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# Short Communication

## Expression of Very Low Density Lipoprotein Receptor mRNA in Rabbit Atherosclerotic Lesions

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***The expression of very low density lipoprotein (VLDL) receptor mRNA in atherosclerotic lesions in rabbits was investigated. To examine the expression of the VLDL receptor in the vascular wall, poly(A)$^+$ RNA was isolated from whole aortas of cholesterol-fed New Zealand White (NZW), Watanabe heritable hyperlipidemic (WHHL), and normal NZW rabbits, and then Northern blot analysis was performed. The VLDL receptor mRNA was detected in aortas from both NZW rabbits fed 0.5% cholesterol for 16 weeks and 12-month-old WHHL rabbits, whereas no expression was seen in normal NZW rabbit aortas. To further determine the localization of the VLDL receptor mRNA, in situ hybridization using digoxigenin-labeled riboprobes and immunohistochemistry using monoclonal antibodies against each cell component were performed. Early atherosclerotic lesions, termed fatty streaks, in the NZW rabbits fed 0.5% cholesterol for 4 weeks demonstrated strong expression of the VLDL receptor mRNA by macrophages. The VLDL receptor mRNA was also expressed in more advanced atherosclerotic lesions from both atherogenic animal models. The predominant origin of the VLDL receptor mRNA-positive cells was macrophages, and some intimal smooth muscle cells appeared to express a weak but significant signal in these advanced lesions. Our findings suggest that the VLDL receptor expression may play a role in the development of atherosclerosis. (Am J Pathol 1996, 149:1831–1838)***

Atherosclerotic lesions are characterized by the accumulation of extra- and intracellular lipids and lipid-loaded foam cells derived from circulating monocytes and medial smooth muscle cells.[1] A major lipid source in atheromatous plaques appears to be low density lipoprotein (LDL) enriched with cholesterol from the circulation. The trapped LDL is thought to generate *in vivo* oxidative products, termed oxidized LDL.[2] Macrophages in atherosclerotic lesions express few LDL receptors that are unable to scavenge oxidized LDL.[3,4] Although the LDL receptor is a key molecule in cholesterol homeostasis, it is not responsible for lipoprotein metabolism in the arterial wall.[4,5]

Several *in vitro* studies have shown that macrophages take up acetylated and oxidized LDL through scavenger receptors. The scavenger receptors, which are not down-regulated by sterol, play a role in foam cell formation in atherosclerotic lesions.[6–8] The LDL-receptor-related protein (LRP) is capable of binding apolipoprotein (apo)E-containing lipoproteins, lipoprotein lipase (LPL) and LPL-triglyceride-rich lipoprotein complexes.[9–11] Luoma et al have reported that *in situ* hybridization using riboprobes reveals expression of the scavenger receptor and LRP mRNAs in human fatty streaks and advanced atherosclerotic lesions.[12] Immunohistochemistry demonstrated expression of the scavenger receptor on macrophages but not on smooth

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muscle cells, whereas LRP was identified on both macrophages and smooth muscle cells in atherosclerotic lesions.[12] The physiological role of these two molecules in atherogenesis is unclear with regard to lipoprotein metabolism in the arterial wall.

The very low density lipoprotein (VLDL) receptor was discovered in 1992.[13] Although this lipoprotein receptor has high structural homology to the LDL receptor, these two receptors have distinct ligand-binding specificity. The VLDL receptor binds the apoE-containing lipoproteins, including VLDL, intermediate density lipoprotein (IDL), and β-migrating VLDL (β-VLDL) with high affinity.[13] Recently, Suzuki et al have reported that rabbit alveolar macrophages express the VLDL receptor mRNA and generate foam cells after exposure to β-VLDL, suggesting that the VLDL receptor may be involved in foam cell formation in atherosclerosis.[14]

In our study, the expression of the VLDL receptor mRNA was examined in early and advanced atherosclerotic lesions by Northern blot analysis and *in situ* hybridization in independent animal models, cholesterol-fed New Zealand White (NZW) rabbits and Watanabe heritable hyperlipidemic (WHHL) rabbits. To identify the cell of origin responsible for the VLDL receptor mRNA expression, immunohistochemistry using monoclonal antibodies against macrophages and smooth muscle cells was performed. We demonstrated the presence and localization of VLDL receptor mRNA in the arterial wall of these atherogenic animal models.

## Materials and Methods

### Animals

Male NZW rabbits, weighing approximately 2.5 kg, were purchased from Japan SLC (Hamamatsu, Japan). WHHL rabbits were raised in the Institute for Experimental Animals, Kobe University School of Medicine.[15] Each rabbit was caged individually and maintained in a room equipped with laminar flow filters at 22°C in the Experimental Animal Laboratory of Fukushima Medical College. This study was carried out in conformance with the Guidelines on Animal Experiments in Fukushima Medical College, the Japanese Government Animal Protection and Management Law (No. 105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

### Animal Study Protocol

A total of 30 NZW rabbits were randomly assigned to five groups. One group of rabbits (n = 6) was fed a standard diet (RC4, Oriental Yeast Co., Tokyo, Japan), and the other four groups of rabbits (n = 6 in each group) were fed an atherogenic diet supplemented with 0.5% cholesterol (Oriental Yeast Co.) for 2, 4, 8, and 16 weeks, respectively. Each rabbit was given a diet of 120 g per day and water *ad libitum*. Food intake was recorded daily and body weight was monitored weekly. WHHL rabbits were fed a standard diet. The WHHL rabbits used for this study were 4, 6, 8, and 12 months old.

### Northern Blot Analysis

Aortas were removed from 16-week-treated cholesterol-fed NZW rabbits, 12-month-old WHHL rabbits, and normal NZW rabbits after $CO_2$ anesthesia and frozen in liquid nitrogen for RNA extraction. Northern blot analysis was carried out as previously described.[16] Total aortic RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method,[17] followed by poly(A)$^+$ RNA isolation. Glyoxal-treated poly(A)$^+$ RNA samples were electrophoresed on a 1.5% agarose gel and transferred to a nylon membrane (Hybond N$^+$, Amersham, Little Chalfont, UK) in 20X SSPE.[18] The membrane was prehybridized at 42°C for 3 hours in a buffer containing 50% formamide, 5X SSPE, 5X Denhardt's solution, 1% sodium dodecyl sulfate (SDS) and 200 μg/ml denatured salmon sperm DNA, followed by hybridization with a $^{32}$P-labeled probe at 42°C for 16 hours. The membrane was washed twice with 1X SSPE containing 0.1% SDS and then twice with 0.1X SSPE containing 0.1% SDS for 15 minutes each at 60°C. The membrane was exposed to x-ray film and an intensifying screen at −70°C. After autoradiography, the nylon membrane was treated with boiling water containing 0.5% SDS and used for rehybridization. The probes used in this study were a 1.2-kb *Eco*RV-*Kpn*I fragment, a 461-bp *Pst*I-*Eco*RV fragment of the rabbit VLDL receptor cDNA,[13] and a 1.9-kb *Bam*HI fragment of the human β-actin cDNA.[19] Each probe was labeled with [α-$^{32}$P]dCTP (3000 ci/mmol; ICN, Costa Mesa, CA) by random hexanucleotide priming.[20]

### In Situ *Hybridization*

The rabbits were sacrificed under $CO_2$ anesthesia and their aortic arteries (from ascending aorta to abdominal aorta) were immediately removed. The aortas were placed in phosphate-buffered saline (PBS) pretreated with 0.1% diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, MO) and the adventitia was gently removed. The aortic arch was fixed

with 4% paraformaldehyde for 24 hours, embedded in paraffin, and sectioned (4 μm thick) onto 3-aminopropyl-triethoxysilane-coated slide glasses (Matsunami Glass Industries, Osaka, Japan). The sections were deparaffinized with xylene, rehydrated through graded ethanol, and treated with 2.5 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for 30 minutes at 37°C. Subsequently, they were post-fixed with 4% paraformaldehyde for 10 minutes, acidified with 0.2 N HCl, and acetylated with 0.25% acetic acid in 0.1 mol/L triethanolamine. The concentration of the digoxigenin-labeled antisense and sense RNA probes was adjusted to 2.5 ng/μl in a hybridization buffer containing 50% formamide, 4X SSPE, 1X Denhardt's solution, 200 μg/ml yeast tRNA, and 10% dextran sulfate. After preincubation with the hybridization buffer, the sections were incubated with the digoxigenin-labeled probe at 50°C for 16 hours and then washed in 2X SSPE at 42°C for 30 minutes and digested with RNAse A (1 μg/ml; Boehringer Mannheim) at 37°C for 30 minutes. The sections were washed with 2X SSPE and then 1X SSPE at 42°C for 20 minutes and incubated with a blocking solution for 30 minutes and then alkaline-phosphatase-labeled anti-digoxigenin antibody (Boehringer Mannheim) for 30 minutes. The sections were washed in buffer and colored with nitroblue tetrazolium (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim).

The above digoxigenin-labeled riboprobe preparation was as follows. A 461-bp *Pst*I-*Eco*RV fragment of the rabbit VLDL receptor cDNA was subcloned into pBluescript SK (Stratagene, La Jolla, CA). This 461-bp fragment encodes a part of the ligand-binding domain of the VLDL receptor. Antisense and sense RNA probes were synthesized by *in vitro* transcription using T7 or T3 RNA polymerase with digoxigenin-labeled UTP (Boehringer Mannheim).[21]

### *Immunohistochemistry*

To determine the cell composition of atheromatous plaques, serial sections (4 μm) were used for immunohistochemical staining with monoclonal antibodies against rabbit macrophages[22] (RAM11, Dako, Carpinteria, CA) and human smooth muscle α-actin[23] (1A4, Dako). A streptavidin-biotinylated horseradish peroxidase system (StreptABComplex/HRP, Dako) was used, and antibody binding was visualized with 3,3′-diaminobenzidine (Wako Pure Chemical, Osaka, Japan) and hydrogen peroxide. The sections were counterstained with hematoxylin.

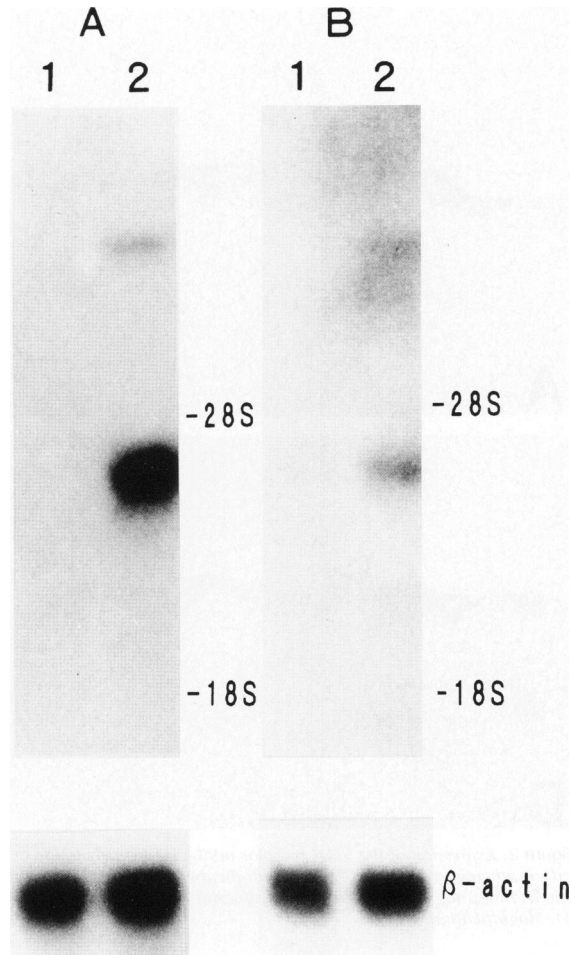


Figure 1. *Northern blot analysis of VLDL receptor. Poly(A)$^+$ RNAs isolated from the aortas of 16-week-treated cholesterol-fed NZW, 12-month-old WHHL, and normal rabbits were hybridized with a $^{32}$P-labeled probe for the rabbit VLDL receptor (6 μg of poly(A)$^+$ RNA per lane). The filter was exposed for 24 hours at −70°C. The same membrane was rehybridized with the human β-actin cDNA probe and exposed for 1 hour. Positions of the 28S and 18S rRNA subunits are indicated. Representative results of three experiments are shown.* A: lane 1, *normal NZW rabbit aorta;* lane 2, *16-week-treated cholesterol-fed NZW rabbit aorta.* B: lane 1, *normal NZW rabbit aorta;* lane 2, *12-month-old WHHL rabbit aorta.*

## *Results*

### *Northern Blot Analysis*

Figure 1 shows Northern blot analysis of the VLDL receptor mRNA expression of the aortas from 16-week-treated cholesterol-fed NZW rabbits and 12-month-old WHHL rabbits having atheromatous plaques using a 1.2-kb *Eco*RV-*Kpn*I cDNA probe. Two transcripts of the VLDL receptor were observed in the aortas from both cholesterol-fed NZW rabbits (Figure 1A) and the WHHL rabbits (Figure 1B). No expression of the VLDL receptor mRNA was detected in normal NZW rabbit aortas (Figure 1, A and

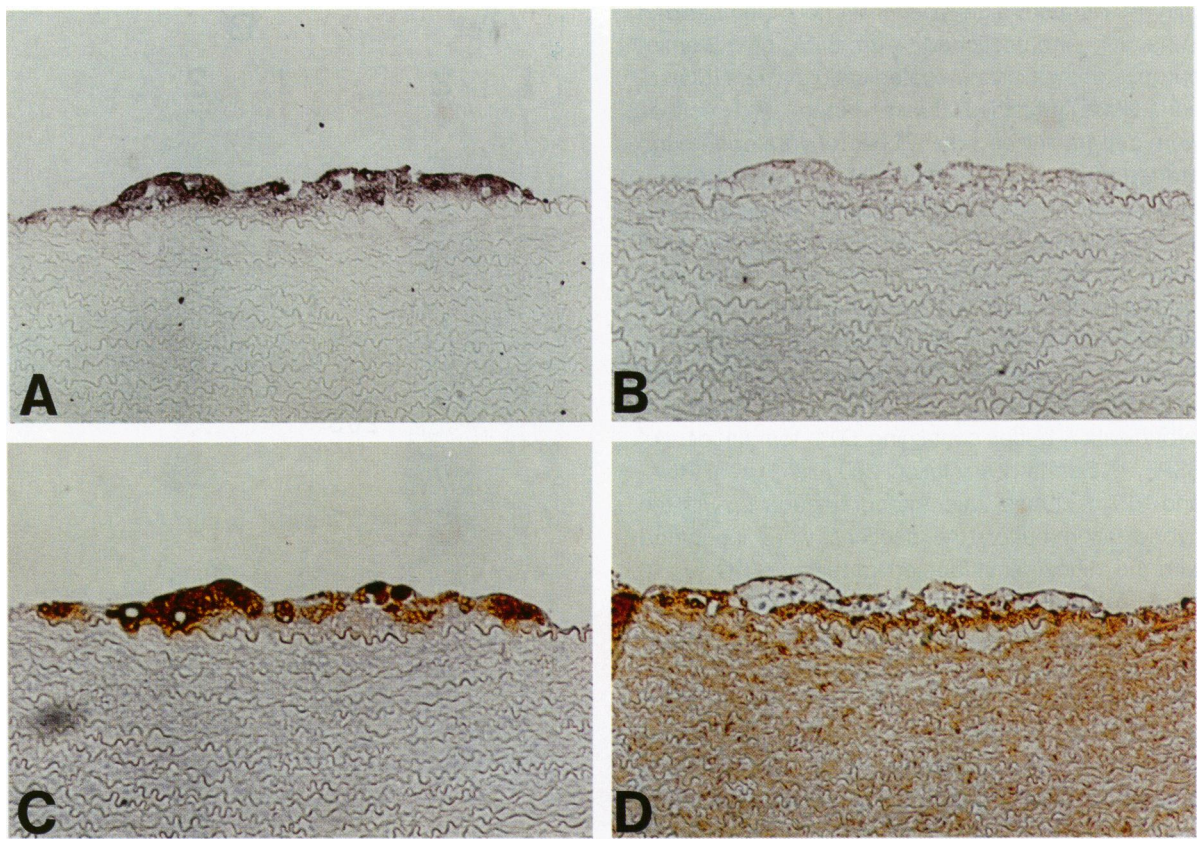


**Figure 2.** *Expression of the VLDL receptor mRNA in an early lesion of diet-induced atherosclerosis.* **A** *and* **B:** In situ *hybridization in a NZW rabbit fed an atherogenic diet for 4 weeks with the digoxigenin-labeled rabbit VLDL receptor antisense probe* (**A**) *and nonhybridizing sense probe* (**B**). **C** *and* **D:** *Immunohistochemical staining of serial sections with the antibodies against macrophages (RAM11;* **C**) *and anti-smooth muscle α-actin (1A4;* **D**). *Magnification, ×200.*

B). The same findings were observed using a 461-bp *Pst*I-*Eco*RV cDNA probe (data not shown).

## In Situ *Hybridization of the VLDL Receptor in Atherosclerotic Lesions in Cholesterol-Fed NZW Rabbits*

To localize the VLDL receptor mRNA expression, aortic arteries from cholesterol-fed NZW rabbits were examined by *in situ* hybridization using the rabbit VLDL receptor antisense complementary riboprobe and the sense noncomplementary riboprobe. To further identify the cells responsible for the VLDL receptor mRNA expression, immunohistochemical staining of serial sections using monoclonal antibodies against macrophages (RAM11) and smooth muscle α-actin (1A4) was concomitantly performed. These NZW rabbits were fed an atherogenic diet containing 0.5% cholesterol for various time periods. The VLDL receptor mRNA was not expressed in aortic walls from rabbits fed the atherogenic diet for 2 weeks or the control diet. Cholesterol feeding for 4 weeks induced an early lesion with accumulation of foam cells, termed fatty streak (Figure 2). *In situ* hybridization (Figure 2, A and B) and immunohistochemistry of serial sections (Figure 2, C and D) demonstrated VLDL receptor mRNA expression in macrophages with early atherosclerotic lesions. Aortic sections from rabbits treated for 8 or 16 weeks showed advanced atherosclerotic lesions and exhibited VLDL receptor mRNA (data not shown). Immunohistochemistry demonstrated that the main origin of the VLDL receptor mRNA-positive cells was macrophages in these animal models. A few intimal smooth muscle cells appeared to express the VLDL receptor mRNA in moderate (8-week treatment) and advanced (16-week treatment) atherosclerotic lesions, whereas no expression was detected in the medial smooth muscle cells (data not shown).

### *VLDL Receptor mRNA Expression in WHHL Rabbit Aorta*

*In situ* hybridization revealed that the VLDL receptor mRNA signal was found in the atheromatous plaques of aortic arteries from 12-month-old WHHL rabbits (Figure 3, A and B). Expression of the VLDL receptor mRNA in the aortic arteries of WHHL rabbits was also found in both the early (4-month-old rabbit) and more advanced (6- and 8-month-old rabbits) atherosclerotic lesions (data not shown). Immunohistochemical study showed that most of the VLDL receptor mRNA-positive cells appeared to be macrophages (Figure 3, C and D). Figure 3, E and F, shows VLDL receptor mRNA expression in macrophages at the border of the thickening intima and media in 12-month-old WHHL rabbits. Some cells in the surface region, enriched with smooth muscle cells, expressed the VLDL receptor mRNA, whereas no expression of the VLDL receptor was observed in the medial smooth muscle cells (Figure 3, A–D). The distribution patterns of the VLDL receptor mRNA-positive cells in younger WHHL rabbits were similar to those in 12-month-old WHHL rabbits.

## *Discussion*

The expression of the VLDL receptor mRNA in rabbit atherosclerotic lesions was examined using Northern blot analysis and *in situ* hybridization. Northern blot analysis demonstrates that VLDL receptor transcripts are present in aortas from cholesterol-fed NZW rabbits and WHHL rabbits (Figure 1). To further determine the localization of the VLDL receptor mRNA in the vascular wall, *in situ* hybridization was performed on aortic sections from the two independent atherosclerotic animal models, cholesterol-fed NZW rabbits and WHHL rabbits (Figures 2 and 3). Immunohistochemistry simultaneously performed with *in situ* hybridization revealed that the main origin of the VLDL receptor mRNA-positive cells was macrophages and that some intimal smooth muscle cells appeared to express the signal in the atherosclerotic lesions. This study is the first to demonstrate the presence of the VLDL receptor message in aortic atherosclerotic lesions.

Recently, macrophages have been shown to express VLDL receptor mRNA, and their levels are not down-regulated by exposure to LDL or β-VLDL.[14,24] Moreover, foam cell formation was induced by the incubation of rabbit resident alveolar macrophages with β-VLDL for 24 hours.[14] Takahashi et al have shown that apoE and LPL affect the binding of triglyceride-rich lipoproteins to the VLDL receptor using Chinese hamster ovary cells transfected with human VLDL receptor cDNA.[25] It has been known that macrophages can produce apoE and LPL,[26,27] suggesting that they may incorporate these lipoproteins via VLDL receptors in atherosclerotic lesions. In addition, Jokinen et al have suggested that aortic VLDL receptors may be located within the vascular wall rather than endothelial cells.[28] Our study proves that VLDL receptors are present on macrophages in early fatty streaks and more advanced atherosclerotic lesions.

We attempted to correlate the expression of the VLDL receptor mRNA and the development of atherosclerosis. Atherosclerotic lesion or VLDL receptor mRNA expression was not identified in NZW rabbits fed 0.5% cholesterol for 2 weeks, although treatment for 4 weeks induced a typical early lesion with VLDL receptor mRNA expression by macrophages (Figure 2). Macrophages predominantly expressed the VLDL receptor mRNA in more advanced atherosclerotic lesions of both rabbits fed 0.5% cholesterol for 8 and 16 weeks, and 4-, 6-, and 8-month-old WHHL rabbits. These findings suggest that the VLDL receptor expression by macrophages may initiate the development of atherosclerosis.

This study also demonstrates that smooth muscle cells in the thickening intima can express VLDL receptor mRNA in advanced atherosclerotic lesions, although the message was not identified in medial smooth muscle cells within the arterial walls. Recently, Ishii et al demonstrated VLDL receptor mRNA expression in cultured intimal smooth muscle cells derived from atherosclerotic lesions in WHHL rabbits and increased the cholesterol ester accumulation when incubated with β-VLDL.[29] Their *in vitro* results support our *in vivo* finding.

Our findings raise a question of what is the ligand(s) for the VLDL receptor in atherosclerotic lesions. Rapp et al have demonstrated that VLDL and IDL could enter atherosclerotic plaques in human aortas.[30] Other studies have demonstrated that the VLDL receptor binds triglyceride-rich lipoproteins such as VLDL, IDL, and β-VLDL,[13,14,24,27] suggesting that some particles of these lipoproteins may bind the VLDL receptor in atherosclerotic lesions.

In conclusion, our data have demonstrated the presence of VLDL receptor mRNA in rabbit atherosclerotic lesions and suggested that the expression of this lipoprotein receptor plays an important role in the rate of atherogenesis. Additional studies are required to clarify the physiological role of the VLDL receptor in atherogenesis.

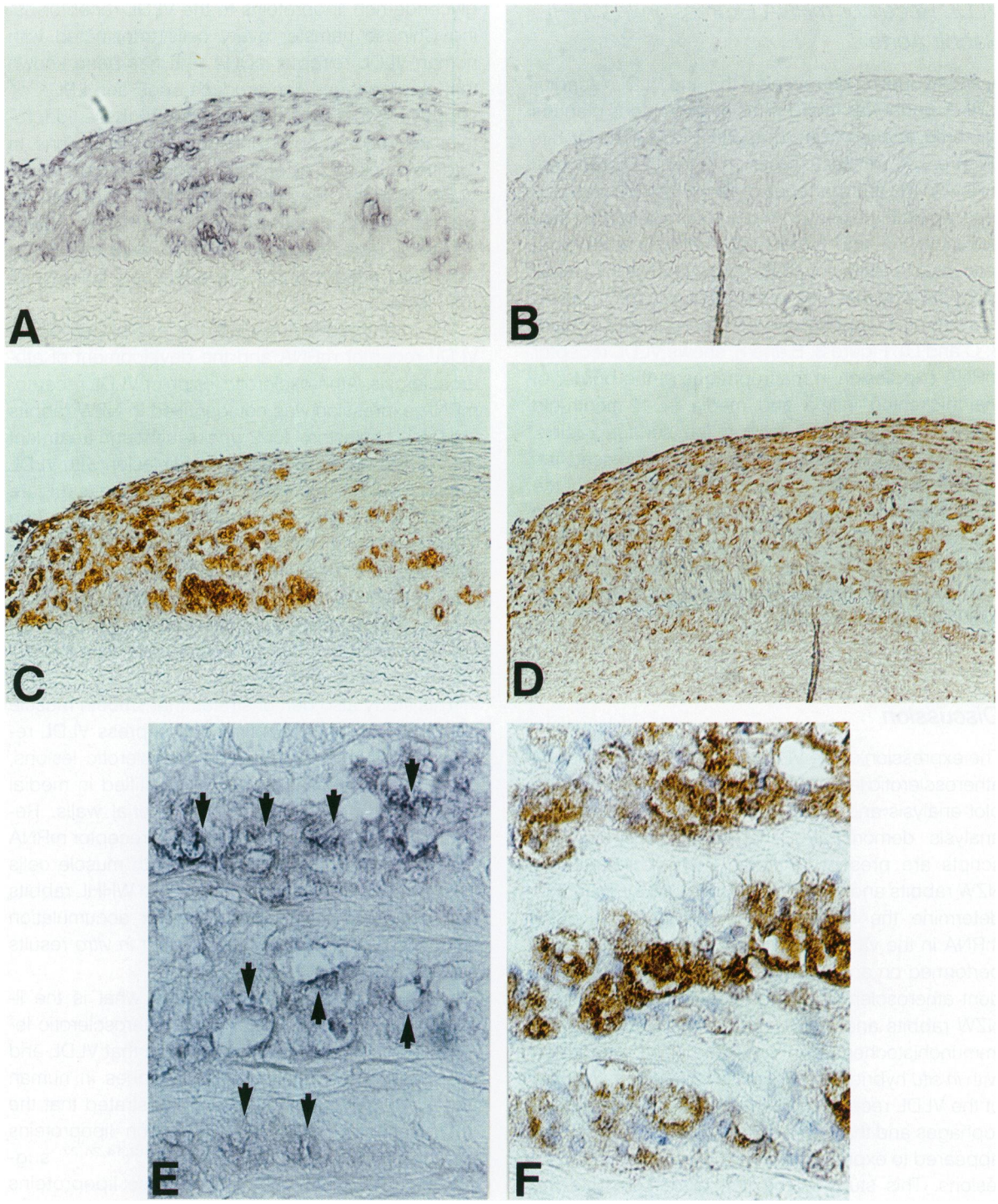


Figure 3. *Expression of the VLDL receptor mRNA in a 12-month-old WHHL rabbit atherosclerotic lesion (aortic arch).* **A** *and* **B:** In situ *hybridization of serial sections with the digoxigenin-labeled rabbit VLDL receptor antisense probe* (**A**) *and the nonhybridizing sense probe* (**B**). **C** *and* **D:** *Immunohistochemical staining of the serial sections with the antibodies against macrophages (RAM11;* **C**) *and smooth muscle α-actin (1A4;* **D**). *Magnification, ×100.* **E** *and* **F:** *Expression of the VLDL receptor mRNA by macrophages in the border region of thickening intima and media of an atherosclerotic lesion, as shown by* in situ *hybridization with the VLDL receptor antisense probe* (**E**) *and immunohistochemical staining with the antibody against macrophages* (**F**). **Arrows** *indicate the VLDL receptor mRNA-positive cells. Magnification, ×300.*

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