of LDL by tagging different parts of those particles with DLH2 together with other mAbs of the series.

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