A Novel Mechanism of γ/δ T-Lymphocyte and Endothelial Activation by Shear Stress

The Role of Ecto-ATP Synthase β Chain

Yi Fu,* Yingjian Hou,* Chenglai Fu, Mingxia Gu, Chenghong Li, Wei Kong, Xian Wang, John Y.-J. Shyy, Yi Zhu

Rationale: Endothelial cells (ECs) have distinct mechanotransduction mechanisms responding to laminar versus disturbed flow patterns. Endothelial dysfunction, affected by imposed flow, is one of the earliest events leading to atherogenesis. The involvement of γ/δ T lymphocytes in endothelial dysfunction under flow is largely unknown.

Objective: To investigate whether shear stress regulates membrane translocation of ATP synthase (ATPSβ) in ECs, leading to the increased γ/δ T-lymphocyte adhesion and the related functions.

Method and Results: We applied different flow patterns to cultured ECs. Laminar flow decreased the level of membrane-bound ATPSβ (ecto-ATPSβ) and depleted membrane cholesterol, whereas oscillatory flow increased the level of ecto-ATPSβ and membrane cholesterol. Incubating ECs with cholesterol or depleting cellular cholesterol with β-cyclodextrin mimicked the effect of oscillatory or laminar flow, respectively. Knockdown caveolin-1 by small interfering RNA prevented ATPSβ translocation in response to laminar flow. Importantly, oscillatory flow or cholesterol treatment elevated the number of γ/δ T cells binding to ECs, which was blocked by anti-ATPSβ antibody. Furthermore, the incubation of γ/δ T cells with ECs increased tumor necrosis factor α and interferon-γ secretion from T cells and vascular cell adhesion molecule-1 expression in ECs. In vivo, γ/δ T-cell adhesion and ATPSβ membrane translocation was elevated in the aortic inner curvature and disturbed flow areas in partially ligated carotid arteries of ApoE−/− mice fed a high-fat diet.

Conclusions: This study provides evidence that disturbed flow and hypercholesterolemia synergistically promote γ/δ T-lymphocyte activation by the membrane translocation of ATPSβ in ECs and in vivo in mice, which is a novel mechanism of endothelial activation. (Circ Res. 2011;108:410-417.)

Key Words: endothelial dysfunction ■ T lymphocyte ■ blood flow ■ mechanotransduction ■ ATP synthase

Fluid shear stress plays a pivotal role in vascular physiology and pathophysiology.1,2 Laminar flow imposed on the straight parts of the arterial tree enhances vascular tone, inhibits cell proliferation and thrombosis, and augments anti-inflammatory effects. In contrast, disturbed flow patterns, such as oscillatory flow, at bifurcations and curvatures predispose the endothelium to become atheroprotective.3 Thus, local flow patterns in combination with other risk factors, such as hyperlipidemia and vascular inflammation, result in the focal nature of atherosclerosis. This thesis is supported by the observation that atheroprotective flow enhances lesion development in atherosclerosis-susceptible regions (eg, aortic root and inner curvature of the aortic arch) of apolipoprotein E knockout (ApoE−/−) mice. However, atheroprotective flow spares atherogenesis in the straight part of vessels (eg, thoracic aorta of ApoE−/− mice).

Located at the mitochondrial inner membrane, F1,F0-ATP synthase (F1,F0) produces ATP via the proton gradient generated by the respiratory chain. As the key subunit in F1,F0 for ATP production, F1 consists of a trimeric αβ heterodimer (αβ), around a central stick-like γ chain. Although engaged in the F1-ATPase catalysis, ATP synthase β chain (ATPSβ) is also located on the surface of the plasma membrane.4 This mitochondrial-dissociated ATPSβ, ecto-ATPSβ, is present in many cell types, including vascular endothelial cells (ECs). Various extracellular ligands can bind to ecto-ATPSβ. In hepatocytes, surface ATPSβ serves as a receptor for ApoA-I- or ApoE-enriched high-density lipoprotein.5–7 In ECs, ecto-ATPSβ binds to angiotatin, which suggests that the membrane-bound ATPS is involved in angiogenesis.8–10 Our previous study demonstrated that ECs incubated with choles-
terol induced the translocation of ATPSβ from mitochondria to membrane caveolae,11 which suggests that ecto-ATPSβ may be involved in cholesterol efflux from the vessel wall. Interestingly, ecto-ATPSβ in tumor cells could be recognized by γδ T lymphocytes, possibly through the antigen receptor T-cell receptor (TCR) in T cells.12

Although endothelial dysfunction is one of the earliest vascular events leading to atherogenesis,13,14 macrophages and T lymphocytes are the 2 major hematopoietic cell types infiltrating the vessel wall during atherogenesis. γδ T cells, named after their expression of TCR, represent ≈5% of the T-cell population. Although the α/β subset represents the major T cells seen in atherosclerotic plaque, γδ T cells are also present in lesions, with a high percentage (10% to 15%) of infiltrated T cells in the early stage of atherogenesis.15 Galea et al reported that γδ T cells could bind to and migrate through ECs,16 and Dyugovskaya et al found that after binding to ECs, the activated γδ T cells release various proinflammatory cytokines, including tumor necrosis factor (TNF)α.17

Because the involvement of γδ T cells in endothelial dysfunction is largely unknown, we investigated the regulation of ATPSβ translocation in ECs under laminar versus oscillatory flow and the consequent effect on the interaction with γδ T cells. Compared with static controls, laminar flow decreased the membrane translocation of ATPSβ in ECs, which reduced γδ T-cell adhesion. In contrast, oscillatory flow increased the level of ecto-ATPSβ and enhanced the interaction with γδ T cells, which initiated endothelial activation.

Methods

Reagents, EC Culture, and Treatment

Human umbilical vein ECs (HUVECs) were isolated and cultured as previously described.18 This investigation conforms to the principles outlined in the Declaration of Helsinki for use of human tissue. All of the cells used were before passage 5. Bovine aortic ECs (BAECs) were purchased from Cell Application Inc (San Diego, CA) and cultured. The flow experiments were performed as described previously.19 The applied laminar flow was steady shear stress of 12 dyne/cm². The oscillatory flow generated by an oscillator was shear stress of 0.5 ± 4 dyne/cm² with a frequency of 1 Hz.20 The detail cell culture and treatment are described in the expanded Methods section (available in the Online Data Supplement at http://circres.ahajournals.org).

Purification of Lipid Raft Protein and Western Blot Analysis

Whole-cell lysates, membrane protein, and mitochondrial protein were isolated from ECs by a multiple-centrifugation procedure.21 Lipid raft fractions were purified from ECs by a modified detergent-free procedure.22 Western blot analysis was performed accordingly.

RNA Interference

The caveolin (Cav)-1 small interfering (si)RNA sequence was 5’-CCA GAA GGA ACA CAG ACU U-dTdT-3’ corresponding to bases 223 to 241 of the bovine Cav-1 mRNA.23

Isolation of γδ T Lymphocytes, Cell Adhesion, and Determination the Level of Cytokines

Human peripheral monocytes were obtained from healthy volunteers and isolated on Ficoll-Hypaque density gradient centrifugation. γδ T cells were separated from the isolated monocytes by magnet separation24 and then labeled magnetically with a hapten-modified anti-TCRγδ antibody and fluorescein isothiocyanate–conjugated anti-hapten microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). In cell adhesion assay, confluent HUVECs in 96-well plates were treated with cholesterol, β-cyclodextrin (βCD), or subjected to the specified flow patterns for 2 hours. Purified human γδ T cells (2 × 10⁵ cells/well) were labeled with fluorescence dye (BECIF; Invitrogen) and then coincubated with HUVECs for 30 minutes. Human TNFα and interferon (IFN)γ were measured in media by use of sandwich ELISA kits.

En Face Immunostaining of Mouse Aorta and Animal Experiment

HUVECs were coincubated with purified human γδ T cells for 24 hours and then stained with rabbit anti–vascular cell adhesion molecule (VCAM)-1 antibody and goat anti-rabbit rhodamine red–conjugated secondary antibody (Jackson Immunoresearch Laboratory, West Grove, PA). Eight-week-old ApoE⁻/⁻ and C57BL/6 male mice were obtained from the Peking University Health Science Center. Mice were fed a high-fat diet or a chow diet for 1 week as indicated. The aortic arch and thoracic aorta were fixed and excised in the intima by en face immunostaining as reported previously.25 Partial ligation of the left carotid artery was carried out as described previously26 with minor modification. Detailed methods are described in Online Methods.

Statistical Analyses

Results are expressed as means±SEM from at least 3 independent experiments. Statistical analysis involved the 2-tailed Student t test, 1-way ANOVA, and Dunnett multiple comparison test. P<0.05 was considered statistically significant.

Results

Shear Stress Causes ATPSβ Translocation

Steady laminar flow or high shear stress (5 to 20 dyne/cm²) is proposed to be antiatherosclerotic and disturbed flow with low mean shear stress (<5 dyne/cm²) atheroprone. We first investigated whether different flow patterns could affect ATPSβ translocation between the plasma membrane and the mitochondria in cultured ECs. Under laminar flow (12 dyne/cm²), the ATPSβ level was decreased in plasma membrane but increased in mitochondria at 30 minutes and was maintained for 2 hours (Figure 1A). Therefore, laminar flow might induce the translocation of ATPSβ from the plasma

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**Non-standard Abbreviations and Acronyms**

- **ApoE** : apolipoprotein E
- **ATPSβ** : ATP synthase β chain
- **BAEC** : bovine aortic endothelial cell
- **Cav-1** : caveolin-1
- **CD** : cyclodextrin
- **EC** : endothelial cell
- **HUVEC** : human umbilical vein endothelial cell
- **IFN** : interferon
- **LSS** : laminar flow
- **OSS** : oscillatory flow
- **siRNA** : small interfering RNA
- **TCR** : T-cell receptor
- **TNF** : tumor necrosis factor
- **VCAM** : vascular cell adhesion molecule

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membrane to mitochondria. Under oscillatory flow (0.5±4 dyn/cm²), the ATPSβ level was increased in plasma membrane but decreased in mitochondria at 30 minutes (Figure 1B). To examine the temporal effect of different flow patterns on ATPSβ redistribution, the flow exposure time was extended to 24 hours. Laminar flow caused a transient reduction of ATPSβ in the membrane fraction (Figure 1C). However, oscillatory flow induced sustained membrane localization (Figure 1D). These effects were not attributable to the upregulation of ATPSβ because the level of total ATPSβ was not changed by laminar or oscillatory flow.

ATPSβ Translocation Is Induced by Alteration of Membrane Cholesterol Content

Because cholesterol plays an important role in protein localization and membrane fluidity, we determined the cholesterol content in the EC plasma membrane subjected to different flow patterns. The level of membrane cholesterol decreased after exposure to laminar flow (Figure 2A). This effect of laminar flow was similar to treatment with βCD, an agent depleting membrane cholesterol. In contrast, oscillatory flow increased the level of membrane cholesterol as early as 30 minutes, an effect mimicked by cholesterol treatment. We then stimulated ECs with βCD or cholesterol and found that βCD decreased but cholesterol increased the level of ATPSβ in the plasma membrane. Reciprocally, βCD augmented and cholesterol decreased the content of ATPSβ in mitochondria (Figure 2B). To investigate further the effects of membrane cholesterol on the flow-induced ATPSβ translocation, we pretreated ECs with βCD or cholesterol before exposure to flow. As shown in Figure 2C, cholesterol and βCD could block the effects of laminar and oscillatory flow, respectively, on ATPSβ translocation. Therefore, the cholesterol content in ECs changed by the applied flow patterns may cause the translocation of ATPSβ between plasma membrane and mitochondria.

Translocation of ATPSβ Depends on Cav-1

Ecto-ATPSβ is present in endothelial caveolae, and our previous study demonstrated that ecto-ATPSβ was associated with Cav-1 in caveolae after cholesterol loading.11 Because cholesterol loading mimicked oscillatory flow in driving ATPSβ translocation, we investigated the role of Cav-1 in this translocation in ECs responding to laminar versus oscillatory flow. As shown in Figure 3A, laminar flow induced the
migration of both ATPSβ and Cav-1 from lipid rafts. However, oscillatory flow caused an opposite effect and drove ATPSβ and Cav-1 into lipid rafts. To further delineate the role of Cav-1 in ecto-ATPSβ migration, we knocked down Cav-1 by siRNA and blocked the laminar flow-induced ATPSβ translocation (Figure 3B). Moreover, βCD or cholesterol-induced translocation of ATPSβ in ECs was inhibited by Cav-1 siRNA (Figure 3C).

Ecto-ATPSβ Affects the Adhesion of γδ T Lymphocytes to ECs

Given that ecto-ATPSβ binds to the TCR of γδ T cells,4 we compared the adhesion of human γδ T cells to HUVECs exposed to laminar or oscillatory flow. Compared with static controls, laminar flow significantly decreased but oscillatory flow greatly increased the adhesion of γδ T cells (Figure 4A and 4B). To test whether the oscillatory flow–enhanced γδ T-cell attachment was due, at least in part, to an increase in ecto-ATPSβ level, we pretreated HUVECs with a blocking antibody against ATPSβ. As expected, the blockade of ecto-ATPSβ abolished the γδ T-cell attachment imposed by oscillatory flow (Figure 4B).

Because cholesterol depletion or enrichment with βCD or cholesterol treatment caused similar effects as those by laminar or oscillatory flow, respectively, on ATPSβ translocation, we investigated the adhesion of γδ T cells to HUVECs treated with βCD or cholesterol. Indeed, γδ T lymphocytes decreased their adhesion to the βCD-treated ECs as compared with untreated controls (Figure 4C). However, the γδ T-cell adhesion increased on pretreatment with cholesterol (Figure 4D). Similar to oscillatory flow, anti-ATPSβ antibody treatment abolished the cholesterol-enhanced γδ T-cell adhesion (Figure 4D). Therefore, laminar and oscillatory flow had opposite effects on the adhesion of γδ T cells, which was mainly attributable to the level of ecto-ATPSβ in the EC membrane.

Binding of Ecto-ATPSβ and γδ TCR Activated Both Endothelium and T Lymphocytes

When the TCR of lymphocytes binds to its ligands present in the target cells, the cognate cells are activated. On binding to ecto-ATPSβ, the activated γδ T cells release various proinflammatory cytokines, including IL-8, IFNγ and TNFα, which are hallmarks of γδ T-cell activation. Consistent with results from our adhesion assay, incubation of ECs with

Figure 3. ATPSβ translocation depends on caveolin-1. A, BAECs were subjected to laminar flow or oscillatory flow for 2 hours. Sucrose gradient ultracentrifugation was used for isolating lipid rafts (fractions 4 to 5) and mitochondrial fractions (fractions 8 to 10). Lipid raft and mitochondrial fractions were examined by Western blot analysis with anti-ATPSβ, anti–Cav-1, and anti–Flotillin-2 antibodies. The images are representative of 3 independent experiments. B and C, BAECs were transfected with scramble or Cav-1 siRNA for 48 hours and then subjected to different flow patterns, βCD, or CHL for 2 hours. The level of ATPSβ and Cav-1 was detected by Western blot analysis. Graph shows the ratio of membrane ATPSβ to total ATPSβ. Data were from 3 independent experiments with static control set as 1. *P<0.05. Mem indicates membrane; Mito, mitochondria; Raft, lipid raft; T, total.

Figure 4. γδ T-cell adhesion to ECs is mediated through endothelial ecto-ATPSβ. HUVECs were subjected to laminar flow (LSS) (A), oscillatory flow (OSS) (B), or βCD (C) for 1 hour or CHL treatment for 2 hours (D). ECs were then incubated with BCECF-labeled γδ T cells for 30 minutes. In the blocking experiment, HUVECs were pretreated with anti-ATPSβ antibody (50 μg/ml) for 30 minutes before γδ T-cell incubation. Adherent T cells were counted using fluorescence microscopy. The representative result on the left shows the attached cells (green) and phase-contrast images. The graphs on the right show the number of bound lymphocytes per EC. The results represent the means±SEM from 3 independent experiments. *P<0.05.
cholosteryl increased the levels of TNFα and IFNγ in the media (Figure 5A). When ECs were cotreated with antibody against ATPSβ and cholesterol, TNFα secretion was prevented (Figure 5B).

TNFα released from activated γδ T cells can induce an inflammatory response of ECs such as increased expression of VCAM-1.27,28 Thus, we detected VCAM-1 expression in ECs incubated with γδ T cells for 2 hours and then incubated with or without γδ T cells for 24 hours. The VCAM-1 expression in ECs was analyzed by real-time PCR (C) and immunostaining (D). E, The collected cocultured medium was incubated with new HUVECs for 24 hours, and VCAM-1 expression was analyzed by immunostaining with anti-VCAM-1 antibody and Hoechst 33258. Pseudocolor images show the merge of VCAM-1 (red) and nucleus (blue). Results are representative from 3 independent experiments.

**Figure 5.** γδ T lymphocytes and ECs were activated by their interaction. A, HUVECs were pretreated with cholesterol for 2 hours and then coincubated with γδ T cells for 24 hours. B, HUVECs were pretreated with cholesterol for 2 hours and then coincubated with γδ T cells in the presence of IgG or anti-ATPSβ antibody (50 μg/mL) for 24 hours. Supernatant was collected, and the levels of TNFα and IFNγ were determined by ELISA. Graphs show amount of TNFα or IFNγ secreted to the media with T cells under different conditions. *P<0.05 vs controls. C and D, HUVECs were pretreated with cholesterol or pretreatment combined with the addition of γδ T cells (Figure 5C and 5D). However, VCAM-1 expression did not increase in ECs treated with cholesterol alone (Figure 5C). This effect of VCAM-1 upregulation in ECs was confirmed by using conditioned media collected from the coculture of γδ T cells and cholesterol-pretreated ECs (Figure 5E).

**Figure 6.** Disturbed flow enhanced endothelial ATPSβ membrane translocation and γδ T cells binding to the aortic endothelium in vivo. Following partial ligation of carotid arteries, 8-week-old male ApoE−/− mice (n=6) were fed a high-fat diet and C57BL/6 mice were fed a normal diet for 1 week. The aortic arch, thoracic aorta, and left (partially ligated) and right (sham operation) carotid arteries were isolated for en face immunofluorescence analysis. A, Representative images of ATPSβ (red), Cav-1 (green), and nucleus (blue) from different aortic regions of ApoE−/− mice. B, Representative images of TCRγ (red) and nucleus (blue) in different regions of aorta of ApoE−/− mice. C and D, Representative images of TCRγ (green), Mac3 (red, top), and TCRα (red, bottom) with their nuclei in blue from distinct aortic segments of C57BL/6 and ApoE−/− mice. E and F, Representative images of ATPSβ (green), TCRγ (red), and nucleus (blue) from different ligated and sham-treated carotid arteries of ApoE−/− and C57BL/6 mice.

Disturbed Flow Induced γδ T Cells Attaching to the Vascular Wall In Vivo

To confirm in vivo the findings obtained from in vitro experiments, we investigated the distribution of ATPSβ and γδ T cells in mouse aorta with hyperlipidemia. At the inner curvature of the aortic arch, blood flow patterns are disturbed and the flows on the outer curvature and thoracic aorta are relatively laminar.3,29 To induce hyperlipidemia, 8-week-old male ApoE−/− mice were fed a high-fat diet for 1 week, and then aortas were isolated for en face immunostaining for ATPSβ and Cav-1. Confocal microscopy showed that ATPSβ and Cav-1 expression on the EC membrane was enhanced in the curvature of the aortic arch, when compared with the thoracic aorta (Figure 6A). As a functional consequence of the ATPSβ surface expression, TCRγ-positive cells were increased in the inner curvature of the aortic arch, which indicates an increase in the adhesion of γδ T lymphocytes to the vessel wall, not seen in the outer curvature or thoracic aorta (Figure 6B).

Given that macrophages and conventional αβ T lymphocytes are associated with endothelial activation and atherogenesis, the adhesion of Mac3- or TCRα-positive cells on the surface of endothelium of mouse aorta was assayed in C57BL/6 and ApoE−/− mice. In C57BL/6 mice, we could not detect any T-cell subpopulation adhered to the thoracic aorta. However, a weak staining of Mac3- and TCRγ-positive cells
was found in the inner curvature of the aortic arch (Figure 6C). Compared with control mice, ApoE/−/− mice fed a high-fat diet showed significantly increased adhesion of Mac3- or TCRγ-positive cells but not TCRα-positive cells in the inner curvature of the aortic arch (Figure 6D). To further investigate the effect of disturbed flow on the adhesion of γδ T cells to endothelium, we used an animal model of changed arterial shear stress and accelerated atherogenesis after partial ligation of carotid arteries of ApoE/−/− mice followed by a high-fat diet. Reducing shear stress by partial ligation markedly promoted ecto-ATPSβ expression in endothelium and γδ T-lymphocyte adhesion in ApoE/−/− mice fed a high-fat diet (Figure 6E and 6F). In control C57BL/6 mice, partial ligation led to a moderate increase in ecto-ATPSβ expression (Figure 6E) but not γδ T-lymphocyte adhesion on the endothelium (Figure 6F).

Discussion

The focal nature of atherosclerotic lesions is largely attributable to the distinct effects of local flow patterns predisposing other atherogenic events. Steady laminar flow is atheroprotective by counteracting hyperlipidemia and inflammation, but disturbed flow is atheropromotive and aggravates these pathological factors. In this study, steady laminar flow reduced ecto-ATPSβ adhesion on the EC membrane in vitro and in vivo, which resulted from the depletion of membrane cholesterol. In contrast, oscillatory flow enhanced the localization of ecto-ATPSβ at the EC membrane. We showed that Cav-1 plays a central role in determining the ecto-ATPSβ distribution. The functional consequence of increased level of ecto-ATPSβ on the EC membrane was the enhanced adhesion of γδ T lymphocytes to ECs, which in turn induced the release of cytokines, including IFNγ and TNFα, by the attacked γδ T cells and hence elevated the expression of VCAM-1 in ECs. This model of action is presented in Figure 7.

In response to oscillatory flow, the increase in ATPSβ bound to the membrane lasted for 24 hours (Figure 1). Thus, the effect of oscillatory flow on ATPSβ translocation was sustained. This notion is consistent with results from en face staining demonstrating a higher level of ecto-ATPSβ in ECs under disturbed flow than under laminar flow (Figure 6). Yamamoto et al showed an increase in ecto-ATPSβ level that led to ATP release within minutes after the onset of a step flow administered to ECs. This experimental condition could be viewed as the EC response to a rapid change in flow environment. In contrast, results seen in Figures 1 and 6 represent the regulation of ATPSβ by physiological or pathophysiological flow conditions.

Our previous work demonstrated that cholesterol incubation enhanced ecto-ATPSβ translocation in ECs. The involved mechanism was cholesterol increasing the lipid raft content, which drove the migration of a complex of ATPSβ and Cav-1 from mitochondria to lipid rafts. These effects could be blocked by cytochalasin B, which suggests that this process depends on the actin-based cytoskeleton. The effect of laminar versus oscillatory flow in determining the localization of ATPSβ was similar to that with cholesterol or βCD incubation. Thus, we hypothesize that the flow-induced ecto-ATPSβ translocation depends on cholesterol content. βCD and cholesterol blocked the distinct effects of oscillatory and laminar flows on ATPSβ translocation. Furthermore, Cav-1 knockdown blocked both flow- and cholesterol-induced ATPSβ translocation. Thus, the intracellular ATPSβ translocation would be highly associated with lipid rafts (eg, caveolae) and Cav-1 protein level. However, we found that the distinct effects of different flows on membrane cholesterol content were greater in short-term experiments (Figure 2A) as on ecto-ATPSβ translocation (Figure 1). The intracellular cholesterol homeostasis may be exquisitely regulated and depends on the balance between cholesterol synthesis and influx, cholesterol ester formation, and translocation to the plasma membrane for efflux. In flow channel experiments, ECs are adapted to the change of membrane cholesterol to maintain homeostasis after prolonged exposure of the applied shear stress. Therefore, the dynamic change of membrane translocation of cholesterol seems more important in initiating the cascade of ATPSβ translocation. More importantly, our in vivo experiments showed that disturbed flow patterns promoted ecto-ATPSβ expression in endothelium, with increased adhesion of γδ T lymphocytes. This result also suggests that the change in membrane cholesterol content by oscillatory flow promotes ATPSβ translocation. When synergistic with hypercholesterolemia, this atheroprotective effect leads to dysfunctional endothelium.

In addition to many inflammatory cells such as macrophages and αβ T cells, lymphocytes bearing γδ TCR are also involved in atherogenesis. Although γδ T cells represent a small portion of CD3+ cells in human peripheral blood (<5%), these cells account for a higher percentage (10%6537415%) among infiltrated T lymphocytes in early lesions. Compared with αβ CD4+ and CD8+ T cells, γδ T cells have a higher potency to transmigrate endothelium.
ATPSβ on the tumor cell surface binds to γδ TCR, which activates γδ T cells.13,23 Our results showed that oscillatory flow or cholesterol incubation potentiated the adhesion of γδ T cells to ECs, whereas laminar flow or βCD attenuated this association. The effect of oscillatory flow or cholesterol was reversed by the blockade of ecto-ATPSβ, which suggests that ecto-ATPSβ mediated the γδ T-cell–EC interaction (Figure 4). In addition to changes in γδ T-cell adhesion, the increase in mitochondrial ATPSβ with a complementary decrease in ecto-ATPSβ may also benefit mitochondrial biogenesis in ECs. This scenario could be attributable to the involvement of ATPase in ATP production and electron transport in mitochondria.

The activation of γδ T cells is manifested by the release of various proinflammatory cytokines, including TNFα, IFNγ, and IL-8.17 The expression of adhesion molecules such as VCAM-1, a marker of endothelial activation, can be induced by these cytokines. Our ELISA experiments revealed that γδ T cells coincubated with cholesterol-treated ECs increased the release of TNFα and IFNγ into the cocultured medium, as compared with in the absence of cholesterol (Figure 5A). The release of those cytokines would be attributable to the increase in the membrane ATPSβ and the activation of γδ T lymphocytes, because a blocking antibody against ATPSβ could attenuate the release of TNFα (Figure 5B). Importantly, TNFα was undetectable in ECs not coincubated with γδ T cells (data not shown). Conversely, coculture of γδ T cells with βCD-treated HUVECs reduced the release of TNFα (data not shown). Furthermore, immunofluorescence assay and RT-PCR showed that VCAM-1 expression was increased in ECs pretreated with cholesterol and prolonged coculture with γδ T cells (Figure 5C). Such an elevated expression of VCAM-1 in ECs was not seen in the absence of γδ T cells, which indicates that the interaction of γδ T cells caused the activation of ECs. Furthermore, this effect was induced by cytokines released in the medium because of VCAM-1 upregulation seen in ECs incubated with the conditioned medium (Figure 5E). In line with the in vitro study, in vivo, the inner curve of the aortic arch, which is presumably under disturbed flow, showed increased membrane ATPSβ level and γδ T lymphocyte adhesion (Figure 6A and 6B). Importantly, ApoE−/− mice with hypercholesterolemia but not control C57BL/6 mice showed increased ATPSβ-mediated adhesion of γδ T lymphocytes (Figure 6C and 6D). Our results agree with previous reports that VCAM-1 and ICAM-1 are highly expressed in the inner curve of the aortic arch.25,26 but are reduced or absent in the outer curve or thoracic aorta.

Disturbed flow, hypercholesterolemia, and vascular inflammation are important pathogenic factors leading to atherosclerosis. Hence, our work provides a novel mechanism of the synergistic effect of these features in atherosclerosis. Local flow patterns predispose other risk factors such as hyperlipidemia to result in the focal nature of atherosclerosis. In atherosclerosis-susceptible regions of ApoE−/− mice (eg, aortic root and inner curvature of the aortic arch), disturbed blood flow enhances lesion development in part through ATPSβ-mediated γδ T-cell adhesion. Similar results were obtained from a model in which the flow pattern was changed by partial ligation of mouse carotid arteries (Figure 6E and 6F). With this animal model, Nam at el reported marked shear stress reduction, as well as significant endothelial dysfunction and atherogenesis in ligated carotid arteries in ApoE−/− mice fed a high-fat diet. However, atheroprotective flow spares atherogenesis in the straight part of vessels under hyperlipidemic conditions (eg, thoracic aorta and unligated sham-treated carotid artery of ApoE−/− mice). In control C57BL/6 mice, partial ligation led to a moderate increase in ecto-ATPSβ expression (Figure 6E) but not adhesion of γδ T lymphocytes on intima (Figure 6F). This finding suggests a synergistic effect between disturbed flow and hypercholesterolemia on the adhesion of γδ T cells to dysfunctional endothelium in the atheroprone areas in vivo.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- Shear stress resulting from blood flow is a key determinant of the focal nature of atherosclerosis.
- Endothelial dysfunction caused by the interaction of flow characteristics and high plasma cholesterol concentrations is an early event in development of atherosclerotic lesions.
- Macrophages and T lymphocytes are 2 major hematopoietic cell types infiltrating the vessel wall during atherosclerosis.
- Cell membrane–bound ATP synthase β chain (ATPSβ) in tumor cells could be recognized by γδ T lymphocytes.

**What New Information Does This Article Contribute?**

- In vitro, membrane-bound ATPSβ is decreased by laminar flow, whereas it is increased by oscillatory flow.
- High cholesterol concentrations and laminar flow induce the translocation of ATPSβ from mitochondria to membrane caveolae in endothelial cells and promote adhesion of γδ T cells.

- In vivo, the γδ T cell adhesion increases in lesion-prone areas of arteries.

Endothelial dysfunction, induced by imposed flow, is one of the earliest events in atherogenesis. The infiltration of T lymphocytes into the vessel wall also contributes to atherosclerotic lesion formation. However, it is unclear whether the detrimental effects γδ T lymphocytes and endothelial dysfunction are synergistic. We found that oscillatory flow increased ATPSβ on the endothelial membrane, as well as membrane cholesterol concentrations. Consequently, the endothelial cells facilitated inflammatory processes including the promotion of γδ T lymphocyte recruitment. This study provides the first line of evidence showing that the combination of disturbed flow and hypercholesterolemia synergistically promote the activation of γδ T-lymphocyte through the membrane translocation of ATPSβ in endothelial cells. This represents a novel mechanism of endothelial activation.
A Novel Mechanism of γδ T-Lymphocyte and Endothelial Activation by Shear Stress: The Role of Ecto-ATP Synthase β Chain

Yi Fu, Yingjian Hou, Chenglai Fu, Mingxia Gu, Chenghong Li, Wei Kong, Xian Wang, John Y.-J. Shyy and Yi Zhu

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Methods and Materials

Reagents, antibodies and EC culture
Human umbilical vein ECs (HUVECs) were isolated and cultured as previously described. The investigation conforms to the principles outlined in the Declaration of Helsinki for the use of human tissue. All the cells used were prior to passage 5. Bovine aortic ECs (BAECs) were purchased from Cell Application, Inc. (San Diego, CA) and cultured in DMEM (Hyclone, Logan, UT) supplemented with 10% FBS (Omega, Tarzana, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells at passages 5-8 were used for experiments. The cholesterol determination kit was from Wako Chemicals (Richmond, VA) or BIOSINO Inc. (Beijing). ELISA kits for hTNFα and IFNγ were from Dakewe Biotech Co. (Shenzhen, China). Cholesterol, β-cyclodextrin (βCD) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies for western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow experiments
The flow experiments were performed as previously described. In brief, glass slides seeded with confluent BAECs or HUVECs were assembled into a parallel-plate flow channel. The flow system was kept at 37°C and ventilated with 95% humidified air and 5% CO₂. The applied laminar flow was steady shear stress of 12 dyne/cm². The oscillatory flow generated by an oscillator was shear stress of 0.5±4 dyne/cm² with a frequency of 1 Hz.

Protein extraction and purification of lipid rafts
Whole cell lysates, membrane protein, and mitochondrial protein were isolated from ECs by a multiple-centrifugation procedure. Briefly, ECs were homogenized. After centrifugation at 3,000×g for 10 min, the supernatant served as the whole-cell protein sample. After centrifugation at 12,000×g for 10 min, the pellet was considered the mitochondrion fraction. The resting supernatant was ultracentrifuged at 130,000×g for 40 min. The final pellet served as the membrane fraction.

Lipid raft fractions were purified from ECs by a modified detergent-free procedure. Twelve fractions were collected from the gradient. Protein in fractions 4-5 and 8-10 were further ultracentrifuged and used as lipid raft protein and mitochondrial protein, respectively.

Western blot analysis
Protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with various primary antibodies, then horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized by use of an ECL kit (GE Healthcare, UK), and the densities of the protein bands were measured by use of NIH Image J software.

Cholesterol determination
Total cholesterol kits were used for cholesterol determination. The membrane fraction was isolated as described. Cholesterol in the membrane fraction normalized to protein concentration was assessed.

RNA interference
The caveolin-1 (Cav-1) siRNA sequence was 5′-CCA GAA GGA ACA CAC AGU U-dTdT-3′ corresponding to bases 223-241 of the bovine caveolin-1 mRNA. The scramble siRNA sequence was used as a negative control. BAECs at 50%-70% confluence were transfected
with siRNA (180 pmol/dish, 45 nM) with RNAiMAX lipofectamine (Invitrogen, Grand Island, NY). After 48 hr, transfected ECs were seeded on slides for shearing or chemical stimulation.

**Isolation of γ/δ T lymphocytes, cell adhesion and determination of TNFα level**

Human peripheral monocytes were obtained from healthy volunteers and isolated on Ficoll-Hypaque density gradient centrifugation. The protocol was approved by the human research committee of Peking University, Health Sciences Center, and volunteers agreed to the use of cells. γ/δ T cells were separated from the isolated monocytes by magnet separation, then labeled magnetically with a hapten-modified anti-TCR γ/δ antibody and fluorescein isothiocyanate-conjugated anti-hapten microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Subsequent positive selection involved MS columns. The isolated γ/δ T cells were more than 95% pure, as assessed by flow cytometry, and cell viability was up to 90%, as determined by trypan blue exclusion.

In cell adhesion assay, confluent HUVECs in 96-well plates were treated with cholesterol, βCD or different flow patterns for 2 hr. Purified human γ/δ T cells (2 x 10^5 cells/well) were labeled with fluorescence dye (BCECF, Invitrogen), then coincubated with HUVECs for 30 min. The nonadhering cells were washed off, and the adhered γ/δ T cells were counted under a fluorescence microscope. Pre-treatment of HUVECs with anti-ATPSβ antibody (50 μg/ml) for 30 min was used in the blocking experiment. To measure the release of TNFα and IFNγ in the cocultured media, the duration of coincubation was 24 hr. Human TNFα and IFNγ were measured in media by use of sandwich ELISA kits.

**Quantitative real-time RT-PCR**

The nucleotide sequences of the primers were as follows: GAPDH: 5’-gagtcacaggtggtgctg-3’ and 5’-tgattctgaggatctc-3’; VCAM-1, 5’-taaaatgcctgggaagatgg-3’ and 5’-ctggtgtgtctgaatct-3’.

**Immunofluorescence and en face immunostaining of mouse aorta**

HUVECs were coincubated with purified human γ/δ T cells for 24 hr, then stained with rabbit anti-VCAM-1 antibody and goat anti-rabbit Rhodamine red-conjugated secondary antibody (Jackson Immunoresearch Lab, West Grove, PA). The nuclei were counterstained with Hoechst 33258.

Eight-week-old ApoE−/− and C57BL/6 male mice were obtained from the Peking University Health Science Center. All experimental protocols were approved by the Peking University Institutional Animal Care and Use Committee. ApoE−/− mice were fed a high-fat diet and C57BL/6 mice a chow diet until partial ligation. Partial ligation of the left carotid artery (LCA) was carried out as previously described with minor modification. After surgery, ApoE−/− mice were fed a high-fat diet until partial ligation.
diet and C57BL/6 mice a chow diet for 1 week. Then, carotid arteries were isolated, fixed and excised for determination of ATPS$\beta$ and TCR$\gamma$ levels in the intima by en face immunostaining.

**Statistical Analyses**

Results are expressed as mean±SEM from at least 3 independent experiments. Statistical analysis involved the 2-tailed Student's t test, one-way ANOVA and Dunnett's multiple comparison test. A P<0.05 was considered statistically significant.

**References**


