Arginase I enhances atherosclerotic plaque stabilization by inhibiting inflammation and promoting smooth muscle cell proliferation

Xu-Ping Wang†, Wei Zhang†, Xiao-Qian Liu, Wen-Ke Wang, Fei Yan, Wen-Qian Dong, Yun Zhang*, and Ming-Xiang Zhang*

The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Public Health, Shandong University, Qilu Hospital, No.107, Wen Hua Xi Road, Jinan, Shandong 250012, P.R. China

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Aims

The aim of this study was to investigate the effect of Arginase I (ArgI) on plaque stabilization in unruptured atherosclerotic plaque and explore its mechanism.

Methods and results

The atherosclerotic plaque model was established in New Zealand rabbits by balloon injury of abdominal arteries and a high cholesterol (1%) diet. Arginase I overexpression reduced the content of macrophages and lipids and increased that of smooth muscle cells and collagen in the atherosclerotic plaque, thus contributing to decreased plaque vulnerability. Arginase I overexpression decreased the expression of the inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) as well as inducible nitric oxide synthase (iNOS) in plaques. In vitro, Arg I overexpression or iNOS inhibition abolished the secretion of TNF-α and IL-6 induced by lipopolysaccharide in Raw264.7 cells. However, exogenous L-arginine restored the expression of inflammatory cytokines. Arginase I overexpression inhibited the activity of iNOS without changing its expression. Moreover, Arg I co-localized with iNOS in both Raw264.7 cells and human aortic atherosclerotic plaques. In addition, the IL-10 level was increased in plaque with Arg I overexpression. Finally, Arg I promoted aortic vascular smooth muscle cell proliferation, which was associated with increased production of intracellular polyamines.

Conclusion

Arg I enhances the stability of atherosclerotic plaque by inhibiting the expression of inflammatory cytokines and stimulating smooth muscle cell proliferation.

Keywords

Arginase I • Atherosclerotic plaque stability • Inflammation • Proliferation

Introduction

Arterial thrombosis triggered by the rupture or erosion of atherosclerotic plaques that have advanced to a state of vulnerability leads to an acute vascular event, which is responsible for the high mortality rate with atherosclerosis.1 Vulnerable plaque rupture involves the interaction of modified lipoproteins, monocyte-derived macrophages or foam cells, mast cells, T-lymphocytes, endothelial cells, and vascular smooth muscle cells (VSMCs). Although various mechanical mechanisms have been proposed to cause plaque rupture, growing evidence still indicates that inflammation and apoptosis are the primary underlying causes of atherosclerotic plaque instability and rupture.2

Macrophages, and to a lesser extent T lymphocytes, are the predominant cells at the immediate site of rupture or superficial erosion in human atherosclerotic plaque.3 Inflammatory cytokines mainly secreted by activated macrophages and T lymphocytes may be responsible for activation of other cells such as neutrophils or mast cells and induction of apoptosis. The procoagulant effects of cytokines are direct or via endothelial dysfunction and may induce plaque vulnerability or rupture.4 Another potential mechanism of plaque rupture involves apoptosis resulting in a decreased structural

† These two authors contributed equally to this work.
* Corresponding author. Tel: +86 5318216 9235, Fax: +86 53186169356, Email: zhangyun@sdu.edu.cn
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integrity of the plaque. Apoptosis of VSMCs has been detected in the shoulder regions of plaques, which appear most likely to rupture, so VSMC apoptosis may predispose to plaque rupture.

Vascular smooth muscle cells are important components of atherosclerotic plaques, responsible for promoting plaque stability in advanced lesions. Atherosclerotic plaque caps consist primarily of VSMC-derived collagen, proteoglycans, elastin, and extracellular matrix. To maintain stability, plaque caps require more collagen and extracellular matrix content than the nearby intima. Fibrous caps of vulnerable plaque contain less collagen and extracellular matrix components and fewer VSMCs than caps from stable lesions, which suggests the VSMC promotion of plaque stability.

Arginase I (ArgI) is a cytosolic enzyme constitutively expressed in the liver, where it functions in nitrogen elimination by hydrolyzing L-arginine to produce urea and L-ornithine. Arginase I activity has also been detected in a number of non-hepatic tissues that lack a complete urea cycle, where the reaction is thought to provide a source of ornithine, the biosynthetic precursor of proline, an important constituent of collagen, and polyamines, which are important for cell proliferation. Arginase I is critically involved in wound healing and fibrosis. The role of ArgI in immunity has been revealed in animal models as well as mouse and in humans. For example, several important immunological functions of murine ArgI have been clarified recently, including a direct and indirect role in inflammation attenuation, T-cell proliferation inhibition, T-cell hyporesponsiveness, and the induction of Th2-mediated immunopathology in schistosomiasis. The ArgI of murine macrophages likely functions by competing with inducible nitric oxide synthase (iNOS) for l-arginine and by providing polyamines. In addition, ArgI is detected in human mononuclear cells after injury and inflammatory cells of bronchoalveolar lavage fluid of asthmatic patients as well as polymorphonuclear leucocytes. Kropf et al. demonstrated the role of ArgI in suppressing T cells via l-arginine depletion in human pregnancy.

We and others have demonstrated the expression of ArgI in SMCs and atherosclerotic plaque. We previously reported that increased ArgI activity may attenuate inflammation in both human VSMCs and rabbit plaque. Thomas and colleagues validated the down-regulation of ArgI in rabbit foam cells and human atherosclerotic plaque. In addition, ArgI was identified as a new candidate gene of atherosclerosis resistance. We hypothesized that ArgI may enhance atherosclerotic plaque stability by inhibiting inflammation and VSMC proliferation. To test this hypothesis, we used the in vivo atherosclerotic plaque model established by New Zealand rabbits and cultured Raw264.7 macrophages and human aortic smooth muscle cells (HASMCs) in vitro.

Methods

Animal treatment

New Zealand white rabbits underwent balloon injury to the abdominal aorta and then continued with a high-cholesterol diet. Lentivirus vector was delivered by local injection to plaques guided by vascular ultrasonography at the end of 12 weeks as described (Supplementary material online, Methods).

Tissue sampling

At autopsy, the left anterior descending artery (LAD) between the vascular bifurcation of the left coronary artery and the first bifurcation of the LAD was dissected and removed.

Cell culture

RAW264.7 cells were cultured in RPMI 1640 medium containing 10% foetal bovine serum (FBS). Human aortic smooth muscle cells were cultured in SMC medium containing 2% FBS and 1% SMC growth supplement (ScienCell, USA). Human aortic smooth muscle cells up to passage 4 were used.

Immunohistochemistry

In brief, after being incubated with a protein blocking agent, sections or cells were incubated with primary antibody overnight at 4 °C, then washed with phosphate buffered saline and secondary antibody for 30 min at 37 °C.

Arginase activity and nitric oxide assay

Arginase activity was assayed as described.

Western blot analysis and ELISA assay

Western blot analysis was performed as described. The ELISA assay involved use of a kit (Bender, Austria).

Measurement of cell proliferation and intracellular polyamines

Staining for 5-ethyl-2′-deoxyuridine (EdU) incorporation was performed according to the manufacturer (Rib Bio, Suzhou, China). Intracellular putrescine, permine, and spermidine levels in HASMCs were determined by a sensitive high-performance liquid chromatography procedure.

Statistical analysis

Data are expressed as means ± SD. Data for multiple comparisons were tested by one-way ANOVA, followed by Tukey’s test, or Kruskal–Wallis H test, followed by the Mann–Whitney U test, with SPSS v16.0 (SPSS, Inc., Chicago, IL, USA). A P < 0.05 was considered statistically significant.

Results

Arginase I expresses in macrophages, SMCs, and atherosclerotic plaque

Immunofluorescence staining revealed the expression of ArgI in Raw264.7 macrophages, HASMCs, and human aortic atherosclerotic plaque (Figure 1A–C). Positive staining for ArgI in tissue was 34.83 ± 8.01% of the total human plaque area. With ArgI lentiviral overexpression in rabbit plaque, ArgI expression was increased 5- to 2.5-fold that with LacZ overexpression at 1–4 weeks after injection (Figure 1D and E).

Arginase I overexpression enhances atherosclerotic plaque stability as evaluated by plaque components

Sirius red staining revealed more pyknotic structure but less necrotic core in rabbit plaque with ArgI lentiviral overexpression than with LacZ or control treatment, but the plaque size did not differ among groups (P > 0.05 vs. LacZ, Figure 2A). With ArgI overexpression,
Figure 1  Arginase I is expressed in macrophages, smooth muscle cells and atherosclerotic plaque. Immunofluorescent staining of Arginase I in (A) Raw264.7 cells, (B) human aortic smooth muscle cells, and (C) human aortic plaque. Nuclei were stained by DAPI (blue). Photographs represent five independent microscope fields for each of three different cultures. (D) Quantification of western blot analysis of the Arginase I protein level in rabbit plaque. Data are means ± SD (horizontal bar and whiskers), dots represent samples of Arginase I expression with Arginase I lentiviral injection relative to LacZ injection set to 1 at each time (n = 5). (E) Immunofluorescence staining of Arginase I in rabbit plaque at 2 weeks after Arginase I lentiviral injection, n = 5.

Figure 2  Arginase I regulates components of rabbit atherosclerotic plaque. Control, physiological saline-injected plaque; LacZ, LacZ lentiviral-overexpressed plaque; ARGI, Arginase I lentiviral-overexpressed plaque. Representative images of Sirius red staining of (A) plaque macroscopic structure and (B) collagen; n = 10 rabbits per group. Immunochemical staining of (C) macrophages and (D) smooth muscle cells, and (E) Oil-red O staining of lipids. (F) Area measurement of plaque components. Data are means ± SD; n = 10 rabbits per group. (G) Vulnerability index of plaques. Data are means ± SD, n = 10.
positive staining for collagen and SMCs was markedly increased (Figure 2B, D, and F), but that of macrophages and lipids was significantly decreased (Figure 2C, E, and F). The vulnerability index of plaque was lower with ArgI overexpression than LacZ treatment (1.56 ± 0.53 vs. 2.65 ± 0.80; P < 0.01; Figure 2G).

**Arginase I overexpression inhibits atherosclerotic plaque inflammation**

To examine the anti-inflammatory ability of ArgI, we determined the level of TNF-α and IL-6 in rabbit plaque. With ArgI overexpression, TNF-α and IL-6 positive staining was decreased significantly (P < 0.05 vs. LacZ; Figure 3A–C) and iNOS expression was decreased (0.72 ± 0.3 vs. 0.98 ± 0.31; P < 0.05 vs. LacZ, Figure 3D). Furthermore, ArgI overexpression increased ArgI protein level and arginase activity (both P < 0.01 vs. LacZ, Figure 3E and F) but decreased ArgII protein level (P < 0.01 vs. LacZ, Figure 3E).

**Arginase I inhibits lipopolysaccharide-induced inflammation in macrophages by competing l-arginine with inducible nitric oxide synthase**

To determine the anti-inflammatory ability of ArgI, we determined the secretion of inflammatory cytokines in Raw264.7 cells. Lipopolysaccharide-increased TNF-α and IL-6 levels were reversed with ArgI overexpression but augmented with ArgI down-regulation or inhibition (Figure 4A). To validate the contribution of iNOS to inflammation, we used iNOS selective inhibitors (1400W and AG). Both inhibitors attenuated the production of the inflammatory cytokines (Figure 4B). To further explore the mechanism of ArgI participating in the inflammatory process, we determined the catalysing activity of arginase and iNOS. To clarify the effect of iNOS on ArgI, we used iNOS inhibitors. The catalysing activity of arginase was increased with ArgI overexpression (71.49 ± 9.43 vs. 25.85 ± 4.88; P < 0.01 vs. LPS) and lower with ArgI down-regulation than with LPS alone (8.31 ± 1.1 vs. 25.85 ± 4.88; P < 0.01 vs. LPS) or inhibition (7.3 ± 1.3 vs. 25.85 ± 4.88; P < 0.01 vs. LPS). Remarkably, 1400W and AG increased the catalysing activity of arginase by ~20% (both P < 0.05 vs. LPS). iNOS activity was decreased significantly with ArgI overexpression (3.96 ± 0.73 vs. 8.86 ± 0.96; P < 0.01 vs. LPS) but was increased with ArgI down-regulation (11.5 ± 1.77 vs. 8.86 ± 0.96; P < 0.05 vs. LPS) and inhibition (12.86 ± 1.33 vs. 8.86 ± 0.96; P < 0.01 vs. LPS) (Figure 4C).

To further explore the effect of ArgI on iNOS, we determined iNOS protein expression by western blot analysis. Lipopolysaccharide induced a ~11-fold increase in iNOS protein expression, which was not affected significantly by ArgI regulation or iNOS inhibition (Figure 4D). Because many enzymatic pathways are compartmentalized in the cell to avoid direct competition for substrates, to validate the competition relationships between ArgI and iNOS, we next...
revealed the localization of the two enzymes. As expected, co-localization of ArgI and iNOS was confirmed by immunofluorescence both in RAW264.7 cells and in human plaque (Figure 4E and F).

To identify which isoform of arginase is involved in the inflammatory process, we determined the expression of both isoforms in LPS-incubated macrophages. Because iNOS was a prior
inflammatory contributor during the inflammatory response in macrophages, we also determined the expression of iNOS during the process. The expression of arginase (Arg I and Arg II) and iNOS were both time-dependently increased during LPS incubation; iNOS expression increased as early as 2 h after LPS incubation, whereas Arg I and Arg II were induced later (Figure 4G). Moreover, the expression of iNOS continued to increase, while that of Arg I and Arg II decreased after 24 h.

Furthermore, to illustrate the effect of LPS on arginase and iNOS, we determined the catalysing activity of the enzymes. In accordance with the protein expression, the LPS-increased arginase activity lagged behind that of iNOS and decreased after 24 h, whereas NOS activity continued to increase slowly (Figure 4H). To confirm the competitive relationship of iNOS and Arg I, we pretreated cells with l-arginine during LPS incubation. The activity of both enzymes increased until 48 h (Figure 4H), which confirms l-arginine competition between the two enzymes.

To further illustrate whether substrate competition is involved in Arg I-inhibited inflammation, we evaluated the effect of l-arginine pre-addition. Without LPS, l-arginine pretreatment had no effect on TNF-α or IL-6 release alone or together with Arg I overexpression, but with LPS incubation, l-arginine augmented the cytokine release and reversed the inflammation-inhibited ability of Arg I (Figure 4I).

**Arginase I increases Th2-type cytokine expression**

To explore the effect of Arg I on other immune cells such as T cells, we determined the production of Th2-type cytokines (IL-4 and IL-10) in plaques. The interleukin -10 protein level was increased in Arg I-overexpressed plaques (1.27 ± 0.33 vs. 0.98 ± 0.22; P < 0.05 vs. LacZ, Figure 5), while that of IL-4 was under the limit of determination.

**Arginase I promotes vascular smooth muscle cell proliferation via increased intracellular polyamine level**

Collagen content and SMC count were higher in plaque with Arg I than LacZ overexpression (Figure 2B and D). We searched for evidence of SMC increase in vivo and in vitro. We detected proliferating cell nuclear antigen (PCNA), an indicator of cell proliferation, by immunocytochemistry in atherosclerotic plaques. In Arg I-overexpressed atherosclerotic plaque, PCNA staining was increased (0.21 ± 0.07 vs. 0.16 ± 0.06; P < 0.01 vs. LacZ) and mainly localized in agreement with SMC distribution (Figure 6A and B). To evaluate whether Arg I can promote cell proliferation in vitro, we determined EdU incorporation in HASMCs. Arginase I overexpression increased EdU incorporation (0.18 ± 0.05 vs. 0.12 ± 0.03; P < 0.01 vs. LacZ) and the arginase inhibitor S-(2-boroenoyethyl)-L-cysteine (BEC) reversed the increase in EdU incorporation (0.14 ± 0.03 vs. 0.18 ± 0.05; P < 0.05 vs. Arg I) (Figure 6C and D).

Polyamine derived from arginine is essential for cell proliferation. To demonstrate the role of polyamines in cell proliferation, we determined their production in Arg I-overexpressed HASMCs. Consistent with the findings of cell proliferation, levels of intracellular putrescine (0.42 ± 0.13 vs. 0.21 ± 0.04; P < 0.01, vs. LacZ), spermidine (11.89 ± 1.6 vs. 6.23 ± 0.44; P < 0.01, vs. LacZ), and spermine (4.52 ± 0.53 vs. 2.19 ± 0.28; P < 0.01, vs. LacZ) were higher in HASMCs with increased Arg I than LacZ overexpression, whereas the arginase inhibitor BEC significantly attenuated the increase in the polyamine level with Arg I overexpression in HASMCs (Figure 6E).

**Discussion**

The present investigation is a continuation and extension of our earlier work about Arg I in HASMCs showing that Arg I inhibited inflammatory cytokine secretion by competing with iNOS for l-arginine and attenuated inflammatory cytokine production in atherosclerotic plaque. The major findings of the present study are that Arg I enhances plaque stability by reducing inflammation, increasing Th2 cytokine levels, and promoting VSMC proliferation. This is the first report demonstrating the protective role of Arg I in atherosclerotic plaque vulnerability.

Macrophage accumulation is one of the characteristics of vulnerable plaque, and macrophage activation increases iNOS and NO production. Previous studies have shown that arginase blockade in vivo enhanced classical macrophage activation and increased iNOS activity. Consistent with these observations, we found that Arg I overexpression decreased the expression of pro-inflammatory iNOS in atherosclerotic plaque. Among the inflammatory cytokines, TNF-α and IL-6 were significantly decreased in the level in plaque with Arg I overexpression. Tumour necrosis factor-α is a pleiotropic cytokine produced chiefly by activated macrophages and by cells such as T-lymphocytes, endothelial cells, and SMCs. Interleukin-6 is produced by antigen-presenting cells such as macrophages and non-haematopoietic cells such as endothelial cells. Tumour necrosis factor-α and IL-6 occupy a central role in
the amplification of the inflammatory cascade. The combination of TNF-α and IL-6 could enhance T-cell proliferation. Tumour necrosis factor-α is also known to cause apoptosis in VSMCs, a possible contributor of plaque rupture.21 These data provide evidence for an anti-inflammatory ability of ArgI in vivo. We used a rabbit model in our study, despite the scarcity of specific antibodies for in vivo-determining techniques.

To mimic the atherosclerotic response of macrophages, we induced inflammation in Raw264.7 macrophage cells with LPS incubation. Consistent with the observations in vivo, the production of TNF-α and IL-6 was significantly decreased in macrophages with ArgI overexpression, which was attributed to competition for L-arginine by ArgI and iNOS. Whether ArgI functions as a competitor of iNOS in murine macrophages is disputed, and we have no direct evidence for active arginase in human macrophages. Nonetheless, recent reviews have shown iNOS activity in human macrophages.22 In the present study, macrophages were decreased in number in plaque with ArgI overexpression. Therefore, other immune cells such as T lymphocytes in addition to macrophages might participate in the ArgI-mediated atherosclerotic process. To examine this hypothesis, we determined the level of Th2 cytokines (IL-4 and IL-10) in plaques. Although we found the level of IL-4 below the limit of detection in plaque, de Boer et al. showed that both interferon (IFN)-γ (Th1-type cytokine) and IL-4 (Th2-type cytokine) were produced by T-cell clones generated from human atherosclerotic plaque, with the production of the Th1-type cytokine predominant in most cell clones.23 However, the level of IL-10, secreted by activated monocytes/macrophages and T lymphocytes, was increased in plaque with ArgI overexpression. Interleukin-10 has multifaceted anti-inflammatory properties, such as inhibition of matrix-degrading metalloproteinases, inhibition of macrophage apoptosis and promotion of the phenotypic switch of T-lymphocytes to the Th2 phenotype.24 Although we could not

Figure 6 Arginase I enhances vascular smooth muscle cell proliferation by increasing intracellular polyamine production (A and B) Arginase I enhances cell proliferation in vivo. (A) Representative immunohistochemical images for proliferating cell nuclear antigen and (B) quantification. Data are means ± SD; n = 10 rabbits per group. (C and D) Arginase I promotes human aortic smooth muscle cell proliferation in vitro. Transfected human aortic smooth muscle cells were incubated at 37°C for 72 h in complete SMC medium containing 0.4 mM L-arginine. Cell proliferation was assessed by EdU incorporation after a 3-day (72 h) growth. BEC was added to cells at Day 2 (24 h). (C) Representative images of EdU incorporation (red) and (D) quantification. Data are means ± SD from five independent microscope fields for each of three independent experiments. (E) Content of polyamines in human aortic smooth muscle cells incubated with 0.4 mM L-arginine. Data are means ± SD of duplicate determinations from three independent experiments.
determine the physiological role of IL-10 in atherosclerotic plaques, IL-10 may play a protective role in plaque progression and instability.

Previous studies have outlined the role of ArgI in VSMC proliferation, which is attributed to increased production of polyamines. In agreement with these observations, we demonstrated that ArgI overexpression promoted the proliferation of HASMCs by increasing the production of intracellular polyamines. Vascular smooth muscle cells in atherosclerotic plaque have been traditionally considered to contribute to the generation of lesions and, therefore, are an undesirable consequence of the atherosclerotic process. In contrast, recent reviews have emphasized the role of VSMCs in maintaining the integrity of plaque and suggested that VSMC proliferation may be beneficial to plaque stability. Thus, VSMC proliferation may be a ‘double-edged sword’ in atherosclerosis. Indeed, disastrous outcomes in atherosclerosis are from rupture of the thinnest part of the fibrous cap region, associated with reduced extracellular matrix synthesized almost exclusively by VSMCs and number of VSMCs in the fibrous cap. The fibrous cap region undergoes continuous damage then repair in complex atherosclerotic plaque. Most occurrences of the erosion and repair are silent. However, any process that prevents VSMC repair of the cap is potentially dangerous, such as failure to undergo cell proliferation or susceptibility to undergo apoptosis. Our results suggest that the increase in collagen content and VSMC number in atherosclerotic plaque is induced by ArgI promotion of VSMC proliferation that compromises their damage or apoptosis within the plaque cap, thus leading to plaque stability.

We developed a mechanistic model describing the role of ArgI in athero-sclerotic plaque stability (Figure 7). Elevated ArgI level suppresses the inflammatory response by competitively inhibiting iNOS, which contributes to the secretion of inflammatory cytokines in macrophages and by increasing Th2-type cytokines, for more of an anti-inflammatory response. In addition, ArgI enhances the proliferation of vascular smooth muscle cells by increasing intracellular polyamine production, which in turn promotes collagen production. The increased vascular smooth muscle cell number and collagen content favour a thick fibrous cap, thereby leading to stable plaque.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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