the viral genome important for replication of the virus. In contrast, the biological properties of the hybrid virus we described are solely the result of surface properties of the virion and therefore are believed to influence steps of virus entry. This conclusion is based on the observation that a recombinant in which the PV-1(M) capsid was exchanged with that of PV-2(L) is as neurovirulent in mice as PV-2(L) and that PV-1(M) RNA replicates efficiently after transfection into cultured mouse cells (*24*). The W1/2–1D-1 hybrid virus, in addition to an altered antigenic phenotype, also expresses a change of host range. It is likely that multiple amino acid replacements in the highly exposed N-AgI loop have occurred during the evolution of the Picornaviridae. Our results suggest that such subtle changes of the virion capsid may in some cases have led to the emergence of new etiological agents with altered tissue tropism, perhaps explaining in part why human enteroviruses and rhinoviruses cause such a bewildering array of different disease syndromes in humans.

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Malondialdehyde-Altered Protein Occurs in Atheroma of Watanabe Heritable Hyperlipidemic Rabbits

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It has been proposed that chemically reactive lipids released during lipid peroxidation convert low density lipoprotein (LDL), the major carrier of plasma cholesterol, to an abnormal form and that receptor-mediated clearance of this altered LDL produces cholesteryl ester deposition in macrophage-derived foam cells of atheroma. Immunocytochemical analyses now reveal the presence of protein modified by malondialdehyde, a peroxidative end product, which colocalizes with the extracellular deposition of apolipoprotein B-100 protein of LDL in atheroma from Watanabe heritable hyperlipidemic rabbits. These findings provide direct evidence for the existence in vivo of protein modified by a physiological product of lipid peroxidation within arterial lesions.

THE SEQUENCE OF CELLULAR changes in the progression of atherosclerosis has now been elucidated through morphologic studies (*1*). The biochemical mechanisms leading to the formation of macrophage-derived foam cells, the major histological markers of this disease, are still unknown. It has been proposed that alteration of low density lipoprotein (LDL) produces clearance by subendothelial macrophages and results in intracellular deposition of lipoprotein-derived cholesterol (*2, 3*). Studies in vitro have implicated modification of LDL by lipid peroxide products as one potential mechanism (*3–5*). Malondialdehyde (MDA) is one end product of lipid peroxidation (*6*). Reaction of MDA with a critical number of lysine residues of the apolipoprotein B (apoB)–100 protein of LDL produces internalization by the scavenger receptor of human monocyte-macrophages and the subsequent intracellular accumulation of lipoprotein-derived cholesteryl ester in vitro (*3, 7, 8*). Whether modification of LDL by MDA or other lipid peroxides occurs in vivo as a prerequisite to the formation of arterial foam cells has yet to be demonstrated.

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One approach to resolving this issue is through study of agents that prevent lipid peroxidation. Recent studies have shown that probucol, an antioxidant, retards atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits (*9*). WHHL rabbits, an animal model of familial hypercholesterolemia, are homozygous for a mutant allele in the LDL receptor and develop spontaneous, premature atherosclerosis (*10*). A second approach is through identification in situ of LDL modified by lipid peroxides. In our studies we have used MDA as an index of lipid peroxidation. We now report the immunocytochemical identification of MDA-altered protein and colocalization with the apoB-100 protein of LDL in atheroma of WHHL rabbits. By contrast, there is no evidence of MDA derivatization of plasma LDL from WHHL rabbits or of protein in arterial walls from normolipidemic New Zealand White (NZW) rabbits. These findings provide direct evidence that protein modification by a physiological product of lipid peroxidation occurs in vivo during both early and late stages of lesion formation.

MDA-altered protein was detected by the monoclonal antibody (mAb) designated MDAlys, an immunoglobulin G2a (IgG2a) produced by a murine myeloma (*11*). The antibody, generated by established procedures (*11–14*), was selected for ability to bind to immobilized MDA-LDL, but not to

native human LDL, in solid phase radioimmunoassay (RIA) (*12, 13*). Previous chemical analyses have shown that the predominant derivative formed by reaction of MDA with LDL is 1-amino-3-iminopropene, a conjugated imine produced by intramolecular cross-linking of peptidyl lysines by MDA in a 2:1 molar ratio (*7, 15*). Antibody binding was dependent on antigen valency (*16*), that is, the density of MDA-derivatized lysine epitopes per particle of LDL (Fig. 1), and demonstrated specificity for LDL selectively modified by MDA (Fig. 2A). Copper oxidation of LDL, which produces alteration of the lysine residues of apoB protein by unidentified, reactive lipid products (*17*) and other changes in LDL (*4, 5*), failed to inhibit antibody binding (Fig. 2A); these results are consistent with the absence or low levels of thiobarbituric acid–reactive substances covalently bound to LDL after transition metal–dependent oxidation (*4, 5, 17, 18*).

MDA derivatization of irrelevant protein also produced competitive inhibitors of antibody binding to either human MDA_{11}-LDL (Fig. 2B) or WHHL rabbit MDA_8-LDL (*18*). Molecules tested included human serum albumin and transferrin (Fig. 2B), or human IgG, high density lipoprotein (HDL), very low density lipoprotein (VLDL), as well as chicken egg ovalbumin, WHHL rabbit VLDL, WHHL rabbit HDL, and poly-L-lysine (*18*). Immunospecificity was confirmed by ability of mAb MDAlys to bind to each of the MDA-modified proteins, but not to the parent molecules, by direct solid-phase radioimmunoassay (RIA) (*18*).

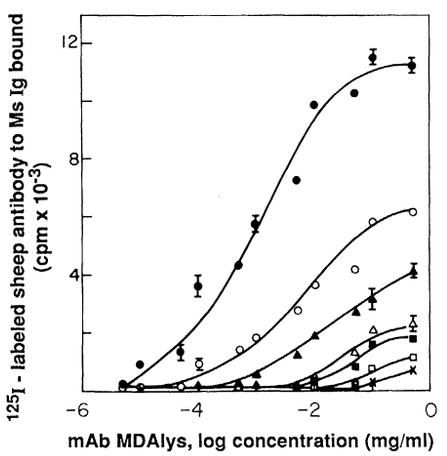

Fig. 1. Solid-phase RIA for binding of mAb MDAlys to human LDL that had been progressively modified by MDA. Each microtiter well contained 38 ng of bound antigen. Ascites fluid was added in 50 μl at the total concentrations indicated and incubated for 18 hours at 4°C. Antibody binding was detected by a second incubation with ^{125}I-labeled sheep antibody to mouse (Ms) Ig (Amersham, 0.8 μCi/ml) for 6 hours at 37°C. Nomenclature utilized is MDA_{zz}-LDL, where zz represents the number of moles of MDA bound per mole of LDL determined by colorimetric assays (*7*). Each point is the average of two values ± SEM for those points in which SEM exceeded 6% of the value determined for each average. Data shown are from a single experiment and have been reproduced in similar experiments conducted with native and modified LDL from individual human subjects (n = 3) or from individual WHHL rabbits (n = 2). ●, MDA_{90}-LDL; ○, MDA_{20}-LDL; ▲, MDA_{14}-LDL; △, MDA_8-LDL; ■, MDA_6-LDL; □, MDA_4-LDL; x, native-LDL.

The apoB-100 protein was detected by the IgG2a murine mAb MB47 (*19*). Monoclonal antibody MB47 selectively binds to LDL from numerous species, including human and rabbit, and cross-reacts with LDL modified by lysine-specific reagents (*19*). The epitope that produces antibody binding has been localized to residues 3350 to 3506 of the apoB-100 protein (*19*).

The presence of MDA-modified LDL was first tested in plasma. LDL was isolated at density (*d*) of 1.019 to 1.063 g/cm^3 (*7*) from the plasma of WHHL rabbits (n = 2) in the presence of 1 m*M* butylated hydroxytoluene and 0.01% EDTA, *p*H 7.4. Samples containing 1 to 10 mg of LDL protein were subjected to heparin-Sepharose affinity chromatography to separate potentially modified, anionic LDL from native LDL (*18, 20*). Lipoprotein from each fraction was tested for ability to bind mAb MDAlys. There was no evidence for MDA-modified LDL, at an estimated sensitivity level of 10 ng (*20*), in as much as 10 mg of plasma LDL.

Grossly visible lesions near intracostal junctions from thoracic aorta of WHHL rabbits were selected for immunocytochemical analyses. A protocol allowing preparation of fresh-frozen samples in antioxidants within 10 minutes after surgical removal was selected to avoid peroxidation of arterial lipids (*21, 22*). Cryostat sections were fixed in buffered formalin (*21*) and probed at 4°C with primary murine mAb in phosphate-buffered saline containing 0.01% EDTA, *p*H 7.4. Detection of primary antibody was accomplished by fluorescein isothiocyanate–conjugated horse antibody to mouse Ig (Becton Dickinson) (30 minutes) or an avidin-biotin complex (ABC) immunoperoxidase method (16 hours) (*21, 23*); each method gave the same results. Three representative lesions from a total of ten subjected to analyses demonstrate stages of lesion development classified by morphology as fatty streak (Fig. 3, A to C), early fibrous plaque (Fig. 3, D to F), and advanced fibrous plaque (Fig. 3, G to I).

Immunostaining of apoB-100 protein in the fatty streak occurred as broad bands within the extracellular matrix of the intimal lesion and as thin striations along the elastic fibers of the media (Fig. 3A). In the early and advanced fibrous plaques, dense foci of apoB-100 protein localized in the middle and deep to the intimal lesion (Fig. 3, D and G, respectively). This distribution is similar to the immunolocalization of apoB protein by polyclonal antisera described in arterial lesions from both humans and animal models of atherosclerosis (*24*).

The presence of MDA-protein epitopes was demonstrated by immunocytochemical analyses of adjacent, serial sections (Fig. 3, B, E, and H). The distribution of MDA-protein epitopes closely parallelled the deposition of apoB-100 protein in these lesions (Fig. 3, B, E, and H compared to Fig. 3, A, D, and G, respectively) as well as in the other seven subjected to analyses (*25*). At

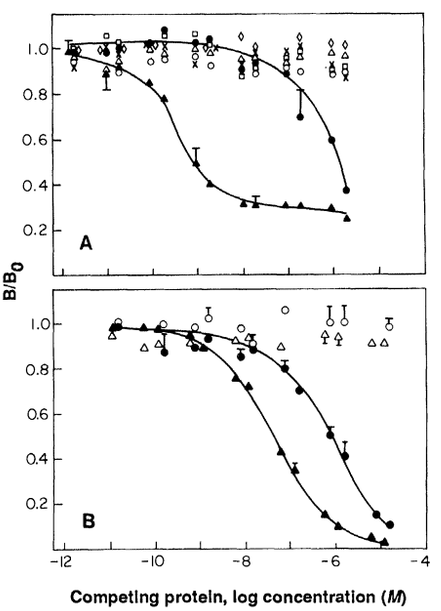

Fig. 2. Competitive displacement of the binding of mAb MDAlys to solid phase–adsorbed human MDA_{11}-LDL by human native or modified LDL (**A**) or by MDA-modified proteins and the parent molecules (**B**). Each microtiter well contained 38 ng of bound MDA_{11}-LDL. Competing proteins were added in 25 μl at the total concentrations indicated together with ascites fluid (25 μl, 10 μg of antibody per milliliter) and incubated 18 hours at 4°C. Antibody binding was determined as in Fig. 1 in the presence (B) or absence (B_0) of added competitor and expressed as B/B_0. Proteins were modified and characterized as previously described (*5, 7, 8*). Each point is the average of two values ± SEM for those points in which SEM exceeded 5% of the value determined for each average. Data shown in each panel are from a single experiment and have been reproduced in similar experiments conducted with native and modified LDL from individual human subjects (n = 6) or from individual WHHL rabbits (n = 4) (A) ○, native LDL; x, copper oxidized LDL; □, succinyl LDL; △, reductively methylated LDL; ◇, acetyl LDL; ●, MDA_{11}-LDL; △, MDA_{91}-LDL; or with modified and parent proteins from different preparations (n = 3) (B) ○, albumin; ●, MDA_{16}-albumin; △, transferrin; ▲, MDA_{15}-transferrin.

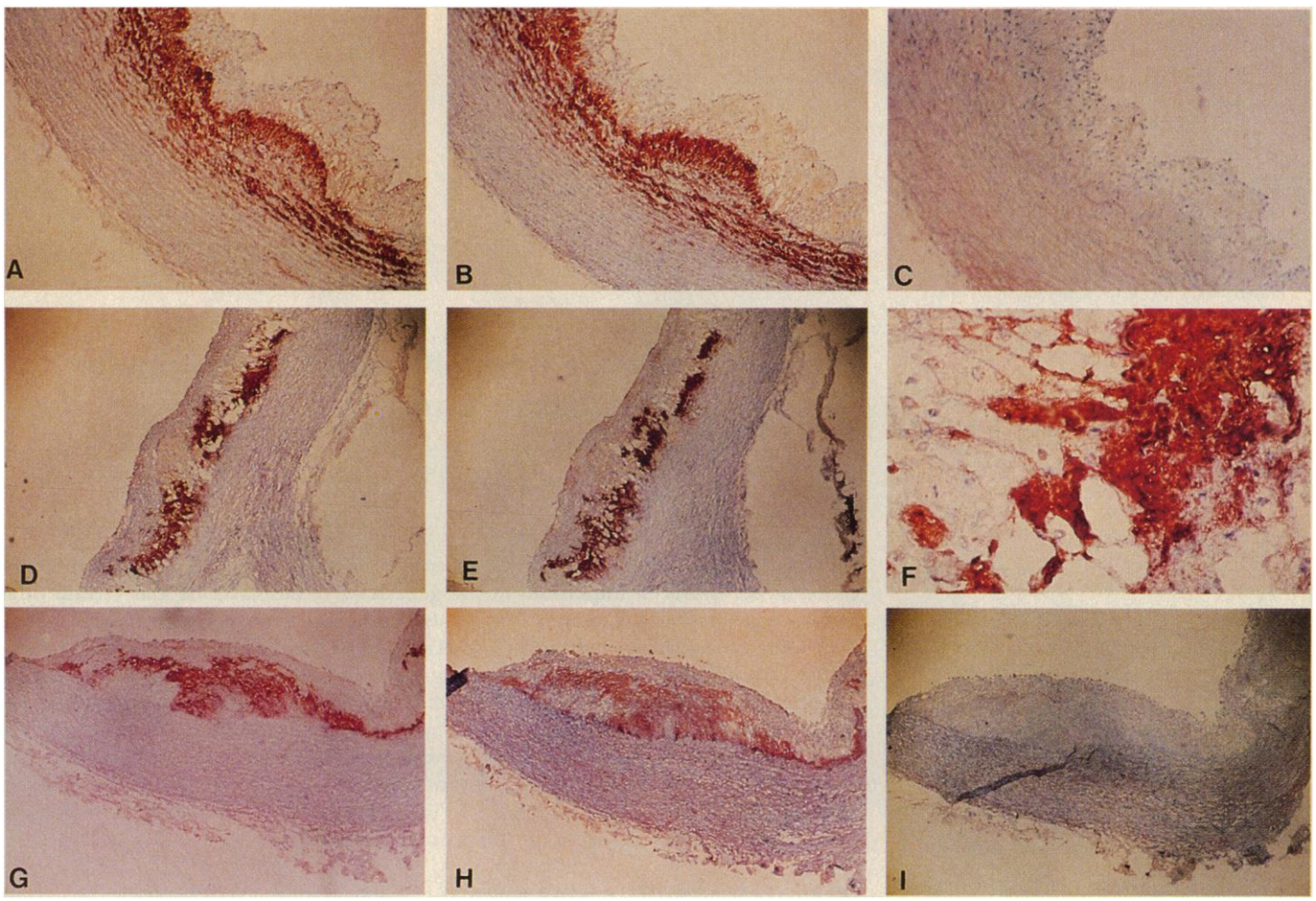

Fig. 3. Immunocytochemical analyses of fresh-frozen sections of upper thoracic rabbit aorta from WHHL rabbits. Tissue identification and microscope magnification factors include lesion 1: (**A** to **C**) (×100); lesion 2: (**D** and **E**) (×40) and (**F**) (×400); lesion 3: (**G** to **I**) (×40). Immunocytochemical analyses were conducted with mAb MB47 specific for apoB-100 protein (**A**, **D**, and **G**); or with mAb MDAlys specific for MDA-protein epitopes in the absence (**E**, **F**, and **H**) or presence of 5 mg of human LDL per milliliter (**B**) or 50 μg of MDA_{90}-LDL per milliliter (**C**); or with an irrelevant IgG2a murine mAb, Thy 1.2 (Becton Dickinson) (**I**). Immunodetection of primary immune complex formation was accomplished by Vectastain ABC kit for murine immunoglobin (PK4002, Vector Laboratories) and enzymic catalysis of 3-amino-9-ethylcarbazole (Sigma) to give the rose-red product indicating antigen localization (*23*). Sections were counterstained with Harris hematoxylin. Optimal murine antibody binding, determined by sequential dilution analyses, was 1:10. Photographs were taken on Kodak color film with a Nikon Optiphot microscope equipped with a Nikon Microflex UFX-II at an additional magnification factor of ×1.25. Analyses are based on samples from 4-month-old normolipidemic NZW rabbits (n = 2; 4 tissue blocks) and 11-month-old WHHL rabbits (n = 2; 16 tissue blocks).

higher magnification, the deposition of MDA-protein epitopes (Fig. 3F) as well as the apoB-100 epitopes (*25*) occurred primarily in the extracellular matrix surrounding the foam cells. Absence of immunostaining within the foam cells in the fresh-frozen tissue provided an internal negative control demonstrating lack of antibody reactivity with unsaturated lipids (Fig. 3F). Immunodetection of MDA-protein epitopes was retained in other sections prepared by precipitation fixation in ethanol to extract lipid components and paraffin embedding (*25, 26*).

Control experiments provided validation of the immunocytochemical analyses (Fig. 3I) and immunoreactivity of mAb MDAlys (Fig. 3, B and C). The addition of LDL (5 mg of protein per milliliter) together with mAb MB47, as expected, abolished immunostaining (*25*).

Immunocytochemical analyses of arterial walls from normolipidemic NZW rabbits demonstrated diffuse patterns of immunostaining with antibody for either MDA-protein epitopes or apoB-100 protein (*25*). Addition of competing antigen abolished the diffuse immunostaining (*25*), indicating that these epitopes occur at low or negligible levels in vivo in normal arterial walls.

In view of these data, it seems reasonable to propose that modification of apoB lipoproteins by peroxidative end products occurs in vivo in arterial lesions. The report that apoB-lipoproteins extracted from aorta of WHHL rabbits contain thiobarbituric acid–reactive substances (*27*) supports this view. The release of MDA is likely to be a local event and to depend upon a number of factors. These include mechanisms initiating lipid peroxidation, for example, cellular pathways of eicosanoid synthesis and free radical oxidation; source of lipid subjected to peroxidation, for example, cellular membranes or lipoprotein-derived lipid; and local oxidant/antioxidant balance (*3, 4, 6, 28*). Entrapment of apoB-lipoproteins by the extracellular matrix of the arterial wall may facilitate modification in situ. We have shown in studies in vitro that MDA derivatization of LDL adsorbed to heparin-Sepharose, after modification of a limited number of apoB-100 lysines, produces release of the modified LDL in a form recognized by the scavenger receptor of macrophages (*7*). We propose that a similar mechanism may operate in vivo to release entrapped, modified LDL for clearance by subendothelial macrophages in atheroma. While the mechanisms require delineation, our findings support the hypothesis that modification of cholesteryl ester–rich lipoproteins by lipid peroxides converts these particles to abnormal forms

which lead to formation of arterial foam cells.

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13. Established protocols were followed for production of murine monoclonal antibody (*11*) and for analyses of immune complex formation by solid-phase RIA (*12*). The immunogen was human LDL (d = 1.019 to 1.063 g/cm^3) modified by 28 mol of MDA per mole of LDL (*7, 8*). Male BALB/c mice were immunized by an intraperitoneal injection (1 mg of immunogen) in complete Freund's adjuvant, and 7 days later, by a tail vein injection (0.1 mg of immunogen) in saline (*14*). On day 10, hybridoma cells were derived from polyethylene glycol–induced fusion of murine myeloma S194/5.XX0.Bu-1 with murine splenic cells (1:10), plated in 96-well microtiter plates in selective medium, screened for antibody production after 14 days, and cloned by limiting dilution (*11, 12*). Clone J8E2B1 secretes mAb MDAlys.
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Inactivation of the Retinoblastoma Susceptibility Gene in Human Breast Cancers

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Mutational inactivation of the retinoblastoma susceptibility (RB) gene, a recessive cancer gene, has been implicated in the genesis of retinoblastoma and certain other human neoplasms. This gene is now shown to be inactivated in two of nine human breast cancer cell lines examined. The RB gene of one cell line had a homozygous internal duplication of a 5-kilobase region containing exons 5 and 6. The RB messenger RNA transcript was correspondingly lengthened, and its translation was probably terminated prematurely due to a shifted reading frame. The other cell line had a homozygous deletion of the RB gene that removed the entire gene beyond exon 2. The RB gene product, pp110^{RB}, was not detectable in either cell line by immunoprecipitation with specific antibodies. These findings are significant in relation to proposed genetic mechanisms of breast cancer formation.

SEVERAL APPROACHES HAVE BEEN APplied to identify genetic elements involved in tumorigenesis. Oncogenes were initially defined in tumor-inducing retroviruses and in tumor DNA capable of transforming nonneoplastic cells in culture (*1*). Most oncogenes are activated homologs of proto-oncogenes that exist in normal cells (*2*). Another class of cancer genes has been proposed for which loss of gene function is associated with oncogenesis (*3*). The existence of such genes was first indirectly suggested by studies with restriction fragment length polymorphisms that indicated a loss of specific chromosomal regions in tumor DNA compared to somatic DNA from the same patients. This "loss of heterozygosity" has been observed in many tumor types including retinoblastoma (*4*), osteosarcoma (*5*), Wilms's tumor (*6*), hepatoblastoma, and rhabdomyosarcoma (*7*). Genes giving rise to tumors by loss of function have been termed "recessive oncogenes" or "cancer suppressor genes," since the presence of one or more normal alleles is sufficient to prevent expression of the cancer phenotype. As a model system for recessive oncogenesis, retinoblastoma has been most intensely studied; recently, the presumptive cancer suppressor gene (RB) that controls retinoblastoma formation has been identified (*8, 9*). Evidence of RB gene inactivation has been found in most retinoblastomas as well as some osteosarcomas and soft tissue sarcomas even without prior retinoblastoma (*10, 11*), indicating a broader role for this gene in oncogenesis than might have been anticipated.

In contrast, the search for specific genetic features in more common human tumors, such as breast cancer, has been hampered by marked genetic and biological heterogeneity in tumor cells and cell lines. Although a specific 1q marker chromosome is sometimes present (*12*), most breast cancer cells demonstrate multiple, complex genetic rearrangements that are refractory to further categorization (*13*). Two recent studies have identified a loss of heterozygosity in breast cancer DNA by means of probes for polymorphic loci on several different chromosomes. In one study of ductal breast carcinomas from men and premenopausal women, loss of heterozygosity was found for markers on chromosome 13, but not on chromosome 11, in four of ten cases (*14*). Another study demonstrated loss of chromosome 11 heterozygosity in 20% of 56 stage II and III breast carcinomas (*15*), but probes on chro-

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