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Expression of Very Low Density Lipoprotein Receptor in the Vascular Wall

Analysis of Human Tissues by in Situ *Hybridization and Immunohistochemistry*

Hinke A. B. Multhaupt,* Mats E. Gåfvels,† Katalin Kariko,‡ Hao Jin,§ Carmen Arenas-Elliott,* Bruce I. Goldman,‖ Jerome F. Strauss III,¶ Bo Angelin,† Micheal J. Warhol,* and Keith R. McCrae§#

From the Department of Pathology and Laboratory Medicine, Pennsylvania Hospital, Philadelphia, Pennsylvania; the Department of Medicine and Molecular Nutrition Unit,† Center for Nutrition and Toxicology, Karolinska Institute at Huddinge University Hospital, Huddinge, Sweden; the Departments of Surgery‡ and Obstetrics and Gynecology,¶ University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and the Sol Sherry Thrombosis Research Center§ and the Departments of Pathology and Laboratory Medicine‖ and Medicine,# Temple University School of Medicine, Philadelphia, Pennsylvania*

The recently cloned very low density lipoprotein (VLDL) receptor binds triglyceride-rich, apolipoprotein-E-containing lipoproteins with high affinity. The observation that VLDL receptor mRNA is abundantly expressed in extracts of tissues such as skeletal muscle and heart, but not liver, has led to the hypothesis that this receptor may facilitate the peripheral uptake of triglyceride-rich lipoproteins. However, little information is available concerning the types of cells that express this receptor* in vivo. *As expression of the VLDL receptor in the vascular wall might have important implications for the uptake and transport of triglyceride-rich lipoproteins, and perhaps facilitate the development of atherosclerosis in hypertriglyceridemic individuals, we used* in situ *hybridization and immunohistochemistry to determine whether VLDL receptor mRNA and protein was expressed in human vascular tissue. We observed expression of the receptor by both endothelial and smooth muscle cells within normal arteries and veins, as well as within atherosclerotic plaques. In the latter, the VLDL receptor was also expressed by macrophage-derived foam cells. The widespread distribution of the VLDL receptor in vascular tissue suggests a potentially important role for this receptor in normal and pathophysiological vascular processes. (Am J Pathol 1996, 148:1985–1997)

The very low density lipoprotein (VLDL) receptor, the newest member of the low density lipoprotein (LDL) receptor gene family,[1] was first cloned by Takahashi and colleagues from a LDL receptor-subtracted rabbit heart cDNA library.[2] Cloning of homologous receptors from human,[3–6] rat,[7] and mouse[8,9] has subsequently been reported. These studies have demonstrated remarkable interspecies conservation (>95%) of the VLDL receptor cDNA sequence, suggesting an important physiological role for this receptor. At present, however, this role remains incompletely defined.

The VLDL receptor bears significant amino acid and structural homology to several other members of the LDL receptor gene family,[1] particularly the LDL receptor.[2,3,10] These receptors both possess five domain structures, which include a ligand-binding domain containing several ~40-amino-acid cysteine-rich repeats, an epidermal growth factor precursor-like domain, an extensively glycosylated serine- and

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Address reprint requests to Dr. Keith R. McCrae, Sol Sherry Thrombosis Research Center, MRB 112, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140.

threonine-rich domain immediately external to the transmembrane domain, and a cytoplasmic domain containing an NPVY sequence, which is required for receptor-mediated endocytosis via clathrin-coated pits.[11] However, an important structural difference between these receptors is the presence of eight cysteine-rich repeats in the ligand-binding domain of the VLDL receptor, in contrast to seven such repeats in the corresponding domain of the LDL receptor.[2,10] This additional cysteine-rich repeat confers upon the VLDL receptor a distinct ligand-binding specificity, as this receptor binds only apolipoprotein (apo)E-containing lipoproteins, whereas the LDL receptor binds those containing apoE and/or apoB-100.[10] In addition, a 39-kd protein that co-purifies with and inhibits the uptake of all known ligands by the LDL receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR)[12,13] and has been termed the LRP/α_2MR receptor-associated protein,[13,14] binds with high affinity to the VLDL receptor but only with low affinity (K_d ~250 nmol/L to the LDL receptor.[15–17] Finally, recent studies have demonstrated that, unlike the LDL receptor, the VLDL receptor mediates the uptake and degradation of additional ligands such as lipoprotein lipase[18,19] and urokinase-plasminogen activator inhibitor type 1 complexes.[18,20]

In addition to their ligand specificities, the tissue distribution of the VLDL and LDL receptors differs as well. Whereas VLDL receptor mRNA is most abundant in heart and skeletal muscle[2] and is also expressed in tissues such as kidney, placenta and brain, it is virtually absent from liver.[2,4,5,7–9] In contrast, LDL receptor mRNA is most abundant in liver, with lesser expression in peripheral tissues.[4,5,8,21] Such observations have led to the hypothesis that the physiological role of the VLDL receptor may be to mediate the peripheral uptake of triglyceride-rich, apoE-containing lipoproteins.[2,4,8–10] However, the lack of coordinate regulation between the VLDL receptor and lipoprotein lipase in cells that produce both of these proteins,[7,8] as well as the minimal alterations in VLDL receptor mRNA levels observed in rat skeletal muscle in response to fasting and refeeding, have led others to speculate that the major role of the VLDL receptor may involve activities independent of lipoprotein metabolism *per se*.[7] This hypothesis is supported by the lack of a significantly altered plasma lipoprotein profile in mice with homozygous deletion of the VLDL receptor gene.[22] In any case, additional information concerning the cellular distribution of this receptor *in vivo* may be of use in further evaluating such hypotheses, as few studies have addressed this issue directly. In one such report, Wittmack et al[23] detected VLDL receptor mRNA in choriocarcinoma and cultured human umbilical vein endothelial cells, and they also used *in situ* hybridization and immunohistochemistry to demonstrate the expression of VLDL receptor mRNA and protein, respectively, by human trophoblast *in situ*. A faint signal resulting from hybridization of the VLDL receptor cDNA probe with mRNA within villous stromal capillary endothelial cells was also detected, although these cells were not stained by anti-VLDL receptor antibodies. These findings have recently been extended by the demonstration that cultured endothelial cells express a functional VLDL receptor.[18]

Although the studies described above suggest that the VLDL receptor might be expressed by endothelium constituting at least some vascular beds, a detailed analysis of the expression of this receptor in normal and pathological specimens of human vascular tissue has not been performed. Addressing this issue may be of significant importance, as the vascular wall, particularly the endothelium, forms a barrier between plasma and parenchymal tissues that regulates the flux of lipoproteins between these compartments. *In vitro*, endothelial cells express LDL[24–29] and scavenger receptors,[29–31] and bovine and rabbit endothelial cells have also been reported, on the basis of ligand-binding studies, to express a receptor for β-VLDL.[29] However, scavenger receptors do not bind native lipoproteins,[32,33] and the expression of LDL receptors by contact-inhibited, confluent endothelium exposed to physiological lipoprotein concentrations is markedly down-regulated,[24–26] raising uncertainty as to the importance of these receptors in mediating lipoprotein uptake by endothelial cells *in vivo*.[27] Therefore, we have used fluorescent *in situ* hybridization and immunohistochemistry to characterize the expression of the VLDL receptor in both normal human vessels as well as atherosclerotic plaques.

Materials and Methods

Tissue Samples

Human umbilical cords and carotid atherectomy specimens were obtained following after deliveries and medically indicated carotid endarterectomies, respectively. Human temporal arteries were obtained from temporal artery biopsy specimens performed for evaluation of suspected temporal arteritis (only vessels in which this diagnosis was excluded were used). Sections of myocardium and normal aorta were obtained from the excised tissue of cardiac allograft recipients. All tissues were fixed in

formalin within 4 to 6 hours after removal and subsequently embedded in paraffin.

In Situ *Hybridization*

The template used to prepare the digoxigenin-labeled VLDL receptor DNA probe was a 3146-nucleotide VLDL receptor cDNA cloned from a human skeletal muscle cDNA library.[4] The cDNA was labeled by the random primer method[34] using the Ready to Go labeling kit (Pharmacia, Piscataway, NJ), with the reaction mixture supplemented with 20 μmol/L digoxigenin-labeled dUTP (Promega, Madison, WI). Labeled probe was purified using Sephadex G-50 spin columns (Boehringer Mannheim, Indianapolis, IN) and then precipitated with ethanol. *In situ* hybridization studies were performed using a modification of previously described methods.[35] Briefly, slides were deparaffinized, digested with proteinase K (Boehringer Mannheim; 5 μg/ml at 37°C for 30 minutes), and acetylated (0.1 mol/L triethanolamine in 0.25% acetic anhydride, pH 8.0, for 10 minutes at 25°C). Slides were then serially washed in 2X standard saline citrate (SSC), 70, 95, and 100% ethanol, chloroform, and 100% ethanol and then air dried. A hybridization solution containing 50% formamide, 10 mmol/L Tris, 1 mmol/L EDTA, 1x Denhardt's solution, 0.5% sodium dodecyl sulfate, 2x SSC, 0.2 mg/ml herring sperm DNA, and 20 ng of labeled probe was added, and the sections were heated to 89°C for 5 minutes before overnight incubation at 42°C in a humidified chamber. After hybridization, slides were washed by incubation in 2X SSC at 37°C for 30 minutes, then digested with RNAse A (5 μg/ml in 0.01 mol/L Tris, 0.5 mol/L NaCl, 1 mmol/L EDTA, pH 8.0) for 30 minutes at 37°C. Finally, slides were washed twice each with 2X SSC and 0.1X SSC at room temperature and incubated for an additional 10 minutes in 0.1X SSC. To detect bound probe, sections were first incubated in 2X SSC containing 2% normal sheep serum and 0.5% Triton X-100 (blocking buffer) and then incubated sequentially for 30 minutes each with fluorescein-isothiocyanate-labeled rabbit anti-digoxigenin, sheep anti-rabbit, and fluorescein-isothiocyanate-labeled rabbit anti-sheep antibodies, all diluted in blocking buffer (all antibodies were from Oncor, Gaithersburg, MD). Slides were then washed five times with phosphate-buffered saline (PBS) at room temperature, mounted in Antifade (Oncor), and viewed using an Olympus AH3 microscope equipped with epifluorescence. All studies included two negative control slides, one in which labeled VLDL receptor DNA was omitted and another in which the tissue under study was treated with RNAse A before the hybridization step.

DNA Sequence Analysis

To verify that digoxigenin-labeled DNA probes generated from random-primer labeling of the VLDL receptor cDNA would not cross-react with mRNA species encoding other members of the LDL receptor gene family, we analyzed the extent of homology between the cDNA sequences of the VLDL and LDL receptors and the VLDL receptor and LRP/α_2MR (see Results). Sequences were obtained from GenBank R86.0 (Hitachi Software, San Bruno, CA) using MacDNASIS version 3.2 (Hitachi Software). The cDNA sequences for the VLDL receptor and LRP/α_2MR (accession numbers L20470 and X13916, respectively) were obtained directly, whereas that of the LDL receptor was derived by linking together 18 exons of the genomic sequence (accession numbers K02573, L00338, and L00352). Amino acid and DNA homology was assessed using MegAlign (LaserGene, DNASTAR, Madison, WI). Dot matrix plots were generated using MacDNASIS. The melting temperatures of DNA oligonucleotides of various lengths was determined using OLIGO version 4.0 (National Biosciences, Plymouth, MN).

Production of VLDL Receptor Antiserum

The VLDL receptor antiserum used in this study was raised against a recombinant polypeptide containing the amino-terminal 160 amino acids of the receptor. The cDNA encoding this fragment was prepared using the polymerase chain reaction (forward primer 5′-CCGCGTGGATCCAAAGCCAAATGTGAACCCTCC-3′; reverse primer 5′-CACGATGAATTCACAGTCCAGCTCATCACTGCC), with the full length VLDL receptor cDNA as template. The polymerase chain reaction product was directionally subcloned into the prokaryotic expression vector pGEX2T (Pharmacia). Recombinant protein was expressed in *Escherichia coli* as a fusion protein with *Schistosoma japonicum* glutathione-S-transferase and purified by affinity chromatography using glutathione-agarose.[36] Rabbits were immunized with the 45-kd fusion protein dissolved in PBS and emulsified with Freund's complete adjuvant and bled 10 to 14 days after booster injections. Sera from five different rabbits were screened for their reactivity on immunoblots against solubilized membrane preparations prepared from VLDL-receptor-overexpressing ldl A7 cells,[15] and IgG from one of the five rabbits was found to recognize proteins of ~120 kd and ~95 kd within these

preparations. When tested against detergent extracts of whole endothelial cells, this antiserum recognized proteins of similar molecular weight and displayed a pattern of reactivity that was identical to that displayed by a previously described, affinity-purified antiserum raised against a synthetic peptide corresponding to the 20 carboxyl-terminal amino acids of the human VLDL receptor.[15] The sensitivity of the amino-terminal antiserum, however, was 5- to 10-fold greater than that of the carboxyl-terminal IgG (data not shown).

Immunofluorescent Staining and Immunohistochemistry

After *in situ* hybridization, selected sections were also examined by using immunofluorescent staining to identify the types of cells that hybridized with the labeled VLDL receptor DNA. Specific antibodies used in these studies included rabbit anti-von Willebrand factor (anti-vWF), the macrophage-reactive IgM_{κ} monoclonal antibody HAM-56[37] (both from Dako, Carpinteria, CA), and monoclonal anti-smooth muscle actin (IgG_{2a}; Biogenics, San Ramon, CA). These antibodies were used at dilutions of 1:1000, 1:100, and 1:2000, respectively. Bound primary antibody was detected using biotinylated goat anti-rabbit or horse anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA), followed by streptavidin-rhodamine (Boehringer Mannheim).

To assess the distribution of VLDL receptor protein, immunohistochemistry was performed using the amino-terminal VLDL receptor antiserum described above at a 1:50 dilution. The specificity of immunostaining was routinely assessed by performing parallel studies in which identical dilutions of nonimmune rabbit serum were substituted for the amino-terminal antiserum. Bound antibody was detected using the avidin-biotin complex (ABC) method, as previously described.[38,39] In selected studies, the expression of VLDL receptors by macrophage-derived foam cells within atherosclerotic plaques was assessed by double immunostaining. In these experiments, sections were concurrently incubated with the amino-terminal VLDL receptor antiserum and HAM-56. Sections were then washed, and HAM-56 binding was detected by sequential incubation of the slides with alkaline-phosphatase-conjugated horse anti-mouse IgG and the alkaline phosphatase substrate new fuchsin (Sigma Chemical Co., St. Louis, MO), which yields a red precipitate. Sections were washed again, and bound anti-VLDL receptor antibodies were detected using the ABC method[38,39] with diaminobenzidine (Sigma), which yields a brown precipitate, as the peroxidase substrate.

Results

Assessment of Amino Acid and Nucleotide Homology between the VLDL and LDL Receptors and LRP/α_2MR

Previous studies, employing Northern blotting techniques, have not demonstrated cross-reactivity between random-primer-labeled VLDL receptor cDNA with mRNA encoding other members of the LDL receptor gene family.[2,23] However, as the stringency conditions used for *in situ* hybridization studies differ from those employed for Northern blots, we extensively analyzed the extent of homology between the VLDL and LDL receptors and the VLDL receptor and LRP/α_2MR to verify that cross-reactivity of our digoxigenin-labeled DNA probe with mRNA encoding these other receptors would not occur. We observed a relatively high degree of amino acid homology (46.2%) between the VLDL and LDL receptors as well as between the VLDL receptor and LRP/α_2MR (24% overall, with local comparisons between the most homologous sub-segments of the two receptors revealing 37.5% homology). Despite these similarities, however, analysis of short sequences containing up to 11 consecutive, perfectly conserved amino acids, revealed poor conservation of corresponding DNA sequences. We also assessed the extent of overall homology between the nucleotide sequences encoding these receptors. These studies revealed a homology of 43% between the cDNA sequences of the VLDL and LDL receptors and 40.1% between the most highly conserved regions of the VLDL receptor (nucleotides 482 to 1129) and LRP/α_2MR (nucleotides 3020 to 3673). As two random DNA sequences would be expected to have ~25% homology, these results suggest that the DNA sequences encoding these three receptors are poorly conserved. Finally, by Clustral[40] and dot-plot analyses,[41] the longest uninterrupted DNA sequences shared between the cDNAs of the VLDL and LDL receptors and the VLDL receptor and LRP/α_2MR were determined to be 16 and 17 nucleotides, respectively.

We next assessed the length of complementary single-stranded DNA that would be capable of binding to target mRNA under the conditions of our *in situ* hybridization studies (42°C, 2X SSC, 50% formamide). We determined that an oligonucleotide with a sequence completely complementary to target

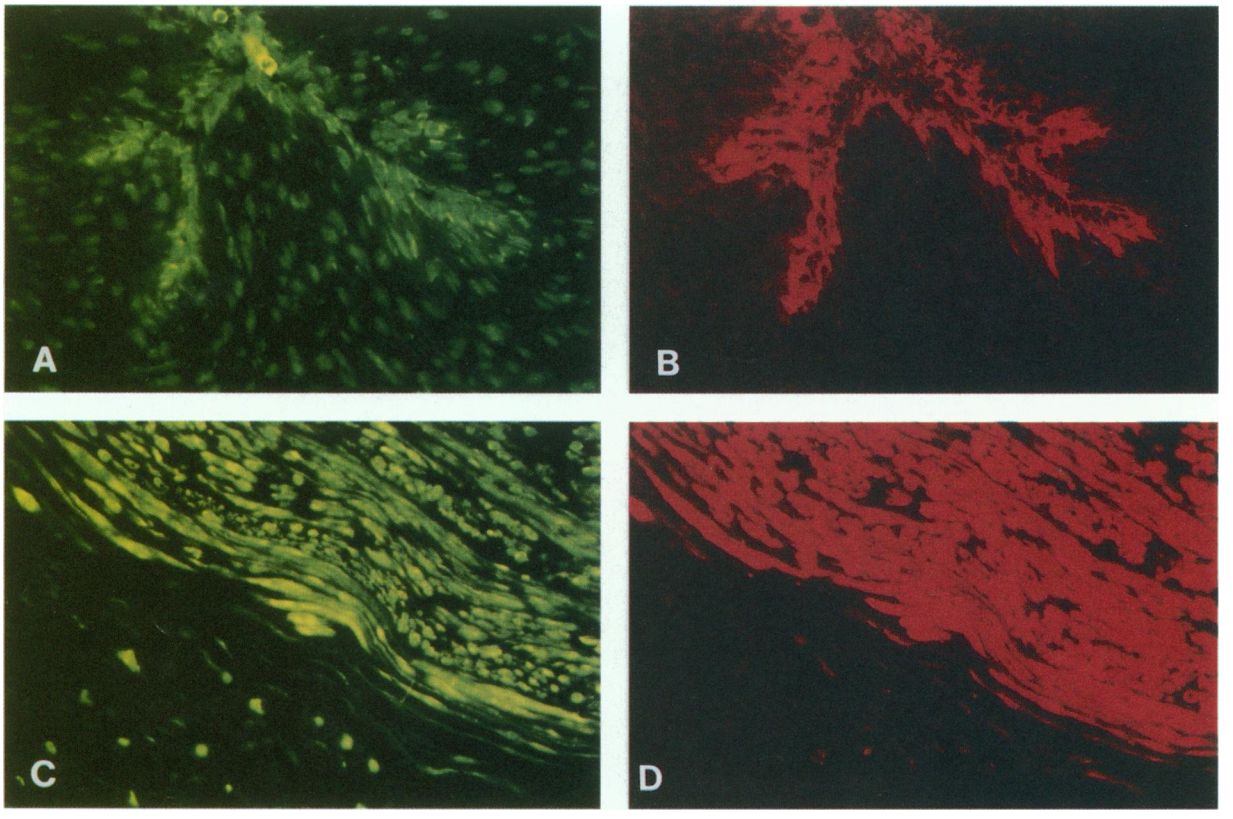

Figure 1. *Expression of VLDL receptor mRNA in the human umbilical vein.* **A** *and* **C**: *Fluorescent* in situ *hybridization using a digoxigenin-labeled VLDL receptor DNA probe. The presence of VLDL receptor mRNA in endothelium* (**A**) *and smooth muscle cells* (**C**) *was confirmed by co-staining sections with antibodies specific for vWF* (**B**) *and α-smooth muscle actin* (**D**). *Original magnification,* ×*50.*

mRNA would have to be at least 36 nucleotides in length to hybridize to its target under these conditions. As the maximal length of completely homologous nucleotide sequences shared by the VLDL and LDL receptors was 16 nucleotides, and between the VLDL receptor and LRP/α_2MR was 17 nucleotides, we concluded that the use of a labeled cDNA probe to specifically detect the presence of VLDL receptor mRNA in tissue sections was appropriate.

Detection of VLDL Receptor mRNA by in Situ *Hybridization*

In initial studies, we determined whether VLDL receptor mRNA was present in cells constituting the wall of the umbilical vein. After *in situ* hybridization of umbilical vein sections with labeled VLDL receptor probe, we observed a pattern of fluorescence that corresponded to the vascular lining of the collapsed vein (Figure 1A). Co-staining of sections with anti-vWF antibodies confirmed the impression that this pattern of fluorescence was largely due to the presence of VLDL receptor mRNA within the endothelial cells lining the umbilical vein (Figure 1B). In tangential sections, we also observed a circumferential layer of VLDL receptor mRNA-containing cells that encircled the vein (Figure 1C). These were determined to be smooth muscle cells, based on the observation that antibodies against α-smooth muscle actin co-localized precisely with the cells that contained VLDL receptor mRNA (Figure 1, C and D).

We next studied arterial tissue to determine whether a similar pattern of VLDL receptor mRNA expression was present in cells constituting the walls of these vessels. Figure 2A depicts a low power view of a section of temporal artery affected by early atherosclerotic change that was hybridized with digoxigenin-labeled VLDL receptor DNA. Hybridization of the DNA with a single layer of cells lining the vessel lumen as well as with a disordered array of cells within the neointima and a more orderly arrangement of cells constituting the vessel media was observed. Co-staining of this tissue with anti-vWF antibodies suggested that the VLDL receptor mRNA-containing cells lining the vessel lumen were endothelium (Figure 2B). Examination of sections at higher power revealed more distinctly the co-localization of VLDL receptor cDNA and anti-vWF antibodies (Figure 2, E and F). The identity of the neointimal and medial cells that contained VLDL receptor mRNA was confirmed in a similar fashion, by co-staining sec-

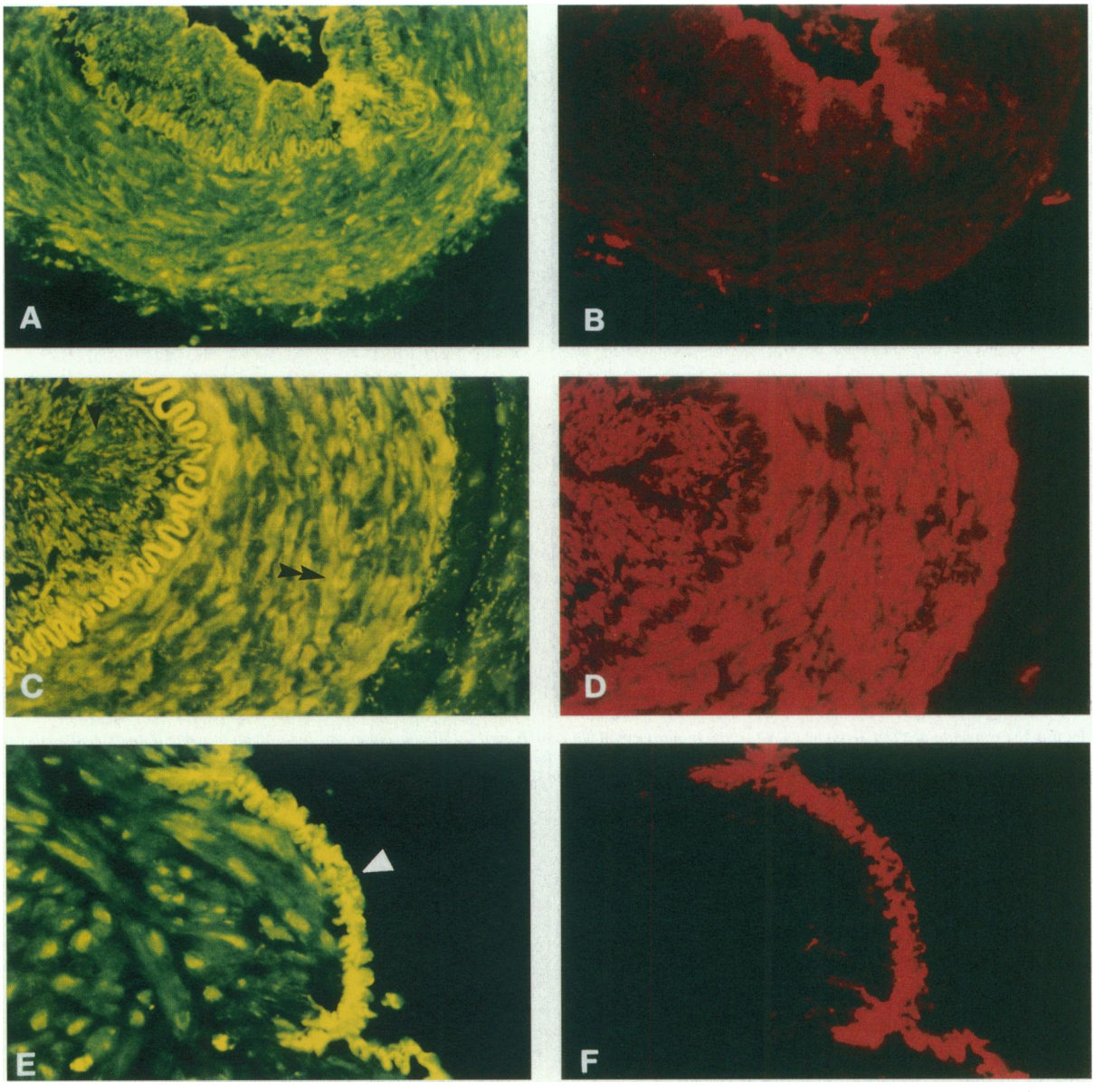

Figure 2. *Expression of VLDL receptor mRNA in atherosclerotic temporal artery.* **A:** *Fluorescent* in situ *hybridization using digoxigenin-labeled VLDL receptor DNA revealed abundant expression of VLDL receptor mRNA in the vascular wall. Note autofluorescence of the internal elastic lamina. Original magnification, ×25.* **C:** *Observation under higher power confirmed the presence of mRNA within both neointimal* (single arrowhead) *and medial* (double arrowhead) *smooth muscle cells. Original magnification, ×50.* **E:** *The presence of VLDL receptor mRNA within individual endothelial cells* (arrowhead). *Original magnification, ×100.* **B** *and* **F:** *Endothelial cells within these sections were localized by co-staining using antibodies specific for vWF. Original magnifications, ×25 and ×100, respectively.* **D:** *Smooth muscle cells were identified by co-staining sections with antibodies specific for α-smooth muscle actin. Original magnification × 50.*

tions hybridized with VLDL receptor cDNA with antibodies to smooth muscle actin. These studies revealed that the VLDL receptor mRNA-containing cells within both of these regions were indeed smooth muscle cells (Figure 2, C and D).

In addition to the tissues described above, a similar pattern of expression of VLDL receptor mRNA was observed in sections of normal aorta and coronary artery (not shown). VLDL receptor mRNA was also present in scalp arterioles (Figure 3), in small venules located in the periphery of the umbilical cord, and in capillaries from several sites. Finally, consistent with reports that have demonstrated the presence of abundant VLDL receptor mRNA within extracts of cardiac tissue, we detected VLDL receptor mRNA within the myocardium. In the latter, the hybridization pattern corresponded to that of individual myofibrils (Figure 4).

In addition to the DNA sequence analyses described above, the specificity of our *in situ* hybridiza-

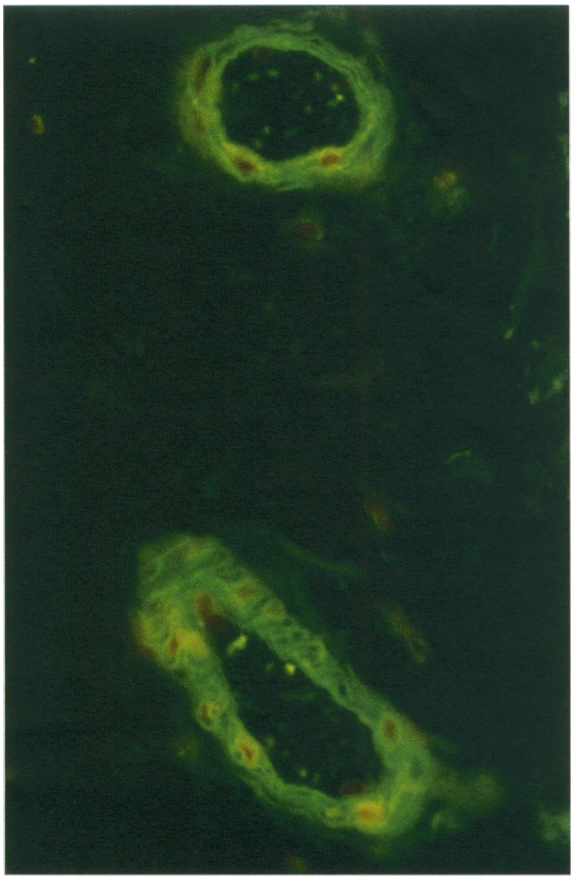

Figure 3. *Expression of VLDL receptor mRNA in scalp arterioles present within a temporal artery biopsy specimen. Nuclei were counterstained with propidium iodide. Original magnification, ×100.*

tion experiments is supported by several observations. First, digestion of sections with RNAse A before incubation with labeled VLDL receptor probe resulted in complete elimination of the positive hybridization signal, demonstrating that the probe hybridized specifically with cellular RNA. Second, Wittmack et al[23] have shown that ^{32}P-labeled DNA probes prepared from the same VLDL receptor cDNA used in these studies hybridized specifically with VLDL receptor mRNA on Northern blots. Third, *in situ* hybridization of the labeled probe with human liver, which contains abundant LDL receptor and LRP/α_2MR mRNA,[42] revealed the presence of VLDL receptor mRNA only within vascular structures and sinusoidal lining cells (presumably Kuppfer cells), with no signal detected in hepatocytes (not shown). These results are consistent with the low level of nucleotide homology between the VLDL and LDL receptors and the VLDL receptor and LRP/α_2MR and provide additional evidence that the digoxigenin-labeled DNA probe used in these studies hybridized specifically with VLDL receptor mRNA.

Immunohistochemical Detection of VLDL Receptor Protein

To determine whether the tissues that expressed VLDL receptor mRNA also expressed the receptor itself, we performed immunohistochemistry using a specific amino-terminal VLDL receptor antiserum. This antiserum stained human vascular tissues in a pattern identical to that observed when *in situ* hybridization was performed using VLDL receptor cDNA. Specifically, both venous and arterial endothelial and smooth muscle cells were consistently stained, with the staining pattern suggesting that the VLDL receptor was distributed on both the surface and within the cytoplasm of both cell types (Figure 5, A and B). The specificity of the observed staining was confirmed by demonstrating that normal rabbit serum used at identical dilutions as the VLDL receptor antiserum did not stain these tissues (Figure 5C). Furthermore, although the antiserum stained vascular structures, Kuppfer cells, and bile duct epithelium within the liver, no staining of hepatocytes was detected, demonstrating that, consistent with prior immunoblot

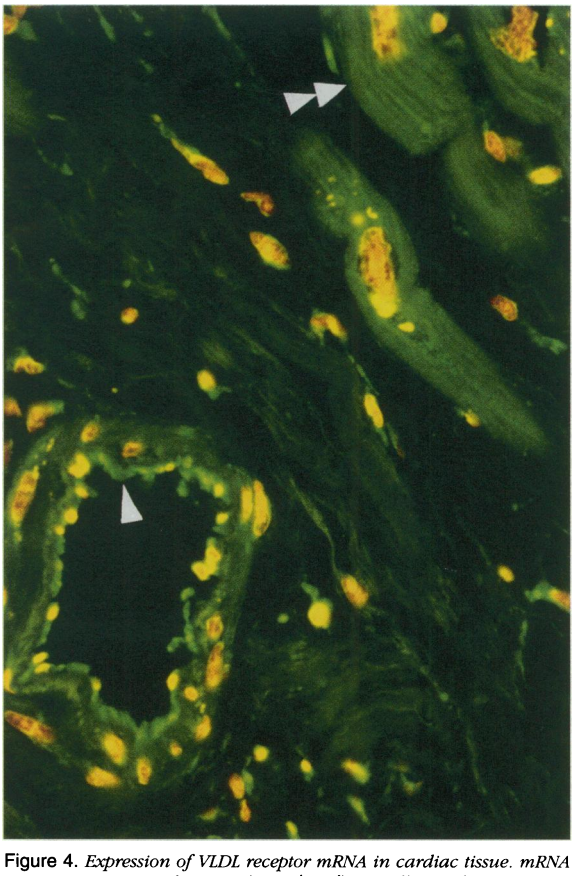

Figure 4. *Expression of VLDL receptor mRNA in cardiac tissue. mRNA was present in a cardiac vein* (**arrowhead**) *as well as within myocytes, in which the hybridization pattern correlated with that of individual myofibrils* (**double arrowheads**). *Nuclei were counterstained with propidium iodide. Original magnification, ×100.*

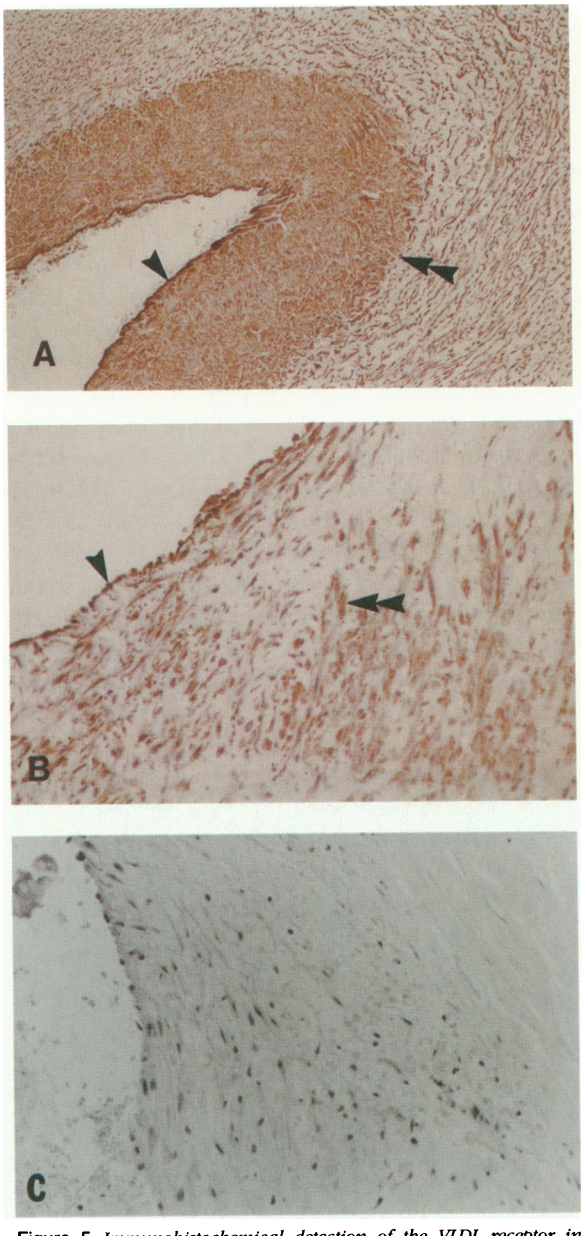

Figure 5. *Immunohistochemical detection of the VLDL receptor in human umbilical vein* (**A**) *and atherosclerotic carotid arteries* (**B** *and* **C**), *using an amino-terminal VLDL receptor antiserum. The receptor was expressed by endothelium lining both vessels* (**arrowheads**) *as well as by normal umbilical vein smooth muscle cells* (**A, double arrowhead**) *and carotid neointimal smooth muscle cells* (**B, double arrowhead**). **C:** *Control immunohistochemical stain in which an identical dilution of nonimmune rabbit serum was substituted for the amino-terminal anti-VLDL receptor antiserum. Original magnifications,* ×*10* (**A**) *and* ×*25* (**B** *and* **C**).

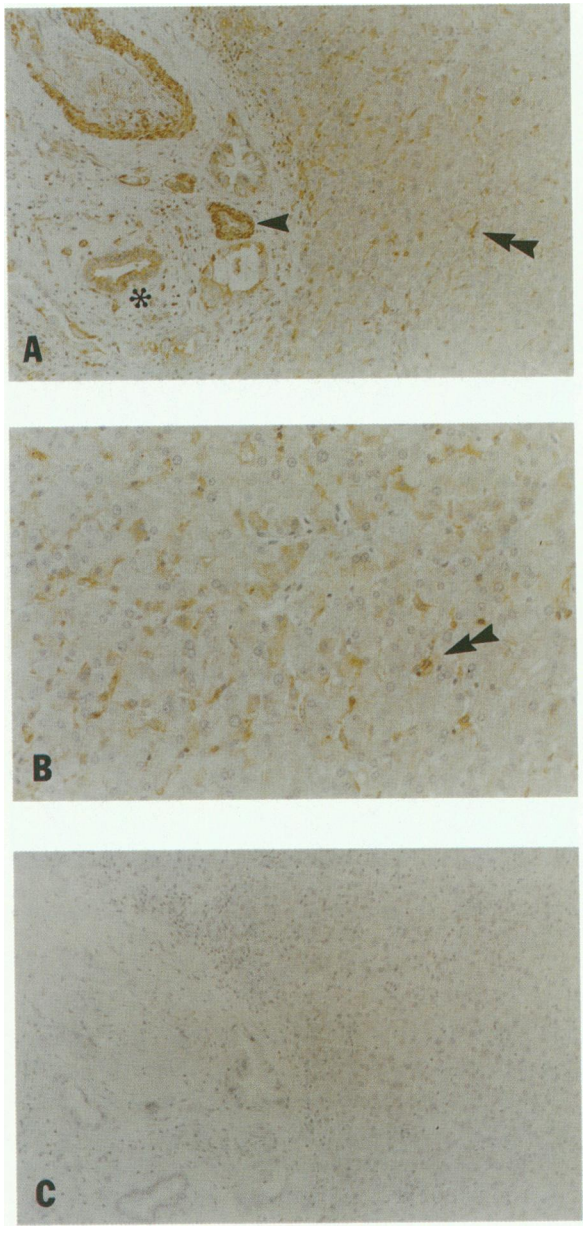

Figure 6. *Expression of the VLDL receptor in human liver.* **A:** *VLDL receptor antisera specifically stained vascular structures* (**single arrow**) *and Kupffer cells* (**double arrows**) *as well as bile duct epithelium* (**asterisk**). **B:** *Under higher magnification, staining of Kupffer cells is more easily observed.* **C:** *Negative control stain of the identical section depicted in* **A**, *in which an equal dilution of nonimmune rabbit serum was substituted for the VLDL receptor antiserum. Original magnifications,* ×*100* (**A** *and* **C**) *and* ×*200* (**B**).

analyses, the antiserum did not recognize hepatocyte LDL receptors or LRP/α_2MR (Figure 6). These observations also demonstrate that the anti-VLDL receptor antiserum did not recognize human glutathione-S-transferase, which is normally found in abundance in liver parenchyma.[43]

Finally, the amino-terminal antiserum was used to investigate whether the VLDL receptor was expressed by foam cells within atherosclerotic lesions. These cells were initially identified by their characteristic appearance in hematoxylin-and-eosin-stained sections of atherosclerotic carotid arteries (Figure 7A) and at least a subpopulation determined to be macrophage derived based upon their reactivity with HAM-56[37] (Figure 7C).

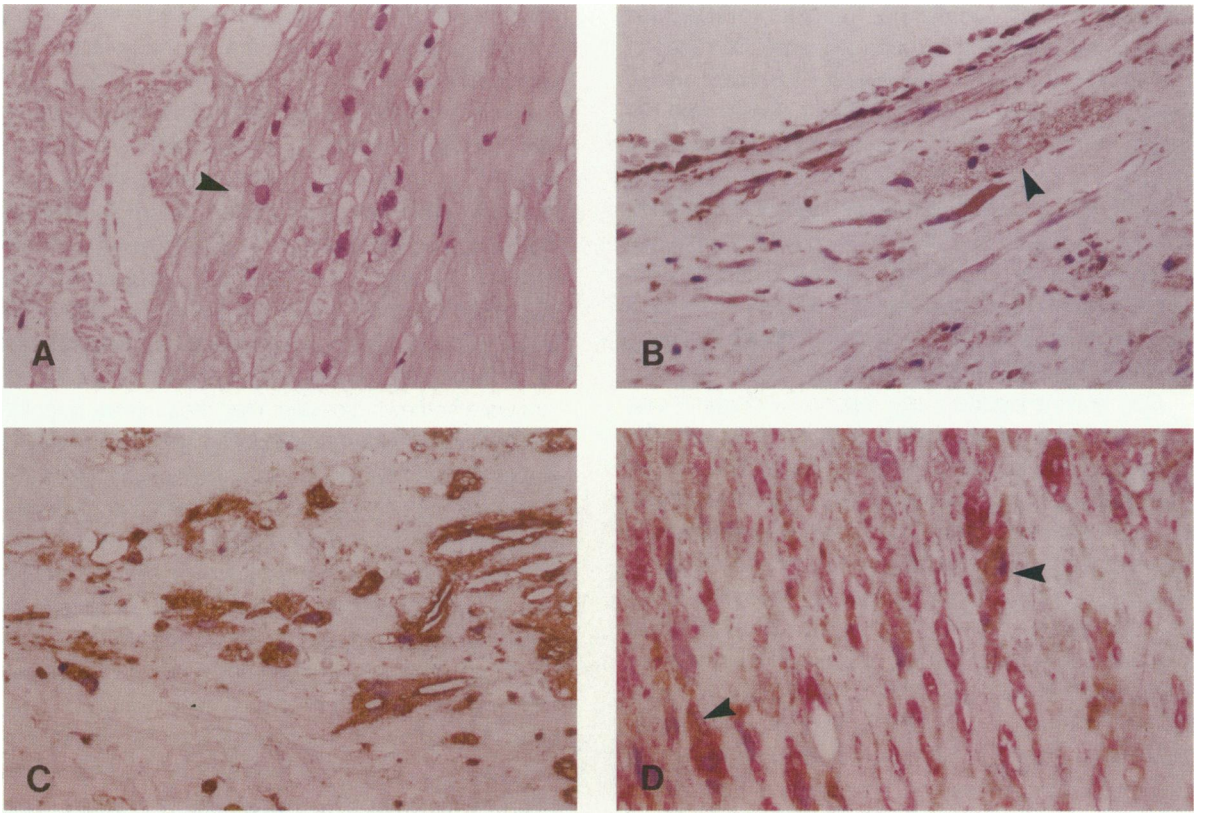

Figure 7. *Presence of VLDL receptor in macrophage-derived foam cells within an atherosclerotic carotid artery.* **A:** *Histological demonstration of foam cells* (**arrowhead**). *Hematoxylin and eosin stain.* **B:** *Immunostaining of foam cells with the amino-terminal VLDL receptor antiserum* (**arrowhead**). **C:** *Immunostaining of foam cells using the macrophage marker HAM-56.* **D:** *Double immunolabeling of foam cells using HAM-56 (red precipitate) and the amino-terminal VLDL receptor antiserum (brown precipitate). Co-localization of both precipitates on individual cells* (**arrowheads**) *confirms the expression of VLDL receptors by macrophage-derived foam cells within this plaque. Original magnification, ×100.*

Foam cells were also stained specifically by the VLDL receptor antiserum (Figures 7B), and double immunostaining experiments performed using both HAM-56 and anti-VLDL receptor antibodies confirmed that at least most of the VLDL receptor-expressing foam cells were indeed macrophage derived (Figure 7D).

Discussion

Our studies have demonstrated that both endothelium and smooth muscle cells composing human venous and arterial tissues express VLDL receptor mRNA and protein. The receptor was expressed in both normal vessels as well as within atherosclerotic plaques and was present in vessels of all calibers. Although the techniques employed in this study do not allow us to accurately quantitate the relative intensity of VLDL receptor expression in vessels from different sites, we hope to address this issue in future studies, with the goal of determining whether VLDL receptor expression is enhanced in the cytokine-rich milieu of the atherosclerotic plaque.[44]

Although an extensive literature exists concerning the expression of lipoprotein receptors by cultured endothelial and smooth muscle cells,[24–31] comparatively few studies have addressed the issue of which of these receptors are expressed by these cells *in vivo*, either within normal vessels or atherosclerotic plaques. The expression of acetyl-LDL receptor mRNA by macrophages within fatty streaks and atherosclerotic plaques has been reported by Yla-Herttuala et al,[45] who demonstrated co-localization of this mRNA species with 15-lipoxygenase mRNA and protein, suggesting a role for macrophage 15-lipoxygenase-induced oxidative modification of LDL in the pathogenesis of atherosclerotic plaque development.[45] However, these investigators did not detect LDL receptor mRNA within these lesions, possibly due to down-regulation of this receptor in response to elevated local cholesterol concentrations. In another study, Luoma et al[46] demonstrated the presence of LRP/α_2MR mRNA and protein in smooth muscle cells within normal aorta as well as within both smooth muscle cells and macrophages within atherosclerotic lesions. Consistent with the findings of Yla-

Herttuala et al,[45] scavenger receptor mRNA was present within lesion macrophages, and LDL receptor mRNA was absent from the plaque.[45] Similar findings were also recently reported by Lupu et al,[47] who detected LRP/α_2MR mRNA within smooth muscle cells of normal aorta and vasa vasorum as well as within smooth muscle cells and macrophages within atherosclerotic lesions. In contrast to previous observations,[48] however, these investigators detected LRP/α_2MR mRNA within endothelium, although endothelial expression of LRP/α_2MR protein could not be demonstrated using immunohistochemistry.[47]

Our findings extend these reports by documenting the expression of an additional lipoprotein receptor within the vascular wall *in vivo*. The current observations demonstrate that, in addition to LRP/α_2MR, smooth muscle cells within both normal and atherosclerotic vessels also express VLDL receptors. Furthermore, in addition to LRP/α_2MR and scavenger receptors, macrophage-derived foam cells within atherosclerotic plaques also express VLDL receptors. Finally, we have demonstrated the expression of both VLDL receptor mRNA and protein in endothelium from numerous vascular sites. Although our study is static and cannot determine with certainty the physiological functions of the VLDL receptor in the vascular wall, the detection of the receptor in this setting is a new observation that provides insight into its potential biological roles. For example, although it is unlikely that the endothelial expression of the VLDL receptor contributes significantly to the overall clearance of chylomicrons and remnants, it may nevertheless promote a limited, constitutive uptake of such triglyceride-rich lipoproteins into the vascular wall. Although chylomicrons have been shown to undergo lipoprotein lipase-mediated hydrolysis on the luminal endothelial surface,[49] the possibility that these and other apoE-containing lipoproteins may, to a limited extent, be directly taken up by the endothelium via receptor-mediated endocytosis has not been fully addressed. After such uptake, these lipoproteins might either undergo intracellular hydrolysis or be directly transported across the endothelium for subsequent uptake by smooth muscle or other parenchymal cells. This hypothesis is supported by additional studies of Yla-Herttuala, who extracted large (35-nm), triglyceride-rich lipoproteins, with the characteristics of β-VLDL, from lesion-free aortic tissue of young trauma victims.[50] Passage of such molecules into the vascular wall in an intact form suggests their direct uptake via a receptor-mediated endocytotic process.

Frykman et al[22] have recently demonstrated that homozygous disruption of the VLDL receptor gene in mice does not impair fertility or cause significant abnormalities in plasma lipoprotein concentrations. These findings, though surprising, are not inconsistent with our observations, for several reasons. First, our techniques are not quantitative, and it is likely, based on *in vitro* studies,[23] that the VLDL receptor is expressed in endothelium in relatively low abundance; therefore, its absence might not grossly affect the metabolism of triglyceride-rich lipoproteins. This hypothesis is consistent with the absence of the VLDL receptor from hepatic tissue and the belief that it does not play a significant role in lipoprotein clearance. Second, the observation that mice lacking functional VLDL receptors were somewhat smaller than their normal counterparts, and that this diminished size was primarily attributable to decreases in adipose tissue, might be explained not only by deficient uptake of triglyceride-rich lipoproteins at the end-organ level but also potentially by impaired transport of such moieties across the vascular wall and decreased delivery to peripheral tissues. However, it must also be appreciated that Jokinen et al[7] were unable to demonstrate VLDL receptor mRNA within mouse endothelial cells using *in situ* hybridization, and thus it is possible that the presence of this receptor on human endothelium is a species-specific phenomenon. This hypothesis will require more detailed investigation; nevertheless, we believe that our findings accurately depict the distribution of the VLDL receptor in human vascular tissues, particularly as they are supported by studies employing cultured human endothelial cells.[20,23]

It is also possible that in some individuals the VLDL receptor may be involved in pathological processes, such as atherosclerotic plaque formation. Taken together with the findings of this study, the observations that the VLDL receptor may mediate foam cell formation[51] and that its expression is not diminished by high cholesterol concentrations[3,10] suggest that the VLDL receptor might facilitate the uptake of excessive amounts of atherogenic, triglyceride-rich lipoproteins into the vascular wall in individuals with clinical syndromes associated with elevated levels of such lipoproteins, such as diabetes mellitus[52–56] or familial hypertriglyceridemia.[57] Indeed, the characterization of an endothelial receptor capable of mediating even limited uptake of chylomicrons or remnants may contribute to a better understanding of the relationship between atherosclerotic cardiovascular disease and postprandial hypertriglyceridemia originally proposed more than 40 years ago,[58] revived by Zilversmit,[59] and supported by data from numerous recent reports.[60–64] Finally, although the VLDL receptor has been found to be expressed by monocytic cell lines, such as THP-1 cells,[3] we believe that our studies are the first to dem-

onstrate the expression of this receptor by macrophages within atherosclerotic plaques.

Much additional work will be required to clearly define the role of the VLDL receptor in normal and abnormal vascular physiology. Indeed, the relative contribution of this receptor to the overall uptake of triglyceride-rich lipoproteins by cultured endothelial or smooth muscle cells has not yet been determined. Furthermore, the observation that lipoprotein lipase promotes the uptake of human VLDL by VLDL receptor-overexpressing ldl A7 cells[19] suggests that similar interactions between triglyceride-rich lipoproteins, the VLDL receptor, and lipoprotein lipase may also occur on the endothelium, which binds lipoprotein lipase with high capacity.[65,66] In addition, whether the ability of VLDL to mediate endothelial cytotoxicity,[67,68] enhance the secretion of plasminogen activator inhibitor type 1,[69,70] or promote the adhesion of monocytes[71,72] to endothelium are mediated through interactions with the VLDL receptor remains to be determined. Finally, recent studies[18,20] demonstrate that the VLDL receptor shares with LRP/α_2MR the capacity to internalize and degrade plasminogen activator-inhibitor complexes[73–75] and thus may be involved in processes such as the regulation of vascular wall fibrinolytic activity and angiogenesis. Our studies document the presence of this intriguing and potentially important receptor in the vascular wall and provide a framework for additional studies through which the roles of the VLDL receptor in this setting may be further examined.

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