Shc coordinates signals from intercellular junctions and integrins to regulate flow-induced inflammation

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Atherosclerotic plaques develop in regions of the vasculature associated with chronic inflammation due to disturbed flow patterns. Endothelial phenotype modulation by flow requires the integration of numerous mechanotransduction pathways, but how this is achieved is not well understood. We show here that, in response to flow, the adaptor protein Shc is activated and associates with cell–cell and cell–matrix adhesions. Shc activation requires the tyrosine kinases vascular endothelial growth factor receptor 2 and Src. Shc activation and its vascular endothelial cadherin (VE-cadherin) association are matrix independent.

In contrast, Shc binding to integrins requires VE-cadherin but occurs only on specific matrices. Silencing Shc results in reduction in both matrix-independent and matrix-dependent signals. Furthermore, Shc regulates flow-induced inflammatory signaling by activating nuclear factor κB-dependent signals that lead to atherogenesis. In vivo, Shc is activated in atherosclerosis-prone regions of arteries, and its activation correlates with areas of atherosclerosis. Our results support a model in which Shc orchestrates signals from cell–cell and cell–matrix adhesions to elicit flow-induced inflammatory signaling.

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Abbreviations used in this paper: AP, atheroprone; AR, atheroresistant; BAEC, bovine aortic endothelial cell; CL, collagen; EC, endothelial cell; ERK, extracellular signal-regulated kinase; FN, fibronectin; LN, laminin; NFκB, nuclear factor κB; VE-cadherin, vascular endothelial cadherin; VEGFR2, vascular endothelial growth factor receptor 2; VIT, 4-[4′-chloro-2′-fluorophenylamino]-6,7-dimethoxyquinazoline.

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Introduction

Fluid shear stress, the frictional force from blood flow, acts directly on the endothelium to modulate vessel structure and function (Davies, 1997). In arterial regions with laminar flow, the endothelial cells (ECs) express various atheroprotective genes and suppress several proatherogenic ones, leading eventually to stability and quiescence (Malek et al., 1999). In contrast, in regions with slow or disturbed flow where low shear stress occurs, the atheroprotective genes are suppressed, whereas the proatherogenic genes are up-regulated, thereby promoting the atherosclerotic process (Malek et al., 1999). Importantly, acute onset of laminar flow stimulates many of the same responses as disturbed shear. However, over longer periods, the cells adapt to the unidirectional shear forces and down-regulate the stress signaling, whereas in the disturbed shear, continual changes in flow magnitude and direction lead to sustained signaling (Orr et al., 2006). Thus, the in vitro protocol in which cells under static conditions are exposed to an abrupt increase in flow has been widely used as a model for disturbed flow and is particularly useful in analyzing temporal responses to flow.

EC surfaces are equipped with numerous mechanoreceptors that are capable of detecting and responding to shear stress (Traub and Berk, 1998; Lehoux et al., 2006). After activation of mechanoreceptors, a complex network of several intracellular pathways is triggered, a process known as mechanotransduction. Forces from the apical surface must be transmitted through the cytoskeleton to points of attachment that resist shear stress and anchor the cell in place (Davies, 1995). In that regard, both cell–cell and cell–ECM adhesions have been implicated in shear stress signal transduction. The junction-localized, endothelial-specific cadherin, vascular endothelial cadherin (VE-cadherin), is required for transducing shear stress–dependent signals into the endothelium (Shay-Salit et al., 2002; Tzima et al., 2005). We recently reported that VE-cadherin forms a mechanosensory complex with the EC adhesion molecule PECAM-1 and tyrosine kinase VEGF receptor 2 (VEGFR2), and this minimal complex is necessary for a subset of endothelial shear stress responses, such as the activation of nuclear factor κB (NFκB) and proinflammatory target genes (Tzima et al., 2005). In addition to cell–cell junctions, cell–matrix adhesions have also been implicated in shear stress signaling. Acute onset of laminar flow stimulates the conversion of integrins to a high-affinity state
(Tzima et al., 2001) followed by their binding to the subendothelial ECM (Jalali et al., 2001; Tzima et al., 2001). The newly occupied integrins subsequently activate multiple signaling pathways that lead to cell and cytoskeletal alignment in the direction of flow as well as the activation of NFκB, which is important for the expression of inflammatory genes in the endothelium (Jalali et al., 2001; Tzima et al., 2001, 2002, 2003). Importantly, the activation of NFκB by flow is dependent on ECM composition (activated on fibronectin [FN] but not collagen [CL]; Orr et al., 2005), and certain types of matrix proteins, such as FN, are deposited at the atherosclerosis-prone sites in vivo (Sechler et al., 1998). Although the biochemical and mechanical consequences of integrin- and cadherin-mediated adhesions each have been described, how these adhesions cross-talk and cooperate, especially in response to flow, is less well understood.

Members of the Shc family of adaptor proteins are key components of the pathways that activate Ras and MAPKs downstream of growth factors, cytokines, integrins, and mechanical forces (Pelici et al., 1992; Chen et al., 1999; Ravichandran, 2001). Shc is phosphorylated at tyrosine residues 239/240 and 317 and recruits the adaptor protein Grb2 and the nucleotide exchange factor SOS (Ravichandran, 2001). The assembly of Shc–Grb2–SOS complex provides a mechanism for the activation of Ras and the MAP kinases (Ravichandran, 2001).

In addition, tyrosine-phosphorylated Shc associates with integrins α5β1, αvβ3, and αvβ1 when they are conjugated to the appropriate ligands (Bhattacharya et al., 1995; Wary et al., 1996). Notably, ShcA is expressed primarily in the cardiovascular system of mouse embryos and is required for normal development of the heart and the vascular system (Lai and Pawson, 2000).

Here, we show that Shc associates with constellations of both cell–cell and cell–matrix adhesions in response to flow. Furthermore, activation of Shc occurs in areas of disturbed flow and correlates with atherosclerosis in vivo. Finally, we reveal a surprising role for Shc in flow-induced inflammatory signaling. Thus, Shc orchestrates signals from junctional and matrix adhesion complexes to mediate inflammatory signaling in response to fluid flow.

Results

Activation of Shc in atheroprone (AP) areas of the vasculature

Areas of disturbed flow in vivo are prone to inflammation and atherogenesis. To determine whether Shc is activated in a flow-dependent manner, we performed immunohistochemical analyses in different locations of C57BL/6 mouse aortas using a Shc phospho-Tyr239/240 antibody as a marker. The arch of the aorta, which corresponds to proinflammatory and AP regions (Suo et al., 2007), showed remarkably pronounced phospho-Shc staining. In contrast, the ascending aorta, which is atheroresistant (AR), displayed barely detectable levels of phospho-Shc (Fig. 1 A). The focal activation of Shc is not caused by differences in Shc expression levels as shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200709176/DC1). Thus, activated Shc is localized in regions where blood vessels exhibit sharp curvatures and are therefore more likely to be susceptible to blood turbulence and to develop atherosclerotic lesions. To further test whether the activation of Shc correlates with atherosclerotic lesions in vivo, the ascending aortas from ApoE−/− and C57BL/6 mice were isolated and processed for immunohistochemistry. ApoE−/− mice develop atherosclerotic lesions throughout the aortic tree, with localization similar to the lesions seen in human atherosclerosis (Daugherty, 2002). Although Shc phosphorylation was robust in ApoE−/− mice, particularly in ECs overlaying the atherosclerotic lesions, it was almost undetectable in C57BL/6 aorta (Fig. 1 B). Thus, Shc phosphorylation correlates with atherogenesis and atherosclerosis-prone regions near bifurcations.

The distinct activation pattern of Shc in areas of atherosclerosis in vivo prompted us to ask whether the activation of Shc is regulated by different flow patterns in vitro. Cells stimulated with prolonged oscillatory flow (which is proinflammatory and atherogenic) showed elevated Shc phosphorylation compared with cells stimulated with extended laminar flow (which is considered antiinflammatory and atheroprotective; Fig. 1 C). Interestingly, acute onset of laminar shear stimulates many of the same responses as disturbed/oscillatory flow (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709176/DC1). However, in a prolonged laminar shear, these events are downregulated as cells adapt, whereas in a disturbed shear, they are sustained (Orr et al., 2005). Thus, a large number of in vitro studies have exploited the acute onset of laminar shear to model AP signaling, and a temporal map of signaling cascades can already be assembled (Chatziziisis et al., 2007). We therefore used the laminar flow protocol to assay the early responses downstream of Shc. To this end, we first examined whether the rapid initiation of flow also regulates Shc activation in vitro. In bovine aortic ECs (BAECs), Shc phosphorylation was readily detected upon the onset of flow, as assessed by immunoblotting the cell lysates with a phospho-Shc Tyr239/240 antibody (Fig. 1 E).

Notably, all three isoforms of Shc are phosphorylated in response to flow. Immunofluorescence staining showed that a fraction of activated Shc localized to cell–cell junctions (Fig. 1 D) and colocalized with β-catenin (Fig. S3 A). Flow-induced phosphorylation of Shc Tyr317 was not observed in parallel experiments (unpublished data), which suggests that the downstream signaling elicited by Shc in response to flow is primarily mediated through its phosphorylation at Tyr 239/240.

Shc associates with components of EC junctions in response to shear stress

The distinct spatial activation of Shc in response to the onset of flow suggested that Shc might associate with components of interendothelial junctions. Recently, we identified a minimal complex necessary for a subset of EC shear stress responses, which requires PECAM-1, VE-cadherin, and VEGFR2 (Tzima et al., 2005). To further investigate the role of Shc in shear stress signaling, the association of Shc with crucial components of the VE-cadherin–VEGFR2 signaling pathway was examined. Rapid onset of flow induced an acute association of Shc with VE-cadherin as assessed by coimmunoprecipitation assays (Fig. 2 A). VEGFR2 was also present in these immune complexes (Fig. 2 A), which suggests its possible role in Shc activation and the existence
Figure 1. **Shc phosphorylation in vivo and in vitro.** (A) Aortas were isolated from C57BL/6 mice. Serial sections were obtained from AR and AP regions and stained for phospho-Tyr239/240 of Shc. Inserts show ECs lining up the aorta lumen. (B) Aortas were removed from ApoE-/- or C57BL/6 mice of the same age and embedded in paraffin. Serial sections were stained for phospho-Tyr239/240 of Shc. Boxes indicate the enlarged views shown in the insets. (C) BAECs were plated on FN-coated slides and subjected to oscillatory or laminar flow for 18 h or kept as static controls. Cell lysates were analyzed by immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. (D) BAECs were left untreated or subjected to laminar flow at 12 dyne/cm² for 5 min as described in the Materials and methods. Cells were subsequently fixed, permeabilized, and immunostained for phospho-Tyr239/240 of Shc. (E) BAECs were plated on FN-coated slides and sheared for 1, 5, or 30 min or kept as static controls. Cell lysates were subjected to SDS-PAGE and immunoblotting with anti-Shc phospho-Tyr239/240 or anti-Shc antibodies. Numbers to the left of the gel blots indicate molecular mass standards in kD.
of a multiprotein complex induced by shear. Stimulation of ECs with oscillatory flow also induced the formation of a Shc–VEGFR2–VE-cadherin complex and the localization of activated Shc to junctions (Fig. S3). Importantly, the association of Shc with VE-cadherin was sustained under oscillatory flow (Fig. S3 C), which is similar to the sustained Shc phosphorylation (Fig. 1 C).

Because VE-cadherin is required for VEGFR2 activation by flow and downstream shear-dependent signaling (Shay-Salit et al., 2002; Tzima et al., 2005), we tested whether VE-cadherin is essential for the interaction of Shc with VEGFR2. VE-cadherin null (VE-KO) and cells reconstituted with human VE-cadherin (VE-RC) were used for these studies (Lampugnani et al., 2002). As shown in Fig. 2 B, application of shear stress to VE-RC cells stimulated Shc association with VEGFR2, which was not observed in VE-KO cells, suggesting that VE-cadherin is required for the formation of Shc-VEGFR2 complex in response to flow.

To investigate whether VE-cadherin is also required for flow-induced Shc activation, we examined Shc phosphorylation in VE-KO and VE-RC cells upon shear. As shown in Fig. 2 C, flow-induced Shc phosphorylation at Tyr 239/240 was not observed in VE-cadherin–null cells (Fig. 2, C and D), which indicates that this event is dependent on VE-cadherin.

We then sought to characterize the tyrosine kinases responsible for the flow-induced Shc activation. Flow rapidly activates several tyrosine kinases, including Src family kinases (Takahashi and Berk, 1996; Jalali et al., 1998; Okuda et al., 1999; Yan et al., 1999) and VEGFR2 (Chen et al., 1999; Jin et al., 2003). Because Src and VEGFR2 both localize to cell–cell junctions in response to flow, we tested their requirement for the flow-induced Shc activation. Pretreatment of ECs with Src inhibitor SU6656 abrogated flow-induced Shc tyrosine phosphorylation and translocation (Fig. 3). Similarly, treatment with the VEGFR2 kinase inhibitor 4-(4′-chloro-2′-fluorophenylamino)-6,7-dimethoxyquinazoline (VTI) abolished flow-induced Shc activation and localization to junctions (Fig. 3). These results indicated that tyrosine phosphorylation of Shc and its translocation
Shc function is required for the activation of MAPKs by shear stress

The role of Shc in the activation of the Ras–MAPK pathway and mitogenic signaling has been well described (Ravichandran, 2001). The MAP kinases are activated by shear stress and mediate some of the effects of shear stress on ECs (Traub and Berk, 1998). To test whether Shc is involved in flow-induced extracellular signal–regulated kinase (ERK) activation, we suppressed cellular levels of Shc using siRNAs. As depicted in Fig. 6 (A and B), transfection of BAECs with Shc-specific siRNA resulted in a 90–95% decrease in the levels of all three isoforms of Shc. Decreasing Shc expression by siRNA dramatically inhibited the flow-induced ERK activation, whereas the control siRNA had no effect (Fig. 6, C and D). Interestingly, ERK activation was impaired to a similar extent in VE-KO cells when compared with VE-RC cells (unpublished data). In parallel experiments, the activation of another MAPK, p38, was also inhibited by attenuating Shc expression levels with siRNA, although p38 was inhibited to a lesser extent compared with ERK (unpublished data). Similar to the activation pattern for ERK, shear stress–induced p38 activation does not occur in ECs lacking VE-cadherin (Shay-Salit et al., 2002). As demonstrated (see Fig. 2 D), VE-cadherin is important for flow-induced Shc phosphorylation. These data are consistent with a model in which VE-cadherin–dependent Shc signaling contributes to the transient activation of ERK and p38 MAP kinases in response to fluid flow.

Shc mediates the flow-induced inflammatory responses

Shear stress regulates the chronic inflammation associated with atherosclerosis (Caro et al., 1969; Ku et al., 1985; Glagov et al., 1988).
Discussion

In the present study, we present evidence that Shc integrates signals from both cell–cell and cell–matrix adhesions to regulate flow-induced inflammatory signaling. Shc activation occurs in vivo and correlates with areas of disturbed flow and atherogenesis. Shc associates with components of the junctional complexes VE-cadherin and VEGFR2 at early times after the onset of flow and with integrin–ECM complexes at later times.
Coordinated changes between cell adhesions to the ECM and those to neighboring cells are crucial for numerous physical transformations that the cells must undergo during development, tissue homeostasis, and wound healing. Although cell–cell junctions and cell–matrix adhesions mediate unique downstream signals, integrin and junctional signaling pathways are highly interwoven into complex signaling networks. In the context of shear stress signaling, there are many levels of cross talk. For instance, both cell–cell and cell–matrix adhesions activate common effectors such as NFκB (Shay-Salit et al., 2002; Tzima et al., 2002; Orr et al., 2005; Tzima et al., 2005), ERK and p38 MAPKs (Takahashi and Berk, 1996; Li et al., 1997; Osawa et al., 2002; Shay-Salit et al., 2002; Tai et al., 2005; Fleming et al., 2005), Akt and Src kinases (Okuda et al., 1999; Fleming et al., 2005; Tai et al., 2005; Tzima et al., 2005), and endothelial nitric oxide synthase (eNOS; Jin et al., 2003; Dusserre et al., 2004; Fleming et al., 2005; Bagi et al., 2005). Another level
of cooperation lies in the commonality of binding partners. VEGFR2 binds to both adherens junctions (through VE-cadherin) and integrins, and thus, at any given time, VEGFR2 may regulate two distinct signaling modules by interacting with either VE-cadherin or αβ3 integrin (Bussolino et al., 2001). More recently, integrins were implicated as intermediates that are activated downstream of junctional signaling that leads to phosphoinositide 3-kinase–induced integrin activation and increased ECM binding (Tzima et al., 2001, 2005). We now provide evidence that the adaptor protein Shc plays a critical role in the cross talk between cell–cell junctions and integrins during flow.

The function of Shc in flow may be tightly regulated by tyrosine phosphorylation/dephosphorylation events. Shear stress stimulates the activation of Src kinases, which transactivate VEGFR2 (Jin et al., 2003). VEGFR2 activation may result in the recruitment and tyrosine phosphorylation of Shc, which is dependent on VE-cadherin. As demonstrated (see Fig. 4), VE-cadherin is required for the association of Shc with integrins, which mediates Ras–ERK activation and the flow-dependent transcriptional responses. It is worth noting here that upon VEGF treatment, VE-cadherin becomes phosphorylated and binds to Shc, which is dephosphorylated (Zanetti et al., 2002). The functional importance of this association may be that it facilitates Shc dephosphorylation through a VE-cadherin–associated phosphatase.

In contrast to the stimulation by VEGF, the temporal response of Shc tyrosine phosphorylation induced by shear stress is sustained (Chen et al., 1999).

The contribution of Shc to both integrin- and growth factor–induced activation of ERK is well documented (Wary et al., 1996; Barberis et al., 2000; Lai and Pawson, 2000), but this is the first study to reveal a role for Shc in the inflammatory signaling through NFκB. It has recently been shown that flow-induced NFκB activation is ECM dependent and is only observed in cells plated on FN but not on CL. ECM composition is a crucial factor in atherogenesis and may regulate the early changes...
inflammation associated with atherogenesis (Orr et al., 2005). Interestingly, NFκB activation in Shc-attenuated cells closely resembles cells plated on CL, whereas cells transfected with control siRNA emulate the FN phenotype observed by Orr et al. (2005). In addition, the association of Shc with integrins in response to flow is ECM specific (Fig. 5). Taken collectively, these data raise the possibility that Shc may function as a molecular switch to translate ECM specificity into ECs through its regulated interaction with integrin receptors engaged with the appropriate ECM.

Mutant mice lacking all three Shc isoforms die at embryonic day 11.5 due to cardiovascular defects (Lai and Pawson, 2000), whereas mice selectively missing the p66 ShcA isoform are long-lived (Migliaccio et al., 1999) due to the role of p66 Shc in oxidative stress signaling (Pinton et al., 2007). The exact contribution of each Shc isoform to development and signaling is still unclear. Most recently, pioneering work has shown that combinatorial differences in ShcA docking interactions may yield multiple signaling mechanisms to support diversity in tissue morphogenesis (Hardy et al., 2007).

In conclusion, our data provide a molecular description of the coordination of mechanochemical signals between cell–cell and cell–ECM adhesions that drive the complex inflammatory signaling elicited by disturbed shear stress. As ECM deposition and leukocyte adhesion to the AP sites are instrumental to early events in atherogenesis, our observations together with previously published results point to Shc as a potential therapeutic target in the treatment of atherosclerosis and coronary artery diseases.

Materials and methods

Cell culture, transfections, and shear stress

BAECs were maintained in DME (Invitrogen) with 10% FBS (Invitrogen), 10 μg/ml penicillin, and 0.25 μg/ml streptomycin (Invitrogen), VE-cadherin null (VE-KO) and reconstituted (VE-RC) cells were prepared as described previously (Carmeliet et al., 1999) and grown in DME containing 10% FBS, 5 μg/ml EC growth serum, and 100 μg/ml heparin and penicillin/streptomycin. THP-1 leukocytes were maintained in RPMI 1640 medium (Invitrogen) with 10% FBS, 10 μg/ml penicillin, 0.25 μg/ml streptomycin, and 2 mM glutamate (Invitrogen). Control siRNA or Shc siRNA (Thermo Fisher Scientific) were transfected into BAECs as described previously (Liu et al., 2005). For shear stress experiments, BAECs were plated on appropriate matrix proteins (10 μg/ml FN or 20 μg/ml Coll I) and allowed to grow for 10 h in medium containing 10% FBS or 4 h in 0.5% FBS. Cells were then starved overnight in medium containing 0.5% FBS. Slides were loaded onto a parallel plate flow chamber in 0.5% FBS, and 12 dynes/cm² of shear stress was applied for indicated times. To examine upstream kinases
required for Shc functions, cells were incubated with 10 μM of the VEGF receptor tyrosine kinase inhibitor VTI or 5 μM SU6656 (EMD) for 30 min at 37°C.

Oscillatory flow
To perform oscillatory flow, cells were cultured on 2 x 3 inch slides. After cells reached 100% confluence, the slides were attached to parallel chambers. The chambers were subsequently connected to an NE-1050 bidirectional pump (New Era Pump Systems, Inc.). Cells were sheared at ±6.5 dyne/cm², 1 Hz.

Immunoprecipitations, Western blotting, and antibodies
Cells were harvested in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS) supplemented with 1 mM aprotinin, 1 μg/mL leupeptin, 1 mM PMSF, 1 mM Na3VO4, 10 mM NaF, 1 mM sodium pyrophosphate, and 1 mM β-glycerophosphate. Lysates were precleared with 50 μL protein A/G-Sepharose beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Supernatants were then incubated with 30 μL of protein A/G-Sepharose previously coupled to the primary antibodies for 2 h at 4°C with continuous agitation. The beads were washed three times with lysis buffer supplemented with protease and phosphatase inhibitors, and the immune complexes were eluted in 2× SDS sample buffer. Associated proteins were subjected to SDS-PAGE and Western blotting using the appropriate primary antibodies and HRP-conjugated anti–mouse or anti–rabbit antibodies (Jackson ImmunoResearch Laboratories). Immunoreactive proteins were visualized by enhanced chemiluminescence (GE Healthcare). The phospho-Shc Tyr239/240 or Tyr317, phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-p65 (Ser536), and ERK antibodies were obtained from Cell Signaling Technology. A phospho-Tyr239/240 antibody from BioSource (Invitrogen) was tested and generated similar results to the Cell Signaling Technology phospho-Shc antibody. VEGFR-2 and p38 antibodies were obtained from Vector Laboratories, Inc. anti-Shc phosphoTyr239/240 antibody from BioSource (Invitrogen) was tested and generated similar results to the Cell Signaling Technology phospho-Shc antibody. VEGFR-2 and p38 antibodies were obtained from Vector Laboratories, Inc. Anti–VE-cadherin was purchased from Qbiogene. Anti-Shc and anti-NFκB (p65) were obtained from BD Biosciences. The ICAM-1 and VCAM-1 antibodies were obtained from Santa Cruz Biotechnology. Anti–VE-cadherin was pur-
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