Primary cilia on endothelial cells : component of the shear stress sensor localized to athero-prone flow areas

Heiden, K. van der

Chapter 5

Flow Reversals Induce Endothelial Primary Cilia. Consequences for Atherosclerosis.

Kim Van der Heiden[1]; Robert E. Poelmann[1]; Simone van de Pas[1]; A. Mieke Mommaas[2]; Adriana C. Gittenberger-de Groot[1]; Beerend P. Hierck[1].

[1]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands; [2]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands.

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Abstract

Primary cilia function as shear stress sensors of a variety of cell types, including endothelial cells. Endothelial ciliation *in vivo* is restricted to regions of low and oscillatory shear stress leading to atherogenesis. The aim of the present study is to analyze the mechanism behind the induction of endothelial ciliation and the microtubular composition of the endothelial cilium. Using transmission electron microscopy we show that endothelial cilia resemble primary cilia, with a 9+0 microtubular core. Endothelial cells were subjected to steady, pulsatile, or oscillatory flow in a bioreactor, followed by quantification of primary cilia and analysis of shear-responsive *Krӧppel-Like Factor-2* expression. We demonstrate that endothelial ciliation is dependent on the *pattern* of shear stress rather than the *level* and that a bidirectional (oscillatory), but not an unidirectional (steady or pulsatile), shear stress *pattern* induces the prevalence of primary cilia on endothelial cells. These data demonstrate that endothelial ciliation is induced by exposure to flow reversals, which characterize athero-prone flow.

Introduction

Atherosclerosis occurs at the inner curvature of arched arteries, at branch points, and at arterial bifurcations[1]. Due to the geometry of these sites blood flow is low and disturbed, resulting in low and oscillatory (multi- or bidirectional) shear stress. Shear stress is caused by the drag of blood on the endothelium in parallel to the direction of flow. Since atherosclerosis develops exclusively at sites of low and oscillatory shear stress this flow profile is termed athero-prone[2]. Shear stress drives gene expression in endothelial cells *in vivo*[3,4] and *in vitro*[5] and as a consequence is considered to be a major player in cardiovascular development and pathology. Shear stress level and pattern, i.e., steady, pulsatile, or oscillatory, differentially affect gene expression. Steady and pulsatile flow are unidirectional, whereas flow reversals (bidirectional flow) characterize oscillatory flow. Expression of the shear-responsive gene *Krüppel-Like Factor-2* (*KLF2*), a zinc finger transcription factor that is restricted to the endothelium, is increased by shear in a dose-dependent manner[4,6]. KLF2 acts as a switch between the quiescent and activated state of the endothelium[7], is upregulated by high steady or pulsatile shear stress[4], but is downregulated by prolonged oscillatory shear after an initial upregulation[8].

To translate alterations in flow into a biological response endothelial cells require a shear stress sensor. Endothelial cells elicit a shear response through activation of several cell components, including the cytoskeleton (for excellent reviews see Resnick *et al.*[5], Helmke and Davies[9], Lehoux and Tedgui[10], Li *et al.*[11]) which links all the other responsive components[9], suggesting that it plays a central role in the shear sensing process. Conformational changes of the cytoskeleton by shear stress result in transduction of the mechanical force throughout the cell to all the cytoskeleton-connected shear-responsive cell components[12]. Recently, primary cilia were shown to function as endothelial shear stress sensors[6,13,14]. These solitary, microtubule containing extensions protrude from the cell surface into the fluid and are connected to the microtubular cytoskeleton at their base. We showed that the endothelial shear response is dependent on the microtubular cytoskeleton and that a primary cilium connected to it sensitizes the cell for shear, as ciliated endothelial cells have a higher flow-induced *KLF2* response than non-ciliated cells[6]. Not all endothelial cells carry a primary cilium[15-17]. Non-ciliated cells retain their capacity to respond to shear stress as inferred by alignment in the direction of flow[9] and by induction of shear-

dependent gene expression[4,6]. We concluded that cytoskeletal strain is essential to endothelial shear sensing and can be amplified by a primary cilium. In addition to cytoskeletal strain, shear stress has been shown to result in activation of mechanoresponsive molecules located in the ciliary membrane, i.e., polycystins, which generate an immediate Ca^{2+} transient[14]. Therefore, a dual role for the endothelial primary cilium in shear stress sensing is evident[18]. (1) In an immediate response to ciliary deformation by flow polycystins mediate a Ca^{2+} transient[14]. (2) The primary cilium aids in cytoskeleton-mediated shear sensing, which leads to a prolonged effect on gene expression[6].

Strikingly, endothelial ciliation is restricted to areas of low and oscillatory shear stress in the chicken embryonic heart[15] and in the adult mouse vasculature[16]. In wild-type and *Apoe*$^{-/-}$ adult mice, the endothelial cells at the atherosclerotic predilection sites are ciliated before and after the onset of atherosclerosis[16]. The mechanism of endothelial ciliation is unknown. We hypothesize that athero-prone flow, i.e., low and oscillatory shear stress, in fact induces endothelial ciliation.

In this study, we uncover the mechanism behind the induction of endothelial ciliation by analyzing the prevalence of ciliated endothelial cells after exposing cultured endothelial cells to different flow profiles. We demonstrate that athero-prone but not athero-protective flow profiles induce endothelial ciliation.

Methods

Isolation and culturing of cells

Fertilized White Leghorn eggs (*Gallus domesticus*) were incubated for 14 days at 37°C and 60% humidity to Hamburger and Hamilton[19] stage (HH) 40. Primary endothelial cells from the atria and ventricles were isolated at high purity (80-95%)[6]. Cells were cultured in medium that consisted of M199/Hepes (Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) Fetal Calf Serum (PAA Laboratories, Linz, Austria), 2% (v/v) Chicken Serum (Invitrogen), 2x mmol/L L-Glutamine (Invitrogen), 0.25 g/L Endothelial Cell Growth Supplement (Sigma-Aldrich Chemie, Steinheim, Germany), 1x antibiotic/antimyotic solution (Invitrogen), and 0.05 g/L Gentamicin (Sigma-Aldrich Chemie) in dishes that were coated with 1% (w/v in milli Q) gelatine (Merck, Darmstadt, Germany). Cells that were grown to confluence were passed once a week and used at p2 or p3.

Electron microscopy

Atrial endothelial cells were isolated and cultured (p0) on coated glass coverslips. Subsequently, cells were fixed with 1.5% glutaraldehyde in 100 mM cacodylate buffer for 15 min at room temperature and overnight at 4°C. For field emission scanning electron microscopy (FESEM) cells were dehydrated in graded ethanol series, critical point dried over CO_2, sputter-coated with gold palladium, and studied in the field emission scanning electron microscope (Jeol JSM 7600F). For transmission electron microscopy (TEM) cells were postfixed with 1% osmium tetroxide in phosphate buffer for 60 min at 4°C, dehydrated in a graded ethanol series, and embedded in epoxy LX-112 resin. Ultrathin sections were contrasted with uranyl acetate for 20 min and lead citrate for 10 min and studied in the transmission electron microscope (Philips CM10).

Dynamic flow model

Confluent ventricular endothelial cells on coated glass coverslips were subjected to different flow patterns and levels at 37°C and 5% CO_2. To this aid, a parallel plate flow chamber with a 2 cm^2 flow area (height of 0.25 mm; Glycotech, Rockville, MD, USA) was connected to a peristaltic pump (Masterflex, Vernon Hills, Il, USA) via a pulse dampener resulting in a steady, nonpulsatile flow[6]. Oscillatory or pulsatile flow at a frequency of 2 Hz was generated by connecting the flow chamber to the peristaltic pump and an osci-flow™ controller (Flex cell™ international corporation, Hillsborough, NC, USA). The flow was recorded with a TS410 Transonic flowmeter (Transonic Systems Inc., Ithaca, NY, USA). Signals were digitized with a Powerlab (AD Instruments, Spechbach, Germany) recorder to calculate the mean, minimal, and maximal shear.

Cells were subjected to unidirectional (steady or pulsatile), or bidirectional (oscillatory) flow with a maximum of 2.5 or 5.0 dyne/cm^2 (0.25 and 0.50 Pa, respectively) for 5 hrs. In detail, oscillatory shear levels were 0.5 ± 2.0 and 1.0 ± 4.0 dyne/cm^2, allowing forward flow, and pulsatile levels were 1.5 ± 1.0 and 3.0 ± 2.0 dyne/cm^2. Directly after flow exposure cells were used for primary cilia quantification (n = 6-9) or analysis of *KLF2* expression (n = 4), as described below.

In a second set of flow experiments, cells were pre-conditioned with a steady flow of 25 dyne/cm^2 (2.5 Pa) for 2 hrs, followed by exposure to steady, pulsatile, or oscillatory flow with a maximum of 2.5 dyne/cm^2 for 5 hrs. These cells were used for primary cilia quantification (n = 4-6).

One-way ANOVA analyses with a LSD post-hoc test were performed to compare the effects of different shear patterns and levels. Data are presented as mean ± standard error of the mean (SEM). *P*-value < 0.05 and a power > 80% were considered significant.

Serum starvation

To increase the prevalence of primary cilia independent of flow, confluent ventricular endothelial cells were cultured in medium without serum supplementation for 24 hrs for primary cilia quantification (n = 24 serum-deprived cells, n = 40 non-deprived cells) or analysis of *KLF2* expression (n = 3 for both groups). Serum starvation induces growth arrest that results in an increase in the prevalence of primary cilia as only non-proliferating cells are able to present a primary cilium[20]. Independent sample *t*-test analyses were performed to compare the static cultures with and without serum supplementation to each other. Data are presented as mean ± SEM. *P*-value < 0.05 and a power > 80% were considered significant.

Primary cilia quantification and analysis of *KLF2* expression

Confluent ventricular endothelial cells were either fixed for 10 min in 4% paraformaldehyde (PFA) in 0.1 M PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$ pH 6.97) at room temperature for immunofluorescence followed by primary cilia quantification or lysed for RNA isolation (RNeasy, Qiagen, Hilden, Germany) for analysis of *KLF2* expression by QPCR.

For the detection of primary cilia a monoclonal antibody directed against acetylated α-tubulin was used (diluted 1:2000, clone 6-11B-1, Sigma-Aldrich Chemie)[21]. Fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (diluted 1:50, DAKO, Glostrup, Denmark) was used as secondary antibody. Fluorescence was detected by confocal laser scanning microscopy (Leica TCS SL). Each data set collected on the confocal microscope

was processed with Image J (http://rsb.info.nih.gov/ij/index.html). The number of ciliated and non-ciliated cells of a minimum number of 200 cells was quantified for each glass coverslip.

KLF2 expression was analyzed by QPCR, as described before[22,23]. Samples were normalized to *β-actin* to compensate for differences in RNA input.

Results

Endothelial cilia are 9+0 primary cilia

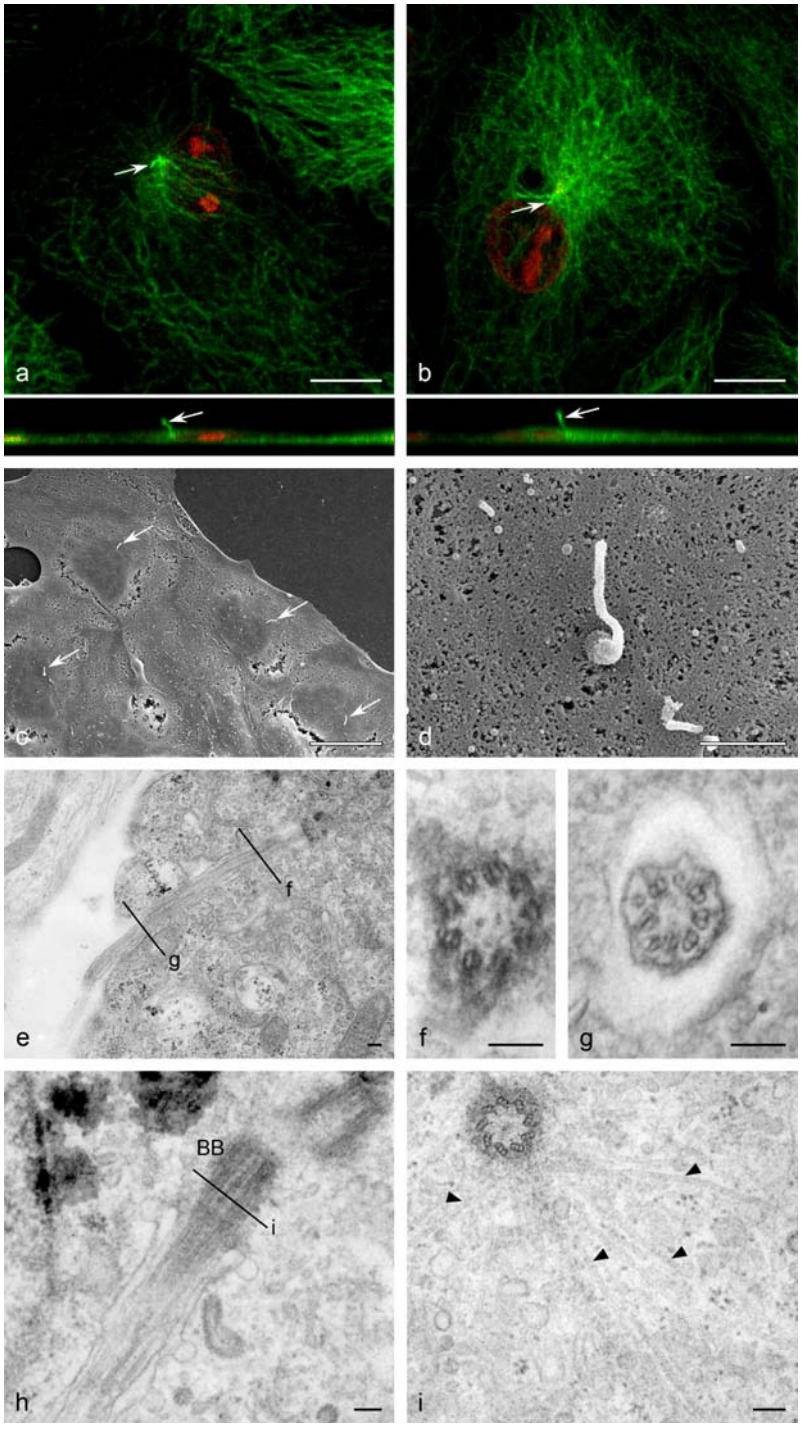
In a previous *in vivo* study, we showed that endothelial cells of the chicken embryonic heart are ciliated[15]. *In vitro* these cells maintain the ability to present a cilium as determined by immunofluorescent staining for acetylated α-tubulin and electron microscopy (Fig. 1). Acetylated α-tubulin is observed in the cilium, the MTOC, and in the cytoskeletal microtubules (Fig. 1a,b). Endothelial cilia protrude from the cell surface as can be observed in the optical cross section of the confocal stack (bottom Fig. 1a,b) and with FESEM (Fig. 1c,d). They are 1-3 µm in length and 0.2 µm in diameter (Fig. 1e-h). To investigate the composition of the ciliary axoneme we performed TEM. Endothelial cilia have a 9+0 configuration of microtubules in their core (Fig. 1f,g). The basal body of the primary cilium is connected to the microtubules of the cytoskeleton (Fig. 1a,b,h,i).

Prevalence of primary cilia is shear *pattern* but not shear *level* dependent

After observing that endothelial ciliation *in vivo* is high specifically in regions exposed to low and oscillatory shear stress and low in regions of high shear stress[15,16] we set out to determine the correlation between flow profile and the prevalence of primary cilia. Under static conditions 23% of the cells is ciliated (Fig. 2a). As the static condition is not comparable to the situation *in vivo*, the percentage of ciliation after exposure to different flow profiles is compared. We subjected cultured endothelial cells to unidirectional (steady or pulsatile) or bidirectional (oscillatory) flow with a maximum of either 2.5 or 5.0 dyne/cm^2 and analyzed endothelial ciliation. Figure 2a shows the percentage of ciliated cells after exposure to the different flow profiles. Under oscillatory shear, both at a maximum of 2.5 and 5.0 dyne/cm^2, significantly more ciliated cells are present than under steady and pulsatile shear. At both shear levels the number of ciliated endothelial cells is more than twofold higher after exposure to oscillatory flow (16% after 2.5 dyne/cm^2 and 11% after 5.0 dyne/cm^2) when compared with steady (6% and 3%, respectively) and pulsatile (7% and 5%, respectively) flow exposure. No significant differences between the effects of steady and pulsatile shear are present at both shear levels. Moreover, a higher maximum shear level does not affect the prevalence of primary cilia at any shear pattern analyzed.

Primary cilia affect the shear stress responsiveness of endothelial cells

To quantify the response of ciliated endothelial cells to different flow profiles, cells were exposed to steady, pulsatile, or oscillatory flow with a maximum of 2.5 dyne/cm^2 and the expression of *KLF2* was analyzed by QPCR. Figure 2b shows the expression of *KLF2* relative to the static controls. Endothelial cells express more *KLF2* after exposure to oscillatory flow than to steady flow, whereas equal amounts of *KLF2* are present in the steady and pulsatile flow samples.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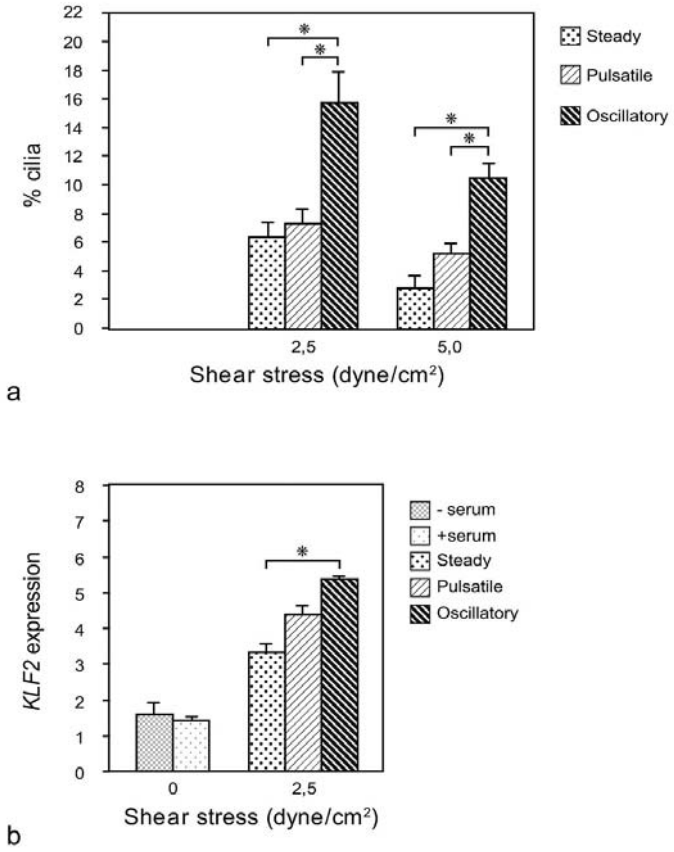

Figure 2. Percentage of ciliated endothelial cells and *KLF2* expression before and after exposure to various flow profiles

a. Ventricular endothelial cells (p2 or p3) were exposed to steady, pulsatile, or oscillatory shear stress with a maximum of 2.5 or 5.0 dyne/cm^2 for 5 hrs. More ciliated cells are present after exposure to oscillatory shear than after exposure to steady or pulsatile shear. Steady and pulsatile shear generate the same amount of ciliated cells. Moreover, a higher maximum shear level does not significantly lower the prevalence of primary cilia. Asterisks indicate $P < 0.05$ and power > 80%. Error bars represent SEM values. b. *KLF2* expression in serum-deprived and non-deprived static endothelial cells and in (non-deprived) endothelial cells exposed to steady, pulsatile, or oscillatory shear stress with a maximum of 2.5 dyne/cm^2 for 5 hrs. *KLF2* expression is normalized to *β-actin* and relative to the, non-deprived, static controls. *KLF2* expression in static endothelial cells does not change upon serum starvation. At a maximum shear level of 2.5 dyne/cm^2 oscillatory flow generates more *KLF2* than steady flow. The asterisk indicates $P < 0.05$ and power > 80%. Error bars represent SEM values.

◄Figure 1. Primary cilia on endothelial cells

Confocal laser scanning microscopic (CLSM) images with optical cross sections (a,b), FESEM images (c,d), and TEM images (e-i) of primary cilia on cultured ventricular endothelial cells at p3 (a,b) and atrial endothelial cells at p0 (c-i). Arrows (a,b,c) depict primary cilia, arrowheads (i) depict microtubules. The approximate locations of sections f and g are marked in e, the location of section i is marked in h. Endothelial primary cilia are 1-3 µm in length (d,e), 0.2 µm in diameter (e-h), protrude from the cell surface (a-e), have a 9+0 configuration of microtubules in their core (f,g), and are connected to the microtubular cytoskeleton (a,b,h,i). CLSM images: acetylated α-tubulin (green), nuclei (red; propidium iodide). BB, basal body. Scale bar a-c = 10 µm, d = 1 µm, e-i = 100nm.

To verify that the level of *KLF2* expression is not directly correlated to the number of ciliated cells, independent of flow, cilia prevalence was experimentally increased under static conditions by serum starvation. Subsequently, the *KLF2* expression in serum-deprived cells was compared to non-deprived cells, showing that the expression level of *KLF2* is unchanged (Fig. 2b), whereas serum starvation significantly increases ciliation from 23% to 34% (not shown).

Athero-prone flow profile induces primary cilia

Figure 2a shows that exposure to a steady or pulsatile shear stress pattern generates a decrease in endothelial ciliation compared with static conditions (Fig. 3). The percentage of ciliated cells after oscillatory shear exposure (16%, see Fig. 2a) does not differ statistically from the 23% under static conditions (Fig. 3). To determine whether the difference between the effect of unidirectional and bidirectional flow conditions reflects induction, a second set of flow experiments was performed. Cells were pre-conditioned to a high level (25 dyne/cm^2) of steady shear stress for 2 hrs to reduce cilium numbers to 5%, followed by exposure to a steady, pulsatile, or oscillatory flow with a maximum of 2.5 dyne/cm^2 for 5 hrs. Figure 3 shows the percentage of ciliated cells after pre-conditioning and after pre-conditioning plus exposure to the different flow patterns. Only oscillatory shear significantly increases the prevalence of primary cilia after pre-conditioning with high steady shear to 10%, which is a doubling of the number of ciliated cells. Steady and pulsatile shear do not increase the prevalence of cilia. The proportions of ciliated endothelial cells after shear with pre-conditioning (Fig. 3) are similar to the proportions after shear without pre-conditioning (Fig. 2a).

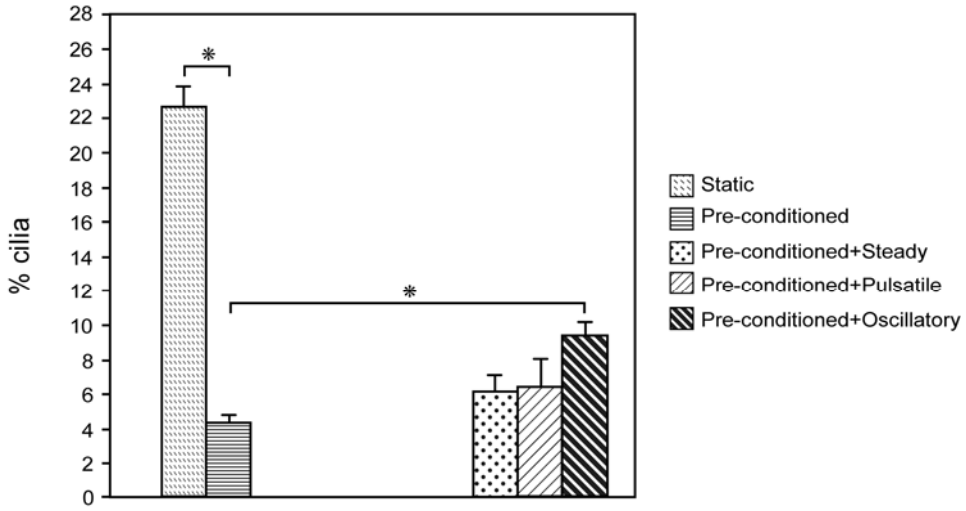

Figure 3. Induction of endothelial ciliation

Percentage of ciliated ventricular endothelial cells (p2 or p3) in static endothelial cells and in endothelial cells after exposure to 2 hrs of pre-conditioning by a steady flow of 25 dyne/cm^2 and after 2 hrs of pre-conditioning followed by a 5 hrs exposure to steady, pulsatile, or oscillatory shear stress with a maximum of 2.5 dyne/cm^2. Note that pre-conditioning reduces primary cilia prevalence and that only subsequent exposure to oscillatory shear induces primary cilia. Asterisks indicate $P < 0.05$ and power > 80%. Error bars represent SEM values.

Discussion

Primary cilia are solitary, microtubule containing extensions that protrude from the cell surface into the fluid. They are connected to the microtubular cytoskeleton at their basal body, as this is the mother centriole of the centrosome[24] that is part of the microtubule organizing center (MTOC) of the cell[25]. As both centrioles of the centrosome are required for mitosis only non-proliferating cells are able to present a primary cilium. In contrast to motile cilia, which have a 9+2 configuration of microtubules, primary cilia have a 9+0 microtubule configuration and are involved in various sensory processes. Primary cilia serve a mechanosensory function in numerous cell types, including the endothelium[6,14,26-29]. We demonstrated that the endothelial shear response is dependent on the cytoskeleton[6], which undergoes a conformational change upon exposure to flow[9,30,31]. The primary cilium is physically connected to the cytoskeleton and sensitizes endothelial cells for shear stress[6], presumably through the amplification of cytoskeletal strain. The endothelium does not solely rely on primary cilia for shear stress sensing as not all endothelial cells carry a primary cilium[15,16]. In this study, we show that endothelial cilia protrude from the cell surface into the fluid, are true primary cilia as they have a 9+0 configuration of microtubules, and that they are connected to the microtubular cytoskeleton. Ciliated endothelial cells in the chicken embryonic heart[15] and in the adult mouse vasculature[16] are predominantly present in areas of low and oscillatory shear stress. Therefore, we hypothesized that the mechanism behind the induction of endothelial ciliation is the exposure to flow reversals. To test this hypothesis we analyzed the prevalence of cilia after exposure to unidirectional (steady or pulsatile) and bidirectional (oscillatory) flow. In all conditions over 50% of the cells is non-ciliated. This is consistent with previous reports on ciliated cells in culture[32] and with the observation that a ciliated cell transfers mechanically induced signals through mechanical or chemical signaling to non-ciliated, neighboring cells. This is probably mediated via cytoskeletal cell-cell linkage and gap junctions[14,26], rendering ciliation of contiguous cells unwarranted. As static conditions do not represent a physiological situation, we compared the percentage of ciliated endothelial cells after exposure to different flow conditions to each other. Endothelial cells exposed to oscillatory flow are proportionally more ciliated than those exposed to steady or pulsatile shear. This is consistent with our previous *in vivo* study in which we observed many ciliated endothelial cells in areas of experimentally-induced low and oscillatory shear stress, whereas very few cilia are present in areas of high pulsatile shear stress[16]. Here we show that shear level on the other hand is not of influence on the prevalence of cilia. Equal numbers of ciliated cells are observed after exposure to a steady flow of 2.5, 5.0, and 25 dyne/cm^2. These data demonstrate that shear *pattern* affects endothelial ciliation rather than shear *level*.

To exclude the possibility that oscillatory flow delays the disassembly in ciliation from static conditions more than steady or pulsatile flow, we pre-conditioned cells with a high steady flow to reduce the prevalence of cilia, and subsequently exposed them to the different flow profiles. Our data demonstrate that oscillatory shear stress truly induces endothelial ciliation, whereas subsequent steady and pulsatile shear stress do not have an inductive effect on ciliation.

To determine the role of the primary cilium in endothelial shear stress sensing, we previously analyzed the shear response of ciliated vs. non-ciliated cells exposed to the same shear pattern and level[6]. In this study, we analyzed the response of ciliated cells to different shear patterns with the same shear level. Ciliated cells are more shear responsive than non-ciliated cells with respect to the expression of the shear-responsive gene *KLF2* upon flow

exposure[6]. *KLF2* expression can be used as a marker for the shear-response of endothelial cells because it is dose-dependent[4]. In this study we demonstrate that the level of *KLF2* expression is not directly correlated to primary cilia number independent of flow, as an increase in ciliation of static endothelial cells does not result in a change in expression. However, *KLF2* expression does alter upon exposure to flow. *KLF2* expression is higher after exposure to 2.5 dyne/cm^2 oscillatory flow than after 2.5 dyne/cm^2 steady flow, confirming the increased shear-responsiveness of highly ciliated cells. It should be noted that this *in vitro* model reflects the capacity to present a cilium and the shear responsive potential of endothelial cells but does not represent the *in vivo* situation. In our experiments, we subjected ciliated endothelial cells to different patterns of shear stress at equal shear levels, whereas *in vivo*, non-ciliated areas[16] are exposed to high levels of pulsatile shear, correlating to a high *KLF2* expression level[4], whereas ciliated areas[16] are subjected to low levels of oscillatory shear, correlating to a low *KLF2* expression level[8]. The high *KLF2* response after exposure to oscillatory shear stress is consistent with the data by Wang *et al*.[8] who observed an initial increase in *KLF2* expression upon exposure to oscillatory shear. Long-term effects of oscillatory shear on *KLF2* expression or ciliation is a subject of further investigation.

The link between oscillatory shear stress and the prevalence of primary cilia (this study), the association between oscillatory shear and atherosclerosis[1], and the occurrence of ciliated endothelial cells at the atherosclerotic predilection sites[16] make a direct relationship between primary cilia and atherosclerosis conceivable. Primary cilia act as shear sensors[14] and as signal amplifiers[6]. The presence of ciliated endothelial cells at the oscillatory flow-exposed and hence athero-prone areas might therefore imply that ciliation is a protective mechanism of the endothelial cells to enhance their shear sensing, thereby protecting them from endothelial dysfunction. In wild-type mice, ciliated endothelial cells are present in the heart[18] and vasculature[16]. Wild-type mice do not develop atherosclerosis spontaneously. We postulate that the introduction of cardiovascular risk factors, such as hypercholesterolemia, diabetes or hypertension, can shift the balance to an atherogenic phenotype. Malfunction of primary cilia has already been associated with atherosclerosis as polycystic kidney disease (PKD) patients, who have dysfunctional kidney epithelial primary cilia[33], display endothelial dysfunction and increased carotid intima-media thickness[34]. It is tempting to postulate that these patients also have dysfunctional endothelial cilia that cause these anomalies.

In conclusion, the endothelial cilium is a primary (9+0) cilium and endothelial ciliation is flow *pattern* but not flow *level* dependent. Flow reversal, a characteristic of athero-prone flow, induces endothelial ciliation. Endothelial cilia hold mechanoresponsive molecules and are likely to augment cytoskeletal strain, rendering ciliated endothelial cells more responsive for shear stress.

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