Identification of Macrophage Arginase I as a New Candidate Gene of Atherosclerosis Resistance

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Objective—Our laboratory has previously created 2 strains of rabbits with genetically determined high-atherosclerotic response (HAR) and low-atherosclerotic response (LAR). The aim of the present study was to identify new genes of atherosclerosis susceptibility in macrophages from the 2 strains.

Methods and Results—Suppression subtractive hybridization was used to screen for genes with higher expression in macrophages from LAR rabbits. We identified a cDNA fragment with high homology to human arginase I (AI; 91%) and subsequently cloned the full-length cDNA of the rabbit homologue. Quantitative RT-PCR revealed a significantly higher macrophage AI mRNA expression in LAR rabbits than in HAR rabbits (77428±10941 versus 34344±4538; P=0.002; copies/10^6 copies β-actin), which also correlated with a significantly higher arginase enzyme activity. Northern blot analysis led to the identification of a size polymorphism of AI mRNA. This was because of a 413 bp C-repeat insertion in the 3’ untranslated region. The shorter transcript variant was predominantly expressed in LAR rabbits and associated with significantly higher AI mRNA expression levels. Transfection experiments indicated decreased mRNA stability of the long AI variant.

Conclusions—High expression of arginase I in macrophages may contribute to atherosclerosis resistance of LAR rabbits, possibly by conferring antiinflammatory effects in the vessel wall. (Arterioscler Thromb Vasc Biol. 2006;26:365-371.)

Key Words: macrophages ■ gene expression ■ arginase I ■ animal models ■ atherosclerosis

Atherosclerotic cardiovascular disease is one of the leading causes of morbidity and mortality in much of the world today. Atherosclerosis is a complex condition, resulting from genetic and environmental contributions.1 Identifying the genetic factors of disease susceptibility is a current challenge requiring animal models, as well as clinical studies. In the last decade, induced mutant mouse models have been created and used to study the genetic mechanisms of lesion formation. Like in humans, susceptibility to atherosclerosis was largely dependent on the genetic background. For example, when crossed to the low-density lipoprotein (LDL) receptor–deficient background, C57BL/6 mice were susceptible to atherosclerosis, whereas FVB/N mice were atherosclerosis resistant.2 Our laboratory has previously created 2 strains of rabbits with genetically determined high- (HAR) and low- (LAR) atherosclerotic response to diet-induced hypercholesterolemia.3 When fed a cholesterol-containing diet, both strains of rabbits develop comparable cholesterol levels of ≈30 mmol/L, and no differences were found in the lipoprotein profile.3,4 However, the 2 strains differed markedly in their susceptibility to develop aortic atherosclerosis. Surface involvement was >70% in HAR rabbits compared with <30% in LAR rabbits.3 In earlier studies, we investigated whether both strains differ in known mechanisms and candidate genes of atherosclerosis. No difference was observed in cell-mediated LDL oxidation and the enhancement of monocyte adhesion to arterial endothelial cells with β-very low-density lipoprotein from LAR and HAR rabbits.4 However, the aortae of HAR rabbits showed greater expression of vascular cell adhesion molecule 1.5 Cultivated aortic smooth muscle cells of HAR rabbits responded with significantly greater induction of scavenger receptor activity when challenged with either phorbol 12-myristate 13-acetate or platelet-derived growth factor BB. In contrast, macrophages from atherosclerosis-resistant LAR rabbits showed significantly higher scavenger receptor expression and activity, paralleled by a higher apolipoprotein E expression.6 Until now, a systematic analysis to elucidate the genetic basis for differences in the susceptibility to atherosclerosis has not been performed in LAR and HAR rabbits.7 Linkage mapping was not feasible, because the 2 strains were outbred, and only few genetic markers have been mapped in the rabbit.7 Another approach to study genetic variation between the 2 strains is expression analysis. In contrast to various other species,
expression arrays are not available for rabbits. Therefore, we used suppression subtractive hybridization (SSH), an unbiased method that allows for identification of differentially expressed genes between 2 cDNA populations and focused on macrophages, because these cells play a key role in atherogenesis. Thus, the aim of the present study was to identify new candidate genes of atherosclerosis susceptibility in macrophages from LAR and HAR rabbits.

Methods

Animals
The 2 strains of rabbits (LAR and HAR) were created by selective outbreeding of progeny of New Zealand White rabbits with <30% (LAR) and >70% (HAR) aortic atherosclerosis surface involvement as described previously. A total of 17 LAR and 15 HAR rabbits were used. Animal care and experimental procedures involving animals conformed to the position of the American Heart Association on research animal use and were approved by the Regierung von Oberbayern and Sachsen.

Isolation of Rabbit Peritoneal Macrophages
Peritoneal macrophages were isolated as described previously. In brief, the rabbits were euthanized 3 days after IP injection of 40 mL of mineral oil, and the peritoneal cavity was washed with 1000 mL of a 154 mmol/L NaCl solution. Cells were centrifuged at 200g for 10 minutes, and the cell pellets were resuspended and washed in Hanks’ balanced salt solution. Morphological examination of stained cell preparations, spun-down on microscope slides, confirmed the high-macrophage yield in the cell preparation and revealed only a minor presence of other cell types.

Immunostaining
Frozen sections of rabbit atherosclerotic tissue were stained as described previously. Mouse anti-arginase 1 antibody 612621 (BD Pharmingen, dilution 1:50) was tested for cross-reactivity with rabbit arginase I by Western blotting and was subsequently used as primary antibody. Mouse anti-rabbit RAM11 (M0633, Dako, 1:500 dilution) antibody. Mouse anti-rabbit IgG HRP, P0260 (Dako) served as secondary antibody (dilution 1:500 for β-actin as a housekeeping gene. Sequence information of primers and probes and conditions of the PCR reaction are provided in the online supplement (Table I, available online at http://atvb.ahajournals.org).

SSH
To minimize the possible variation between individual rabbits, we pooled total RNA extracted from macrophages of 3 sex- and age-matched LAR and HAR rabbits in the first experiment and from 4 sex- and age-matched LAR and HAR rabbits in a second experiment. Poly A+ RNA was isolated from total RNA using oligo(dT)-latex bead spin columns (Nucleo Trap HAR or LAR macrophages.

Cloning and Analysis of Subtracted cDNA Libraries
The PCR products resulting from the subtracted libraries were screened for differential expression using colony arrays. To this end, the subtracted cDNAs were ligated into the TOPO TA Cloning Vector (Invitrogen). Individual colonies were picked, grown in Luria–Bertani–ampicillin medium and spotted onto 2 nylon mem-

Quantitative Fluorogenic RT-PCR (TaqMan)
cDNA from macrophages of 17 LAR and 15 HAR rabbits was synthesized from 2 μg of total RNA by reverse transcription. Quantitative fluorogenic PCR was performed in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). Specific TaqMan primers and probes for fibroactin, OC2-protein, arginase I, ferritin H-chain, interleukin (IL) 6, and rabbit β-actin were selected to span exon junctions (according to their human and mouse homologues) to avoid coamplification of genomic DNA. To determine absolute mRNA copy numbers, standard curves were generated for each gene using a plasmid dilution series containing the target sequences. The mRNA expression levels of the genes were normalized to 10 copies of β-actin as a housekeeping gene. Sequence information of primers and probes and conditions of the PCR reaction are provided in the online supplement (Table II, available online at http://atvb.ahajournals.org).

Arginase Assay
Arginase activity was measured in cell lysates, as described by Corraliza et al, with modifications. Briefly, macrophages (5×106) from 6 LAR and 5 HAR rabbits were lysed for 30 minutes with 1 mL of 0.2% Triton X-100. Thereafter, 50 μL of 10 mmol/L MnCl2 and 50 mmol/L of Tris-HCl (pH 7.5) were added to a 50-μL aliquot of the cell lysate and the enzyme activated by incubating the mixture for 10 minutes at 55°C. Arginine hydrolysis was conducted by adding 25 μL of 0.5 mol/L L-arginine (pH 9.7) to a 25-μL aliquot of the activated sample after incubation at 37°C for 60 minutes. The reaction was stopped with 400 μL of H2SO4 (96%)/H3PO4 (85%)/H2O(1/3/7, volume/volume/volume). The generated urea was measured at 540 nm after addition of 25 μL of 9% isonitrosopropiophenone (dissolved in 100% ethanol) and incubation at 100°C for 45 minutes. The optical density was determined in a microplate reader (SpectraFlur, Tecan) using 200-μL aliquots. Known amounts of urea (Merck; 0 to 30 μg) were used to generate a standard curve. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol urea/min. To normalize arginase enzyme activity, values are given per milligram of cell protein as determined by BCA Protein Assay (Pierce).

Rabbit Arginase I Bacterial Artificial Chromosome Clone
By screening a rabbit bacterial artificial chromosome (BAC) library, we obtained an arginase I-containing BAC clone. BAC DNA was isolated with the Large Construct kit (Qiagen), and 2 μg were used to sequence the promoter region of the arginase I gene (~1000 bp) via a primer located in the proximal arginase I cDNA fragment. Thereafter, PCR primers were selected to amplify and sequence the promoter region in 14 LAR and 14 HAR rabbits. Primer information and conditions of the PCR reactions are provided in the online supplement (Table II, available online at http://atvb.ahajournals.org).

Northern Blot Analysis
RNA (15 μg) from macrophages of 5 LAR and 7 HAR rabbits was electrophoresed on a 1% agarose gel containing 2.2 mol/L formaldehyde transferred to a Nytran-N membrane (Schleicher & Schuell) and cross-linked by UV irradiation. The membranes were prehybridized for 2 hours at 42°C followed by an overnight hybridization with a 819-bp fragment of the arginase I cDNA radiolabeled with 32P by random priming (Multiprime, Amersham). The membranes were washed and exposed to storage phosphor screens. The blots were
quantified by phosphorimage densitometry (Fuji FLA3000). Arginase I mRNA expression was normalized to 28s RNA.

Rapid Amplification of cDNA Ends and Sequencing of Arginase I cDNA
5’ and 3’ rapid amplification of cDNA ends (RACE) was used to identify the arginase I transcription start site and polyadenylation site in LAR and HAR rabbits (First Choice RLM RACE kit, Ambion). Primers for PCR amplification and sequencing of arginase I full-length cDNA were then selected based on the nucleotide sequences obtained by 5’RACE, SSH, and 3’RACE (all of the primer sequences are presented in Table III, available online at http://atvb.ahajournals.org).

Transfection Study for mRNA Stability
The short and the long variant of rabbit arginase I full-length cDNA were cloned into the expression plasmid pCMS-enhanced green fluorescent protein (Clontech). We transfected 0.5 μg of each plasmid into RAW cells using DEAE dextran as described.11 Rabbit arginase I mRNA expression was determined at baseline and 2 hours after the addition of 5 μg/mL of actinomycin D.

Statistical Analysis
Values are given as mean±SEM unless noted otherwise. Statistical analysis was done by t test and ANOVA using the Prism software, version 4.0.

Results
Differential Gene Expression in Macrophages
SSH was used to clone differentially expressed genes in macrophages of atherosclerosis-resistant and susceptible LAR and HAR rabbits. We focused on genes with increased expression in LAR macrophages, which may represent atheroprotective genes. In the initial screening, a total of 42 clones showed increased expression levels in macrophages from LAR rabbits compared with HAR rabbits. We next sequenced these clones and determined whether they were homologous to known genes. Of these clones, 59% (25 of 42) showed homology to known rabbit genes, 29% (12 of 42) were homologous to genes described in other species, and the remaining 12% (5 of 42) showed no homology to sequences in the database. Some of the cDNA sequences were identified more than once (Table).

Arginase I Expression
Based on their potential role in atherogenesis, we aimed to confirm differential expression of 4 of the identified sequences using quantitative RT-PCR as an independent method. These cDNAs showed a high degree of homology to human arginase I (91%), rabbit OC2 protein (99%), rabbit fibronectin (99%), and rabbit ferritin H-chain mRNA (100%). Ferritin H-chain was found in 9 clones identified in independent rounds of hybridization, whereas the other 3 candidates were isolated only once. OC2 protein, fibronectin, and ferritin H-chain were highly expressed in macrophages of both strains but failed to show a difference between LAR and HAR rabbits (OC2 protein: 494568 ±49740 versus 629186 ±73509, P=0.81; fibronectin: 494568 ±49740 versus 629186 ±73509, P=0.11; ferritin H-chain 9138133 ±3043125 versus 8633819 ±2695121, P=0.36; copies/10⁶ copies of β actin in LAR and HAR macrophages, respectively). The failure to confirm differential expression may be related to the high-expression levels of these genes leading to false positives in SSH. In contrast, the arginase I message was less abundant, but the gene showed a significantly higher expression in LAR macrophages than in HAR macrophages (77428 ±10941 versus 34344 ±4538, P=0.002; copies/10⁶ copies of β actin, respectively; Figure 1A).
Arginase Enzyme Activity

To investigate whether arginase was also differentially expressed on a functional level, we next determined arginase enzyme activity in cell extracts from LAR and HAR macrophages. We confirmed that arginase enzyme activity was significantly increased in LAR macrophages compared with HAR macrophages (1123 ± 149 versus 667 ± 63, P = 0.018; mU/mg cell protein; Figure 1B). Linear regression analysis showed a strong positive correlation between arginase enzyme activity and mRNA expression (r² = 0.73; P = 0.008; Figure 1C). Two isoenzymes, arginase I and arginase II, contribute to arginase activity. Because only arginase I but not arginase II mRNA was expressed in macrophages (Figure I, available online at http://atvb.ahajournals.org), we conclude that differences in arginase activity were conferred by arginase I.

Localization of Arginase I in Atherosclerotic Tissue

We then performed immunostaining to determine whether arginase I was expressed in atherosclerotic lesions. As shown in Figure 2, arginase I was detected in the atherosclerotic intima and colocalized to macrophages in the lesion core.

Determination of Arginase I Promoter Sequence

Because differential expression of arginase may have resulted from promoter variants between LAR and HAR rabbits, we next determined the promoter sequence. Screening of a rabbit BAC library resulted in the identification of a clone containing the previously identified arginase I sequence. We sequenced ∼1 kb of the promoter (accession no. AY870253). Resequencing of this region in LAR and HAR rabbits led to the identification of 4 polymorphic sites, occurring in linkage with each other (Figure II, available online at http://atvb.ahajournals.org). The rare alleles of the polymorphic sites were present only in LAR rabbits (1 homozygous and 4 heterozygous), whereas the remaining 10 LAR and all of the HAR rabbit sequences were identical to the identified BAC sequence. Arginase I promoter variants were not associated with arginase I expression levels. Thus, arginase I promoter variants did not appear to be causative for the expression differences between LAR and HAR rabbits.

Identification of Arginase I Full-Length cDNA and Size Variants

Another possible explanation for the observed differences in expression and activity of arginase I was the presence of variations in the arginase I sequence. We, thus, performed Northern blotting to test for possible differences in arginase I transcript size. As shown in Figure 3, 2 transcript variants of ∼1.4kb and 1.8kb were expressed in LAR and HAR rabbits. Densitometric analysis confirmed the previously observed differences in transcript abundance. To elucidate the reason for the size difference of the 2 transcript variants, we went on to determine the full-length sequence of arginase I cDNA, which had not been described previously (accession no. AF365403). Rapid identification of cDNA-ends (5’ and 3’ RACE) revealed that the long- and short-arginase cDNA variants used the same transcriptional start site and polyadenylation signal and were
identical except for a 413-bp insertion in the 3’ untranslated region (Figure 4). The insertion was identified as a member of the rabbit C-repeat family by homology search.

Association of Arginase I mRNA Size Variants With Expression Levels

We then determined arginase I size variants in all of the LAR and HAR rabbits included in the study. Homozygosity for the long variant was present only in HAR rabbits, whereas heterozygosity (long and short variants) was present in both LAR and HAR rabbits, and homozygosity for the short variant was primarily found in LAR rabbits. The distribution of the genotypes is shown in Figure 5A and was significantly different between the strains ($\chi^2$ test, $P=0.002$). We next tested whether the expression level of arginase I mRNA was associated with arginase I transcript size. As shown in Figure 5B, animals homozygous for the short variant showed significantly higher arginase I expression levels than animals homozygous for the long variant (74921±11332 versus 21171±3183, $P<0.01$; copies/10^6 copies of $\beta$ actin). Heterozygous animals showed intermediate expression levels (47893±8630 copies/10^6 copies of $\beta$ actin). Because increased arginase I expression may exert antiinflammatory properties, we also investigated IL-6 expression in macrophages of LAR and HAR rabbits. Expression was decreased in macrophages from LAR rabbits compared with HAR rabbits (2332±276 versus 4212±1085 copies/10^6 copies of $\beta$ actin), although this trend did not reach statistical significance ($P=0.09$).

Differences in mRNA Stability Between Arginase I Size Variants

To determine whether the observed expression differences may result from increased decay of the long arginase I mRNA variant, expression plasmids containing either the long or short variant were transfected into RAW cells. After the addition of actinomycin D, we observed a reduction of arginase I expression to 73% of baseline for the short variant (69948±13246 versus 51288±7954 mRNA copies) and a greater reduction to 32% of baseline for the long variant (37469±4924 versus 11882±4217 mRNA copies). Thus, the long arginase I mRNA size variant was associated with decreased mRNA stability compared with the short size variant.

Discussion

The question addressed in the present study was whether genes in macrophages from rabbits with HAR and LAR were differentially expressed. As a main finding, arginase I was identified as a new candidate gene of atherosclerosis susceptibility. Macrophages of atherosclerosis-resistant LAR rabbits showed increased mRNA expression and enzyme activity compared with macrophages of atherosclerosis-susceptible HAR rabbits.

Arginase I may play an important role in modulating atherosclerosis susceptibility in the vessel wall. In macrophages, L-arginine can be metabolized by NO synthase and arginase to form NO and urea, respectively. NO production in macrophages is induced in response to inflammatory stimuli. There are several lines of evidence pointing to a proatherogenic role of NO secretion from macrophages. For example, inducible nitric oxide synthase–deficient mice on the apolipoprotein E–deficient background developed significantly less atherosclerosis than their wild-type counterparts.12 Chronic treatment of hypercholesterolemic rabbits with the selective inducible nitric oxide synthase inhibitor N-\((\)i-niminoethyl-L-lysine limited the progression of preexisting atherosclerosis.13 Thus, high expression of arginase I in macrophages from LAR rabbits may be an effective mechanism of reducing macrophage NO production through substrate competition.14,15 However, this process is complex and also involves translational control of NO synthase expression.16 Importantly, ornithine, the primary metabolite of the arginase pathway, may itself play an important role in atherogenesis. Ornithine feeds into the synthesis of L-proline and, thus, is a precursor of collagen biosynthesis.17 In addition, metabolism of ornithine by ornithine decarboxylase leads to the formation of polyamines, which play an integral role in the mitogenic response of vascular smooth muscle cells.18 Thus, metabolites of ornithine may contribute to arterial remodeling at sites of vascular damage.18,19 Moreover, polyamines display a distinct antiinflammatory effect by downregulating the proinflammatory cell response of mononuclear cells stimulated with lipopolysaccharide.20 Thus, increased arginase activity may be antiinflammatory and be associated with a decrease of proinflammatory cytokine expression. To this end, we investigated IL-6 expression in macrophages of LAR and HAR rabbits. Macrophages from LAR rabbits showed a trend toward decreased expression of IL-6, although this did not reach statistical significance ($P=0.09$). However, we do not know whether arginase may exert stronger antiinflammatory effects at other time points, on other inflammatory mediators, or within specific compartments of atherosclerotic lesions in the vessel wall.

Arginase I gene expression is regulated by different mediators, including T-helper 2 cytokines, cAMP, C/EBP-β, and glucocorticoids.21–23 The concept of arginase I as an antiinflammatory mediator is supported by the induction of the gene by the Th2 cytokines IL-4, IL-10, and IL-13.24 Sequencing of the rabbit arginase I promoter revealed a high degree of conservation with humans and mice. However, sequence differences between LAR and HAR rabbits did not affect
specific binding sites of known regulators of arginase expression. This finding was supported by a lack of correlation between the presence of promoter variants and arginase I expression levels. In contrast, variation of arginase transcript size was tightly correlated with arginase I expression. The long variant of arginase I, associated with decreased expression levels, was predominantly found in HAR rabbits and caused by the insertion of a 413-bp rabbit C-repeat in the 3' untranslated region. Rabbit C-repeats are typical representatives of short interspersed nucleotide elements (SINEs) and are similar to Alu repeats in humans. Although the structural and transcriptional properties of rabbit C-repeats are similar to the primate Alu-SINEs, C-repeat sequences are not homologous to Alu-repeat sequences. Although the functional properties of SINEs have not been studied in rabbits, studies in humans provide strong evidence that these elements can affect translation and stability of mRNA at the 3' untranslated region. Indeed, transfection experiments in our study indicate that the presence of the insertion in the longer transcript variant of arginase leads to enhanced mRNA degradation and, thus, may provide a mechanism for decreased arginase I expression levels.

In conclusion, arginase I was identified as a new candidate gene of atherosclerosis resistance in macrophages of rabbits with LAR and HAR. High expression of arginase I in macrophages may contribute to atherosclerosis resistance, possibly by conferring antiinflammatory effects in the vessel wall. Future studies using induced mutant mouse models of arginase I will provide conclusive evidence about the role of macrophage arginase I in atherogenesis. Understanding the

Figure 4. Nucleotide sequence of rabbit arginase I mRNA of the short (A1short) and long (A1long) transcript variant (accession no. AF365403). Potential start sites of translation (ATG) and the stop codon (TAA) are framed. The 3' untranslated region (UTR) of the long transcript variant contains a 413-bp insertion of a rabbit C-repeat sequence.
Figure 5. (A) Arginase I transcript size variants in LAR and HAR rabbits. s/s indicates homozygous short/short; s/l, heterozygous short/long variant; l/l, homozygous long/long. (B) Arginase I mRNA expression in regard to arginase I transcript variant.

References


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