Lipoprotein(a) Enhances Advanced Atherosclerosis and Vascular Calcification in WHHL Transgenic Rabbits Expressing Human Apolipoprotein(a)*

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High lipoprotein(a) (Lp(a)) levels are a major risk factor for the development of atherosclerosis. The risk of elevated Lp(a) concentration is increased significantly in patients who also have high levels of low density lipoprotein (LDL) cholesterol. To test the hypothesis that increased plasma levels of Lp(a) may enhance the development of atherosclerosis in the setting of hypercholesterolemia, we generated Watanabe heritable hyperlipidemic (WHHL) transgenic (Tg) rabbits expressing human apolipoprotein(a) (apo(a)). We report here that Tg WHHL rabbits developed more extensive advanced atherosclerotic lesions than did non-Tg WHHL rabbits. In particular, the advanced atherosclerotic lesions in Tg WHHL rabbits were frequently associated with calcification, which was barely evident in non-Tg WHHL rabbits. To investigate the molecular mechanism of Lp(a)-induced vascular calcification, we examined the effect of human Lp(a) on cultured rabbit aortic smooth muscle cells and found that smooth muscle cells treated with Lp(a) showed increased alkaline phosphatase activity and enhanced calcium accumulation. These results demonstrate for the first time that Lp(a) accelerates advanced atherosclerotic lesion formation and may play an important role in vascular calcification.

Since lipoprotein (a) (Lp(a)) was discovered in 1963 by Berg (1), numerous clinical, epidemiological, and genetic (cross-sectional and prospective) studies have revealed that high plasma levels of Lp(a) are associated with human cardiovascular disease, including coronary heart disease, stroke, and restenosis (2–4), although several studies have failed to demonstrate such an association (5, 6). The involvement of Lp(a) in the pathogenesis of atherosclerosis was suggested initially by the presence of Lp(a) in human atherosclerotic lesions (7, 8), and accumulating evidence indicates that Lp(a) deposition in atherosclerotic plaques is associated with the severity of unstable coronary syndrome (9). Nevertheless, the mechanism(s) by which Lp(a) increases the risk of atherosclerotic vascular disease are largely unknown.

The major difficulties in defining the functional roles of Lp(a) in vivo are attributed to the lack of appropriate experimental animals; Lp(a) is naturally present exclusively in Old World monkeys and humans, although one nonprimate species, the hedgehog, has independently evolved an Lp(a)-like protein (10). Four reports using Tg mice showed that apolipoprotein (a) (apo(a)) may increase aortic fatty streak formation when the apo(a) Tg mice are fed a high fat diet (11–14); however, two other studies failed to detect an atherogenic effect of apo(a) in Tg mice expressing either apo(a) alone or human apo(a) and apoB (15, 16).

Our laboratory (17) along with others (18) generated Tg rabbits expressing human apo(a) and showed that human apo(a) is efficiently assembled into Lp(a) in rabbit plasma; this is contrast to human apo(a) Tg mice, in which human apo(a) is not associated with murine LDL (19). Recently we reported that Lp(a) substantially increases the development of aortic and coronary atherosclerosis in Tg rabbits fed a cholesterol-rich diet (20, 21). Although these studies in Tg rabbits or in Tg mice revealed that apo(a), regardless of association with apoB (as in Tg rabbits) or the lack of such association (as in Tg mice), may be proatherogenic in the case of cholesterol-rich diet; they did not provide an answer to the question of whether Lp(a) increases the risk of advanced atherosclerotic lesion progression. This is a difficult issue to address in these models because the lesions formed in cholesterol-fed animals are basically fatty streak and defined as early-stage lesions, in contrast to the lesions in human atherosclerosis, which are more advanced and often associated with rupture and calcification (22). It is these advanced atherosclerotic lesions that increase the risk of ischemic stroke and coronary heart disease and produce many clinical manifestations. Another confounding factor is that the major atherogenic lipoproteins present in cholesterol-fed animals are heparically and intestinally derived remnant lipoproteins (so-called β-VLDLs) rather than LDLs as in humans (23).

To further study Lp(a) atherogenicity, we cross-bred human apo(a) Tg rabbits with WHHL rabbits, an animal model for human familial hypercholesterolemia, to produce apo(a) Tg WHHL rabbits. WHHL rabbits have defective LDL receptor...
Lesions with calcification. The apo(a) Tg WHHL model provides an ideal opportunity for gaining insight into the atherogenicity of Lp(a) because these rabbits have high plasma levels of LDL cholesterol on a chow diet and develop spontaneous atherosclerosis resembling that of humans (25). Moreover, Tg WHHL rabbits have 4-fold higher levels of Lp(a) in plasma than wild-type apo(a) Tg rabbits due to an LDL receptor defect (26). Finally, abnormalities from clinical studies shows that hypercholesterolemia patients have higher levels of Lp(a) than normal populations, and the risk of elevated Lp(a) concentration is increased significantly in the presence of high levels of LDL cholesterol (27, 28). Some researchers have suggested that elevated Lp(a) may be a risk factor for coronary heart disease only in patients with elevated LDL cholesterol levels (29).

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We characterized the atherosclerotic lesions in Tg WHHL rabbits: non-Tg (wild-type) homozygous WHHL rabbits previously (26). Using serial breeding, we obtained two groups of apo(a) were cross-bred with homozygous WHHL rabbits as described (26). In total, eight Tg and seven littermate non-Tg WHHL rabbits were obtained and studied at age 7–8 months.

Analyses of Plasma Lipids, Lipoproteins, and Atherosclerotic Lesions—The plasma lipid and lipoprotein profiles of Tg WHHL rabbits were compared with those of age-matched littermate non-Tg WHHL rabbits at age 7 months (30). The plasma Lp(a) in Tg WHHL rabbits was determined (26). The whole aorta was stained with Sudan IV for evaluation of the gross size of the atherosclerosis (20). For microscopic evaluation of the lesion areas of an aorta, each segment of an aorta was cut in cross sections from three nonoverlapping regions: the aortic arch, the thoracic aorta, and the abdominal aorta (21). All sections were embedded in paraffin and stained with hematoxylin-eosin and Elasticin-van Gieson. The intimas lesions in each section were measured using a computerized image analysis system and expressed as microscopical lesion areas.

To study cellular components and lipoprotein deposits in the lesions, we performed immunohistochemical staining as described (21). In addition, we obtained 12 autopsy coronary artery specimens from patients who died from acute myocardial infarction from the University Hospital of Teukuba and evaluated them for the interaction of calcification and Lp(a) deposition as described above.

In Vitro Study of Smooth Muscle Cells (SMC) Calcification—Rabbit aortic SMCs were obtained and maintained in minimum essential medium (Invitrogen) containing 20% fetal bovine serum and used between passages 3 and 7. Human plasma was obtained from healthy volunteers who were members of our laboratory, and human Lp(a) was isolated by sequential ultracentrifugation and gel filtration chromatography (Sepharcl S-400, Amersham Biosciences) as described previously (31). The isolated Lp(a) was assessed by Western blotting of proteins separated by SDS-PAGE using anti-apo(a) and anti-apoB antibodies. Lp(a) was confirmed to be free from endotoxin contamination (<10 pg/ml).

Cell-associated alkaline phosphatase activity of SMCs and 45Ca accumulation after treatment with Lp(a) were analyzed according to a method described by others (32). To visualize the calcium deposition method described by others (32). To visualize the calcium deposition, we performed immunohistochemical staining as described (21). In addition, we obtained 12 autopsy coronary artery specimens from patients who died from acute myocardial infarction from the University Hospital of Teukuba and evaluated them for the interaction of calcification and Lp(a) deposition as described above.

Reverse Transcription (RT)-PCR Analysis—The oligonucleotide primers used for quantitative RT-PCR were as follows: 1) osteopontin (OPN) 5'-TTC ACT GAA GTC GTT CCC AC-3' and 5'-TTT CAT ATT GGC TGG CAT CTT G-3'; 2) osteoblast-specific factor-2 (Osf2) 5'-ACA TAT TCC GGG AGA TCA TC-3' and 5'-ATT GTG TCT TCT CTT GTC TTC-3'; 3) matrix Gla protein (MGP) 5'-GCC TGC TGC TTC TCA CTT GTC TTC-3' and 5'-GTA CAT ATC ATC GTG CCC GC-3'; 4) β-actin 5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3'; and 5'-CAG ACT CCT CTT GTC TGA TCA ACA TCT GC-3'.

Total RNAs were isolated from SMCs using Trizol reagent (Invitrogen) and then analyzed by RT-PCR using a Qiagen OneStep RT-PCR kit. An aliquot of each RT-PCR mixture was electrophoresed on a 1.2% agarose gel and stained with Vistra Green (Amersham Biosciences).

The signal intensity of the RT-PCR products was determined using FluorImager 595 (Amersham Biosciences). The nucleotide sequences of the RT-PCR products were verified.

Statistical Analysis—Statistical significance was determined using Student’s t test for unpaired data of plasma lipids and in vitro studies. Lesion analyses were compared using the Mann-Whitney U-test for nonparametric analysis. In all cases, statistical significance was set at p < 0.05.

RESULTS

Plasma Lipids and Lipoproteins—On a regular chow diet, Tg and non-Tg WHHL rabbits developed similar hyperlipidemia (total cholesterol: 791 ± 141 mg/dl, Tg (n = 8) versus 717 ± 135 mg/dl, non-Tg (n = 7); triglycerides: 216 ± 52 mg/dl, Tg versus 250 ± 55 mg/dl, non-Tg, p > 0.05). The average plasma level of Lp(a) in Tg WHHL rabbits was 15.4 ± 2.2 mg/dl. Analysis of lipoprotein profiles by agarose gel electrophoresis revealed that WHHL rabbits, both Tg and non-Tg, showed a marked increase of β-migrating lipoproteins and a reduction of α-migrating lipoproteins in comparison to normal wild-type rabbits (Fig. 1A).

Non-denaturing polyacrylamide gel electrophoresis followed by immunoblotting showed that human apo(a) was associated with rabbit LDL to form Lp(a) complexes (Fig. 1B). Under non-reducing conditions, human apo(a) existed as a high molecular weight form co-localized with rabbit apoB or a lower molecular weight form without association with rabbit apoB, suggesting that Lp(a)-like particles (human apo(a)/rabbit apoB) in Tg rabbits are formed through both covalent (~20%) and noncovalent bondages (~80%) (Fig. 1C). Analysis of fractions of lipoproteins with different densities revealed that Tg WHHL rabbits had almost identical amounts of apoB, apoE, and apoAI to those of non-Tg rabbits (Fig. 1D). In Tg WHHL rabbits, human apo(a) was distributed mainly in the range of d = 1.02–1.08 g/ml (F3–F5), in which apoB-containing lipoproteins were also distributed (Fig. 1D). Of note, small amounts of human apo(a) were also found in lighter density fractions, such as in the VLDL (d < 1.006 g/ml, F1) and intermediate density lipoproteins (d = 1.006–1.02g/ml, F2) fractions, indicating that in addition to LDL, apo(a) can bind to other large apoB-containing particles. Quantification of plasma density fraction lipids showed that Tg WHHL rabbits had a similar lipoprotein profile pattern to that of non-Tg WHHL rabbits (data not shown).

Aortic Atherosclerosis—To make a topographic and spatial evaluation of lesion development, we measured sudanophilic en face lesion areas and microscopic lesion areas per section. The en face lesion area of the whole aorta was not significantly different between Tg and non-Tg rabbits: 27.7 ± 8.4%, Tg WHHL, n = 7 versus 26.6 ± 8.4%, non-Tg, n = 8, p = 0.3. The sudanophilic lesions in Tg WHHL rabbits were grossly thicker than those in non-Tg WHHL rabbits, therefore, the intimal lesions from each segment were further evaluated quantitatively under microscopy. The intimal lesion area in Tg WHHL rabbits was increased compared with that in non-Tg WHHL rabbits: there were 3.3-fold and 2-fold increases in the aortic arch (p < 0.01) and thoracic/abdominal aortas (p = 0.08), respectively (Fig. 2A). Under light microscopy, the morphological features of the atherosclerotic lesions of Tg WHHL rabbits differed markedly from those of non-Tg WHHL rabbits. In non-Tg WHHL rabbits, the atherosclerotic lesions were of fatty
streak type and thus were enriched in macrophage-derived foam cells with a small population of SMCs mixed in (Fig. 2B, upper panel). In sharp contrast, Tg WHHL rabbits developed more advanced lesions, including atheroma, fibroatheroma, and calcification (Fig. 2B, lower three panels). These lesions were covered by a layer of fibrotic cap and contained a necrotic or lipid core often associated with calcium deposition or calcification, and were thus defined as advanced atherosclerotic lesions. Immunohistochemical staining showed that the necrotic core contained macrophage-derived foam cells, whereas the thick fibrous caps were composed mainly of SMCs (Fig. 2B).

Because the lesion quality was dramatically different between Tg and non-Tg WHHL rabbits, we further quantified the area occupied by each type of lesion in the arch and compared the lesion distribution of Tg WHHL to that of non-Tg WHHL rabbits. For this purpose, we arbitrarily divided the lesions into three categories based on the American Heart Association classification (22): fatty streak (Type II), fibrous plaque, including both atheroma (Type IV) and fibroatheroma (Type Va), and complicated plaque, which contains either calcium deposition or calcification (Type Vb). As shown in Fig. 2C, the absolute lesion area of the fatty streak was not significantly different between Tg and non-Tg WHHL rabbits; however, the lesion areas of fibrous plaque and advanced lesions (Type IV and V) were strikingly greater in Tg than in non-Tg WHHL rabbits.

In this study, we were particularly interested in the striking vascular calcification associated with the advanced lesions in Tg WHHL rabbits since in some lesions of Tg WHHL rabbits, vascular calcification may evidently potentiate lesion erosion or rupture (Fig. 3A). Immunostaining with antibodies against apo(a) and apoB showed that apo(a) was frequently deposited around the calcified areas and co-localized with apoB (Fig. 3B). Apo(a) was also found in the lipid core of fibrous plaques in Tg WHHL rabbits, which is often associated with calcification (Fig. 3C). X-ray analysis of the whole aorta revealed that in Tg WHHL rabbits, there were increased calcified sites in the aorta, especially in the lesion-prone area (the intercostal ostia) compared with non-Tg WHHL rabbits (Fig. 3D). To make a quantitative evaluation of the increased calcification of the aorta in Tg WHHL rabbits, we scanned the x-ray films and measured the high-density area. As shown in Fig. 3E, Tg WHHL rabbits had apparently more high-density sites than did non-Tg WHHL rabbits.

**Lp(a) Effects on Calcification in SMCs**—To address the issue of whether Lp(a) may act as a potential regulator of the acceleration of vascular calcification found in Tg WHHL rabbits, we examined the effects of human Lp(a) on the calcium accumulation rate in cultured SMCs. Incubation with Lp(a) at physiological concentrations led to significantly increased calcium incorporation in SMCs, and this effect was dose- and time-dependent (Fig. 4A). Furthermore, SMCs treated with Lp(a) showed increased mRNA expression of a calcium-binding protein, MGP, at 2 and 4 days (Fig. 4B, left). OPN expression was slightly increased at 2 days but declined significantly at 4 days

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**Fig. 1. Analysis of plasma lipoproteins.** A, agarose gel electrophoresis of the plasma from chow-fed WHHL and normal rabbits. Plasma (2 μl) was either stained with neutral lipids (top) or subjected to Western blotting with monoclonal antibodies against human apo(a) (bottom). B and C, immunoblotting analysis of Tg WHHL rabbit and human plasma apo(a). Aliquots of plasma were separated by either 3.5% non-denaturing polyacrylamide gel electrophoresis (B) or 4% SDS-PAGE under non-reducing (C, left) or reducing (C, right) conditions. The same immunoblot membranes were reprobed with apoB Ab after stripping. * indicates Lp(a) formed through covalent binding between human apo(a) and rabbit apoB; ** indicates noncovalently associated Lp(a). D, seven density fractions (F1–F7) of plasma lipoproteins were separated and resolved by 1% agarose gel electrophoresis. F1, d < 1.006 g/ml; F2, d = 1.006–1.02 g/ml; F3, d = 1.02–1.04 g/ml; F4, d = 1.04–1.06; F5, d = 1.06–1.08; F6, d = 1.08–1.10 g/ml; F7, d = 1.10–1.21 g/ml. Lipoproteins were stained with Fat Red 7B, and apolipoproteins were detected by immunoblotting with specific antibodies (30). Immunoblots were scanned using a GS-700 imaging densitometer (Bio-Rad), and proteins contents were compared.

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**Fig. 2.** Immunohistochemical analysis of Tg WHHL rabbit and human plasma apo(a). Aliquots of plasma were separated by either 3.5% non-denaturing polyacrylamide gel electrophoresis or 4% SDS-PAGE under non-reducing or reducing conditions. The same immunoblot membranes were reprobed with apoB Ab after stripping. * indicates Lp(a) formed through covalent binding between human apo(a) and rabbit apoB; ** indicates noncovalently associated Lp(a). D, seven density fractions (F1–F7) of plasma lipoproteins were separated and resolved by 1% agarose gel electrophoresis. F1, d < 1.006 g/ml; F2, d = 1.006–1.02 g/ml; F3, d = 1.02–1.04 g/ml; F4, d = 1.04–1.06; F5, d = 1.06–1.08; F6, d = 1.08–1.10 g/ml; F7, d = 1.10–1.21 g/ml. Lipoproteins were stained with Fat Red 7B, and apolipoproteins were detected by immunoblotting with specific antibodies (30). Immunoblots were scanned using a GS-700 imaging densitometer (Bio-Rad), and proteins contents were compared.
In addition, Lp(a) tended to stimulate SMCs toward osteoblastic differentiation by inducing Osf2 mRNA expression accompanied by increased cellular alkaline phosphatase activity (Fig. 4C). When SMCs were incubated in the presence of Lp(a) at the relatively high concentration of 30 mg/dl for 3 days, the SMCs showed an increased number of nodular formations (Fig. 4D) compared with the control. After 13 days of incubation, calcium deposition was clearly demonstrated by von Kossa staining (Fig. 4E).

**Lp(a) Deposition and Calcification in Human Atherosclerosis**—The finding that Tg WHHL rabbits had more extensive advanced lesions and the capacity of Lp(a) to induce calcium deposition in cultured SMCs prompted us to examine whether calcification is associated with Lp(a) deposition in the vascular wall of human atherosclerosis. Three representative coronary atherosclerosis specimens from different patients showed a varying degree of calcification, and we found that Lp(a) was detected in the vicinity of areas with either diffuse calcification.
or ossification (Fig. 5, A and B) or sparse calcium deposition associated with cellular components (Fig. 5 C).

**DISCUSSION**

In this study, we demonstrated for the first time that Lp(a) enhances advanced lesion development in Tg WHHL rabbits. Tg WHHL rabbits have high levels of plasma LDL cholesterol, as do non-Tg WHHL rabbits, but they also have relatively high levels of human Lp(a). In this regard, one could state that the two models (apo(a) Tg and non-Tg WHHL) enabled us to compare the atherogenicities of one risk factor versus two risk factors, namely high LDL cholesterol alone and both high LDL cholesterol and Lp(a) levels. Tg WHHL rabbits showed increased atherosclerotic lesions in the aortic arch and thoracic/abdominal aortas, respectively, in comparison to non-Tg WHHL rabbits, and their lesions consisted of advanced lesions, including atheroma, fibroatheroma, and calcification. The finding of more extensive advanced atherosclerotic lesions in Tg WHHL rabbits not only supports the notion that Lp(a) is a risk factor for the development of atherosclerosis but also strengthens the prevailing view that the risk of hypercholesterolemia for atherosclerosis is increased significantly in the setting of elevated Lp(a). It is surprising that Lp(a) does not lead to a significant increase in the extent of sudanophilic lesions in Tg WHHL rabbits, which is different from what we found in cholesterol-fed apo(a) Tg rabbits (20). We believe that this discrepancy between cholesterol-fed and WHHL Tg rabbits may be due to differences in their atherogenic particles (large β-VLDL versus small LDL) (33), or it is possible that the very high plasma levels of LDL in WHHL rabbits may overwhelm the effect of Lp(a) on the early stage lesion formation. In humans, Lp(a) levels over 30 mg/dl are considered to be atherogenic (34). Even though the plasma Lp(a) levels (15 mg/dl) in Tg WHHL rabbits are lower than this arbitrary threshold, this level is still significantly proatherogenic in rabbits, which normally do not have endogenous Lp(a). The lipoprotein profiles of Tg and non-Tg WHHL rabbits were essentially identical, and the amounts of cholesterol and apoB in LDLs do not appear to explain the occurrence of increased advanced atherosclerosis in Tg WHHL rabbits. Therefore, it is likely that the presence of

**FIG. 4.** Effects of Lp(a) on calcium deposition in SMCs. A, Lp(a) increases calcium deposition in a dose- and time-dependent manner. Rabbit aortic SMCs grown to confluency were treated with the indicated concentrations of Lp(a) for 48 h (left panel), or were incubated in the presence of Lp(a) 10 mg/dl or a similar amount of albumin for the indicated time periods (right panel). *, p < 0.01, versus the control. B, effects of Lp(a) on MGP (left) and OPN (right) mRNA expression. SMCs were incubated in the presence of 10 mg/dl Lp(a) for the indicated time periods. Results are presented as the ratio of MGP to β-actin and OPN to β-actin. *, p < 0.05 versus the control. C, Lp(a) enhances expression of Osf2 mRNA (left) and cellular alkaline phosphatase activity (right) in SMCs. SMCs were incubated in the presence of the indicated concentrations of Lp(a) for 3 days. Osf2 mRNA expression was measured by quantitative RT-PCR analyses. Results are presented as the level of Osf2 relative to the control. Gel electrophoresis of the PCR product is shown on the top. *, p < 0.01 versus the control. D, SMCs treated with 30 mg/dl Lp(a) for 3 days showed an increased number of nodular formations (right). Scale bar represents 200 μm. E, von Kossa staining reveals calcium deposits after SMCs were treated with Lp(a) for 13 days (right). Scale bar represents 200 μm.

**FIG. 5.** Intimate association of Lp(a) with calcification in human coronary arteries. A, Lp(a) deposition along the surface of calcified area on the right side (indicated with arrowheads). B, Lp(a) around ossification. C, in this lesion, calcium deposition is sparsely distributed beneath the foam cells and Lp(a) deposition is intermingled with SMCs. Scale bars represent 200 μm.
plasma Lp(a) was the major factor underlying the significant enhancement of the lesions in Tg WHHL rabbits. The presence of lipid core and a layer of fibrous cap in these advanced lesions in Tg WHHL mimics the features in human atherosclerosis (22). An important finding of the current study was the demonstration of striking vascular calcification in Tg WHHL rabbits, which was barely noted in non-Tg WHHL rabbits. We have excluded the possibility that calcification in Tg WHHL rabbits may be caused by increased plasma phosphate or calcium and/or increased alkaline phosphatase activity (data not shown). It is noteworthy that vascular calcification has not been observed in either human apo(a) Tg mice (11) or cholesterol-fed Tg rabbits (20). In normal rabbits fed a diet containing fairly high cholesterol (2%), only calcifiable matrix vesicles, rather than calcified lesions, were reported in aortas (35), whereas wild-type WHHL rabbits were found to develop vascular calcification when fed a diet containing high cholesterol, vitamin D, and calcium (36) or upon reaching average age of over 15 months. Vascular calcification is a common feature of human atherosclerotic lesions and contributes to a multitude of clinical problems such as coronary heart disease and aortic ruptures (37). In this aspect, the lesions of Tg WHHL rabbits share many features of the calcification seen in human advanced atherosclerosis, and thus they may serve as an ideal model for studying vascular calcification associated with atherosclerosis. The presence of calcification and the intimate association between Lp(a) and calcification in the lesions of Tg WHHL rabbits led us to propose a hypothesis that Lp(a) is a potential mediator of the process of vascular calcification. On a preliminary basis (three representative specimens from the 12 patients), we also found that Lp(a) deposition is closely localized in the calcified areas of atherosclerotic lesions. It is important to note that Yamada recently reported that oxidized Lp(a) is frequently deposited in calcification-associated atherosclerotic lesions by analyzing carotid arterial specimens from eight patients. The etiology of vascular calcification has been now recognized as an active process rather than an end-stage, passive, or degenerative process of aging (38, 39).

A critical question is whether Lp(a) can really induce calcification in vascular cells such as SMCs. To address this issue, we have taken several steps to examine the effect of Lp(a) on calcification of cultured SMCs in vitro. First, treatment of SMCs with Lp(a) at physiological concentrations resulted in an enhanced calcium accumulation in a dose- and time-dependent manner. Secondly, Lp(a) significantly up-regulated MGP mRNA expression, whereas it down-regulated OPN expression of SMCs at 4 days. This pattern of osteogenic protein expression (increased MGP and decreased OPN) is compatible with the notion that calcification of SMCs is associated with high levels of MGP and low levels of OPN in human vascular SMCs (40). Furthermore, Lp(a) may enhance calcification by inducing the differentiation of SMCs to osteoblasts since Osf2 was up-regulated and alkaline phosphatase activity was concomitantly increased in SMCs treated with Lp(a). Taken together, these results strongly suggest that Lp(a) may participate in the process of vascular calcification. In Tg WHHL rabbits, about 20% of Lp(a) was covalently linked with apoB, and remaining Lp(a) was noncovalently associated, which is consistent with the previous studies (18, 20). This may suggest that although rabbit apoB lacks a compatible Cys-4326, which is required for covalent binding with apo(a) in humans, there may be another cysteine residue in rabbit apoB for such covalent linkage. It is currently unknown whether apo(a) needs to be associated with apoB either in covalent or noncovalent linkage to exert its functions such as inducing calcification. In a separate study, we have found that Lp(a) isolated from Tg rabbits showed a similar effect on SMC calcium uptake to human Lp(a). Further studies are needed to address free apo(a) versus Lp(a), covalently associated Lp(a) versus noncovalently associated Lp(a), and native Lp(a) versus oxidized Lp(a) regarding the effect on calcification.

While the mechanism(s) of the increased formation of advanced atherosclerotic lesions caused by Lp(a) in Tg WHHL rabbits remains unclear, the presence of necrotic or lipid cores in the lesions indicates that cell death, either necrosis or apoptosis, occurs, which raises an intriguing question as to whether Lp(a) may directly or indirectly induce such cell death or whether cell death is also involved or required in the subsequent calcification process. Previous studies showed that Lp(a) increases the production of superoxide in monocytes (41) and induces apoptosis in human endothelial cells and rabbit aorta (42). If this mechanism is really operating in vivo, it may certainly help explain the findings observed in Tg WHHL rabbits. Consistent with this possibility, some studies using cultured SMCs showed that apoptosis precedes SMC calcification, and apoptotic bodies are capable of initiating vascular calcification (43).

In conclusion, our current study provides further evidence that Lp(a) is not only a risk factor for the initiation of atherosclerosis but is also an inducer of the acceleration of the progression of lesion development. Furthermore, we have shown that Lp(a) is a potential regulator in the vascular calcification associated with atherosclerosis. Whereas the molecular mechanism(s) of Lp(a) atherogenicity and calcification are not fully understood, the occurrence of advanced atherosclerosis in Tg WHHL rabbits will undoubtedly prove useful for the study of human atherosclerosis and its complications such as plaque rupture and atheromus. In future studies, it will be interesting to determine whether Tg WHHL rabbits have increased incidence of myocardial infarction. We believe that the Tg WHHL model with high levels of Lp(a) and advanced atherosclerosis may be an extremely useful model for studying the benefits of some drugs in the treatment of atherosclerosis in patients with high levels of Lp(a).

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