Accumulation of plasma cells in atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits

(low density lipoprotein receptor deficient/IgG)

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ABSTRACT By screening a cDNA library constructed from aortic total RNA derived from Watanabe heritable hyperlipidemic (WHHL) rabbits by differential hybridization, we have obtained a cDNA encoding the \( \kappa \) light chain of immunoglobulin. Northern blot analysis of total RNA prepared from aortas of WHHL and normal rabbits of various ages revealed that this light-chain mRNA accumulates gradually with age in aortas in WHHL rabbits. Northern blotting and in situ hybridization with an antisense oligonucleotide specific to rabbit immunoglobulin \( \gamma \) heavy-chain mRNA also detected accumulation of this heavy-chain mRNA in advanced lesions of WHHL rabbit aortas. Moreover, immunohistochemical and electron microscopic analyses demonstrated the presence of plasma cells in the atherosclerotic lesions.

The pathogenesis of atherosclerosis has not been fully clarified. This arterial disease is characterized by smooth muscle cell proliferation, cholesterol deposition, and accumulation of monocyte-derived macrophages and T lymphocytes. The development of atherosclerosis has been proposed to be a result of an excessive inflammatory fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the arterial wall (1). Although numerous growth factors, cytokines, and vasoreactive molecules have been considered to be involved in this complex process, the exact mechanisms by which proliferation of smooth muscle cells and lipid accumulation in macrophages are mediated are still unclear.

Familial hypercholesterolemia (FH), one of the most common genetic diseases in humans, is characterized by an elevated concentration of low density lipoprotein (LDL) and deposition of LDL-derived cholesterol in tendons, skin, and arteries. The primary defect in FH is a mutation in the gene encoding the receptor for plasma LDL (2). Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model of FH, have severe hypercholesterolemia, cutaneous xanthomas, and fulminant atherosclerosis caused by a deficiency of LDL receptor (3, 4). WHHL rabbits are homozygous for a mutant allele for the LDL receptor. The mutant LDL receptor in WHHL rabbits has an in-frame deletion of 12 nucleotides that eliminate four amino acids from the cysteine-rich binding domain of the LDL receptor (5). This mutation impedes transportation of the receptor to the cell surface at only 1/10th the normal rate (6). In the aortas of WHHL rabbits, small foci of intimal thickening containing lipid and lipid deposition are detected at 2–3 months, numerous visible lesions develop gradually between 4 and 15 months, and severe atherosclerotic lesions are detectable at 15–35 months of age (3, 7).

Using WHHL rabbits, we have been isolating cDNAs for mRNAs that are highly accumulated in atherosclerotic lesions. In previous studies, we have isolated cDNAs for matrix Glα protein and lysosomal membrane antigen CD63 from WHHL rabbit aortas (8, 9). Analysis of matrix Gla protein and CD63 antigen mRNAs by Northern blotting revealed that the expression of these two mRNAs in aortas of WHHL rabbits is markedly increased during the progression of atherosclerosis, and in situ hybridization with specific antisense oligonucleotides showed that they accumulate in atherosclerotic lesions in WHHL rabbits (8, 9).

Further screening of a WHHL rabbit aortic cDNA library by differential hybridization revealed a cDNA encoding immunoglobulin \( \kappa \) light chain. Here we describe the accumulation of mRNAs for IgG in the aortas of WHHL rabbits during atherosclerosis. We also describe the presence of plasma cells in atherosclerotic lesions.

MATERIALS AND METHODS

Animals. Homozygous WHHL rabbits (3–30 months old) and Japanese white rabbits (3–30 months old) were kindly supplied by Japan Laboratory Animal (Tokyo) and fed on a standard rabbit diet (CLEA Japan, Tokyo). All rabbits were exposed to 12 h of light (6 a.m. to 6 p.m.) and 12 h of darkness (6 p.m. to 6 a.m.) daily for 2 weeks before use.

General Methods. Standard molecular biology techniques were performed essentially as described by Sambrook et al. (10). Nucleotide sequences were determined by the dideoxy-nucleotide chain-termination method (11) using M13 primers T7 and T3 or specific internal primers. Sequence reactions were performed using Taq DNA polymerase with fluorescently labeled nucleotides on an Applied Biosystems DNA sequencer (model 373A).

Northern Blot Analysis. Total cellular RNA was isolated from solid organs by the guanidinium thiocyanate/CsCl centrifugation procedure (12). Total RNA (5 \( \mu \)g) denatured with glyoxal was subjected to electrophoresis on a 1.5% agarose gel and transferred to a nylon membrane for hybridization. Blot hybridization was carried out with either double-stranded 32P-labeled probes primed with random hexanucleotides (13) or 32P-labeled oligonucleotides. Oligonucleotides were labeled with \( \gamma \)-32P]ATP using T4 polynucleotide kinase. Oligonucleotides used for the blot hybridization were 50-mer of the following oligonucleotide sequences: immunoglobulin \( \alpha \) chain, 5'-GGGC-TCTGTCGGAAGGGCTTTCGTCGGCTTGGGCCTCAAGTGAAAGG-3'; immunoglobulin \( \gamma \) chain, 5'-CCGTAAGCAGCGGCGGCGGGCGGATCCTGCTGTTATGACCATG-3'; immunoglobulin \( \mu \) chain, 5'-

Abbreviations: LDL, low density lipoprotein; WHHL, rabbit; Watanabe heritable hyperlipidemic rabbit.

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The sequence reported in this paper has been deposited in the GenBank data base (accession no. D38376).
TGGACTTGGAGATGTTGCTTCACGGAAGGGCAGGGCGGCTGTCGTCGCT-3' for hybridization under stringent conditions, autoradiograms were analyzed on an image analyzer (BAS2000; Fuji).

**Construction of cDNA Library and Differential Screening.** Total aortic RNA prepared from 24- to 30-month-old WHHL rabbits was used to construct a cDNA library. Double-stranded cDNA was synthesized by the method of Gubler and Hoffman (14) with NorI thymidine linker primer 5'-CTCTAGAGGCGGGCAG-3'. The double-stranded cDNA was treated with T4 DNA polymerase, followed by digestion with NorI, and inserted into pBluescript II SK+ (Stratagene). Approximately 2500 recombinants were screened by the differential hybridization procedure (10). 32P-labeled cDNA probes were prepared from total RNAs from the aortas of 24- to 30-month-old WHHL rabbits or from 3- to 6-month-old normal rabbits, using oligo(dt)18 primer and Superscript.

**Anchored PCR.** To analyze the variable region of immunoglobulin \( \kappa \) light-chain mRNA, anchored PCR (15) was carried out. cDNA was synthesized from 5 \( \mu \)g of total RNA using an oligo(dt)18 primer and 200 units of Superscript (Life Technologies, Grand Island, NY) in 20 \( \mu \)l of reverse transcription buffer (50 mM Tris-HCl, pH 8.3/10 mM MgCl2/50 mM KCl/3 mM dithiothreitol/0.1% Nonidet P-40/0.45 mM dNTP) at 37°C for 1 h. A poly(dG) tail was then added to the 3' ends of the cDNAs in 20 \( \mu \)l of reaction mixture containing 30 mM Tris-HCl (pH 6.8), 140 mM potassium cacodylate, 1 mM CoCl2, 0.1 mM dithiothreitol, and 0.25 mM DATP with 25 units of terminal deoxynucleotidyltransferase at 37°C for 10 min. The poly(dG)-tailed cDNA was amplified with an oligo(dC)14 linker primer 5'-AGAGCGGGCGCC(C)14-3' and an antisense primer (5'-TATTGGCCACACACACGATGGTGAC-3') that corresponds to the constant region of rabbit \( \kappa \) light chain (16). One-tenth of the poly(dG)-tailed cDNA was amplified in standard PCR buffer containing 25 pmol of each primer and 0.75 unit of Taq DNA polymerase in 100 \( \mu \)l of standard PCR buffer (10 mM Tris-HCl, pH 8.8/50 mM KCl/1.5 mM MgCl2/0.1% Triton X-100/0.2 mM each dNTP). The thermal profile used was 94°C for 30 s, 55°C for 1 min, and then 72°C for 2 min. After 33 cycles, the PCR products were loaded onto a 5% polyacrylamide gel. A major reaction product was eluted from the gel and subcloned into pBluescript vectors for sequencing.

**In Situ Hybridization.** In situ hybridization was carried out with rabbit immunoglobulin \( \gamma \) heavy-chain-specific oligonucleotides as described (8). Aortas were obtained from 3- and 17-month-old WHHL and 3-month-old normal rabbits. The tissue specimens were fixed in phosphate-buffered saline (PBS) containing 10% formaldehyde for 48 h at 4°C. The specimens were then embedded in paraffin, sectioned at 2.5 \( \mu \)m, and placed on glass slides. Oligonucleotides used for the in situ hybridization were 50-mer sequences: sense primer, 5'-ATGGTACATATAACACAGGCAGGTGCAGGACCCCGCCGCGCTACGGG-3'; antisense primer, 5'-CCCGTACGCGCCGGCGCGCGCGGTGCGCACCCTGGTGGTGTTATGACCAT-3'. The oligonucleotides were labeled at the 3' end with cytidine 5'-[\( ^{32} \)P]thio)triphosphate using terminal deoxynucleotidytransferase. The antisense oligonucleotide probe successfully detected rabbit \( \gamma \) heavy-chain mRNA by Northern blotting.

**Immunohistochemistry.** Aortas from WHHL and normal rabbits were fixed in PBS containing 10% formaldehyde for 48 h at 4°C. The specimens were then embedded in paraffin, sectioned at 2.5 \( \mu \)m, and placed on glass slides. The sections were placed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity and then treated with 1% normal goat serum for 40 min at room temperature. After washing with PBS, the specimens were incubated with goat biotinylated anti-rabbit IgG (Nichirei, Tokyo) for 30 min at room temperature and then incubated with peroxidase-conjugated streptavidin (Nichirei) for 20 min at room temperature. After extensive washing with PBS, the antigen–antibody complexes were visualized by immersion in a solution containing 50 mM Tris-HCl (pH 7.6), 1 mM 3,3'-diaminobenzidine, 10 mM sodium azide, and 0.006% hydrogen peroxide. The sections were counterstained with methyl green.

**Electron Microscopic Analysis.** An aorta obtained from a 17-month-old WHHL rabbit was fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The tissue samples were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead nitrate. The electron microscope was a JEM 1200-EX (Nihon Densi, Tokyo).

**RESULTS**

cDNAs encoding mRNAs abundant in WHHL rabbit aortas were obtained by a differential hybridization procedure. Of ~2500 recombinants screened with 32P-labeled cDNA probes prepared from total RNA from aortas of WHHL or normal rabbits, we obtained one positive clone that hybridized strongly with the cDNA probe prepared from WHHL rabbits. The cDNA, designated pSY66, was sequenced entirely, revealing that it encodes a protein of 238 amino acids with a calculated M, of 25,080 (Fig. 1). Comparison of the amino acid sequence deduced from the nucleotide sequence of the cDNA with those in the data base of the National Biomedical Research Foundation identified it as that of a rabbit immunoglobulin \( \kappa \) light-chain of allotype b4.

The isolation of immunoglobulin \( \kappa \) light-chain cDNA from WHHL rabbit aortas was unexpected and suggested the accumulation of B lymphocytes or plasma cells in atherosclerotic lesions in WHHL rabbits. To test this possibility, we analyzed the levels of the \( \kappa \) light-chain mRNA in the aortas of WHHL and normal rabbits by Northern blotting. Consistent with the isolation of the \( \kappa \) light-chain cDNA by differential hybridization screening, extremely high levels of the mRNA were detected in the aortas of WHHL rabbits but not in those of normal rabbits (Fig. 2A). The mRNA was detectable in the aortas of 2-month-old WHHL rabbits and increased steadily with advancing age; the mRNA levels in 22- and 30-month-old WHHL rabbits were >100-fold higher than those at 2 months (Fig. 2B). There was a significant correlation between the aortic levels of \( \kappa \) light-chain mRNA and aging in WHHL rabbits (r = 0.91; P < 0.01) (Fig. 2B). We also analyzed the mRNA in heterozygous WHHL rabbits that develop no hypercholesterolemia or atherosclerosis, but we could not detect the mRNA in their aortas (data not shown). Although we could not quantify the lesion-covered areas of the aortas or quantify the lesion-covered areas of the aortas in WHHL rabbits because of rapid disruption of the tissues to fix the aortas. The mRNA expression is steadily increased with advancing age in WHHL rabbits.

In the next experiment, we determined the class of immunoglobulin \( \kappa \) light-chain cDNA from WHHL rabbit aortas.

variable regions were deduced by nucleotide sequencing of the PCR products after subcloning into pUC vectors. The nucleotide sequences of six independent clones revealed that none were matched to each other, indicating that the IgG expressed in the WHHL rabbit aorta were of polyclonal origin or hypermutated (data not shown).

To exclude the possibility that the mRNAs for IgG were overexpressed in the whole body of WHHL rabbit due to infection, the levels of κ light-chain mRNAs in other tissues of WHHL rabbits were compared with those of normal rabbits. The levels of κ light-chain mRNA in spleen and mesenteric lymph nodes of WHHL and normal rabbits were not altered significantly, as revealed by Northern blot analysis (Fig. 4). Trace amounts of the mRNA were detectable in the livers of both normal and WHHL rabbits but their levels were not significantly different between the two rabbit strains. These data indicate that the accumulation of immunoglobulin κ light-chain mRNA is specific in aortas of WHHL rabbits and suggest that no systemic immune response is involved in accumulation of the mRNAs for IgG in WHHL rabbit aortas.

![Diagram](image)

**Fig. 2.** Northern blot analysis of immunoglobulin κ light-chain mRNA in aortas of WHHL and normal rabbits. (A) Total RNAs (5 μg) isolated from aortas of WHHL and normal rabbits (2–30 months old) were hybridized with a 32P-labeled rabbit immunoglobulin κ light-chain cDNA probe. Each lane is from an individual animal. (B) Same filter was then reprobed with a 32P-labeled human cyclophilin cDNA (17). Relative levels of immunoglobulin κ light-chain mRNA were estimated by densitometric scanning of the blots; the intensity of the band in rabbit A was assigned a value of 1. Abundance of immunoglobulin κ light-chain mRNA in each sample was determined by the ratio of the light-chain mRNA scanning value to cyclophilin mRNA in 5 μg of total RNA. •, WHHL rabbits; ○, normal rabbits. Solid line was determined by linear regression (r = 0.91; P < 0.01).

To localize the IgG-producing cells in atherosclerotic lesions of WHHL rabbits, we carried out in situ hybridization with sense or antisense oligonucleotide probes for rabbit γ heavy-chain mRNA. With the antisense oligonucleotide, cells expressing γ heavy-chain mRNA were detected in the intima of advanced lesions of WHHL rabbit aorta (Fig. 5 B and C), whereas no signal was detected by the sense oligonucleotide (Fig. 5 A). IgG-positive cells were not detectable in aortas of normal rabbits (Fig. 5 E). Although we detected the mRNA in aortas of 2- to 3-month-old WHHL rabbits by Northern blotting, we could not detect the mRNA-producing cells by in situ hybridization (Fig. 5 D), which may be explained by a relatively small population of IgG-positive cells in the early stages of atherosclerosis.

To detect IgG-producing cells in the aortas of WHHL rabbits, we carried out immunohistochemical analysis with anti-rabbit IgG. Consistent with the in situ hybridization,
IgG-producing cells were detected in the intima of advanced lesions (Fig. 6A). Furthermore, electron microscopic analysis revealed the presence of plasma cells with characteristic cogwheel chromatin and abundant rough-surfaced endoplasmic reticulum in the intima of atherosclerotic lesions (Fig. 6 B-E). Relatively large numbers of plasma cells were detected with numerous lipid-laden cells in the intima of the lesions. No plasma cell was detectable in the adventitia of WHHL rabbit aortas (data not shown).

**DISCUSSION**

In the current study, we have shown that mRNAs for κ light and γ heavy chain of IgG accumulate markedly in the aortas of WHHL rabbits. This accumulation is found only in homozygous WHHL rabbits and is dependent on aging. The in situ hybridization and immunohistochemical and electron microscopic analyses of the atherosclerotic lesions in WHHL rabbits demonstrated the presence of plasma cells, which is of considerable pathological significance.

In WHHL rabbits, small foci of intimal thickening containing lipids are detectable in the aortas at 2–3 months of age, numerous visible lesions develop from 4 to 15 months, and severe atherosclerotic lesions are detected at 15–35 months (3, 7). Similarly, immunoglobulin κ light-chain mRNA accumulates in the aortas of WHHL rabbits with advancing age. The mRNA is detectable at 2–3 months of age, and extremely high

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**FIG. 4.** Expression of immunoglobulin κ light-chain mRNA in various rabbit tissues. (A) Total RNAs (5 µg) isolated from aortas, livers, spleens, and mesenteric lymph nodes of WHHL (lanes a–c, 10 months old) and normal (lanes d–f, 10 months old) rabbits were hybridized with a 32P-labeled rabbit immunoglobulin κ light-chain cDNA probe. Each lane is from an individual animal. (B) Same filter was then reprobed with a 32P-labeled human cyclophilin cDNA. (C) Volume of total RNA in each lane was confirmed by ethidium bromide (EtBr) staining.

**FIG. 5.** *In situ* hybridization of rabbit aortas with oligonucleotide probes for immunoglobulin γ heavy-chain mRNA. Tissue sections prepared from aortas of 17-month-old WHHL (A–C), 3-month-old WHHL (D), and 3-month-old normal (E) rabbits were analyzed by *in situ* hybridization. Hybridization with sense oligonucleotide (A) and with antisense oligonucleotide (B–E) probes specific to rabbit immunoglobulin γ heavy-chain mRNA. Arrows in C show mRNA-expressing cells in B at higher magnification. (A, B, D, and E, ×70; C, ×135.)
levels of the mRNA were detected in aortas of older WHHL rabbits (24 to 30 months old). This mRNA was not detectable in heterozygous WHHL or normal rabbits, which develop no atherosclerotic lesions if given a normal diet. The accumulation of this mRNA is found not only in the atherosclerotic aortas of WHHL rabbits but also in human atherosclerotic aortas (Y.S. and T.Y., unpublished data). This is strong evidence that the accumulation of mRNAs for IgG in the aorta is highly correlated with the development of atherosclerosis.

Electron microscopy directly showed the presence of plasma cells in the intima of advanced atherosclerotic lesions. This suggests that these cells secrete IgG in the lesions. In the mouse hybridoma cells expressing IgG2a, the secreted γ heavy chain is encoded by the 1.8-kb mRNA that is a major transcript, whereas that of the membrane-bound form is a minor transcript of 3.9 kb (18). The 1.8-kb mRNA for the secreted form is also abundant in the mouse spleen, whereas the 3.9-kb mRNA for the membrane-bound form is undetectable by Northern blotting (ref. 19; M.N. and S. Takahashi, unpublished data). Therefore, it is possible that the 2.4-kb γ heavy-chain mRNA in the atherosclerotic lesions, mesenteric lymph nodes, and spleens encode the secreted γ heavy chain. Consistent with the accumulation of the immunoglobulin mRNAs and plasma cells in the lesions, we detected a high concentration of soluble IgG in the extracts of WHHL rabbit aortas (R. Shiga and T.Y., unpublished data). The accumulation of plasma cells in the atherosclerotic lesions may be mediated by the major B-lymphocyte proliferating and differentiation factor interleukin (IL) 6. Lopppnow and Libby (20) have shown that proliferating or IL-1-activated vascular smooth muscle cells secrete very high amounts of IL-6. This overproduction of IL-6 by vascular smooth muscle cells may promote the proliferation of B lymphocytes derived from the circulation and lead to the maturation of B lymphocytes to plasma cells in the atherosclerotic lesions.

The presence of increased numbers of plasma cells in atherosclerotic lesions is unexpected but not altogether surprising because increased numbers of T lymphocytes are also detectable in atherosclerotic lesions in humans and in cholesterol-fed rabbits (21, 22). The presence of plasma cells together with T lymphocytes and macrophages in atherosclerotic lesions suggests that an immunological reaction is involved during the progress of atherosclerosis.

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