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Fluid shear stress-mediated mechanotransduction in circulating leukocytes and its defect in microvascular dysfunction

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ABSTRACT

Leukocytes (neutrophils, monocytes) in the active circulation exhibit multiple phenotypic indicators for a low level of cellular activity, like lack of pseudopods and minimal amounts of activated, cell-adhesive integrins on their surfaces. In contrast, before these cells enter the circulation in the bone marrow or when they recross the endothelium into extravascular tissues of peripheral organs they are fully activated. We review here a multifaceted mechanism mediated by fluid shear stress that can serve to deactivate leukocytes in the circulation. The fluid shear stress controls pseudopod formation via the FPR receptor, the same receptor responsible for pseudopod projection by localized actin polymerization. The bioactivity of macromolecular factors in the blood plasma that interfere with receptor stimulation by fluid flow, such as proteolytic cleavage in the extracellular domain of the receptor or the membrane actions of cholesterol, leads to a defective ability to respond to fluid shear stress by actin depolymerization. The cell reaction to fluid shear involves CD18 integrins, nitric oxide, cGMP and Rho GTPases, is attenuated in the presence of inflammatory mediators and modified by glucocorticoids. The mechanism is abolished in disease models (genetic hypertension and hypercholesterolemia) leading to an increased number of activated leukocytes in the circulation with enhanced microvascular resistance and cell entrapment. In addition to their role in binding to biochemical agonists/antagonists, membrane receptors appear to play a second role: to monitor local fluid shear stress levels. The fluid shear stress control of many circulating cell types such as lymphocytes, stem cells, tumor cells remains to be elucidated.

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Contents

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1. Introduction

The majority of leukocytes in the circulation have an almost perfectly spherical shape with fine membrane folds. In the bone marrow after maturation from hematopoietic stem cells, however, leukocytes migrate across the endothelium into the circulation and assume new cell shapes. They may adopt shapes that consist of just small surface projections to completely distorted cell morphologies (Wolosewick, 1984). One typical structure they develop are pseudopods which are part of their migratory machinery that allows the cells to extend cytoplasm and membrane beyond the perimeter of just a spherical cell. They have been designated by other names (filipodia, uropods); in the following we will refer to them as *pseudopods*. In the bone marrow pseudopods are required for passage across the endothelium into the circulation. Yet once leukocytes entered into the active circulation, the majority return into their round shape without pseudopods. In the circulation of a healthy individual, leukocytes in the blood stream have no pseudopods until they reattach to the endothelium, spread again on the luminal membrane of the endothelium and migrate this time outwards across the vascular barrier into peripheral tissue. Thus, there exists a mechanism by which pseudopod formation on leukocytes can be turned off in the circulation.

A principal evidence that neutrophils require a flowing environment to remain deactivated is that fluid stasis with zero fluid shear stress (e.g. due to a vessel occlusion or ischemia) facilitates cell adhesion and pseudopod extension. The most overt manifestation of the neutrophil responses to fluid shear stress is its impact on pseudopod activity. In-vivo and in-vitro experiments have shown that fluid shear stresses typical of those present in the microcirculation (in the order of 10 dyn/cm^2) in the absence of other biochemical or hormonal mediators, has the ability to block pseudopod formation (Mitchell and King, 2012; Moazzam et al., 1997). The ability to retract pseudopods in the presence of even

this low fluid shear stress is essential to allow free movement through the microcirculation. This is especially important in organs with capillary diameters that are equal to, or below, the dimensions of the leukocytes. Passage through narrow capillaries (with diameters less than ~8 μm) requires leukocyte deformation, which in the presence of pseudopods is reduced due to the stiff cytoplasmic properties of pseudopods. A capillary network with leukocytes that have pseudopods is impaired if not completely blocked (Sutton and Schmid-Schönbein, 1992).

In recent work it has also been shown that this shear stress-dependent mechanism may fail, raising the numbers of leukocytes in the circulation that do not completely retract their pseudopods. In the following we will discuss the effect of fluid shear stress on pseudopod formation and cell membrane adhesion molecules in circulating leukocytes (neutrophils, monocytes). We will discuss cell regulatory mechanisms, the magnitude of shear stress in different regions on the plasma membrane of leukocytes, molecular membrane sensors for fluid shear, and review current understanding of the impairment of the fluid shear stress response in selected pathologies (hypertension and hypercholesterolemia).

2. The pseudopods under fluid shear stress

Pseudopods are formed by polymerization of actin into cytoplasmic extensions that are membrane covered. Actin polymerization pushes the membrane outwards causing the cell to adopt different shapes (Fig. 1). These cytoplasmic extensions are a requirement for all active migration or spreading on a substrate, as in phagocytosis. During pseudopod projection, G-actin polymerizes into F-actin in the pseudopod (Zhu and Skalak, 1988) and in the process generates a stiff elastic (i.e. strain-dependent) cytoplasm inside the pseudopod (Schmid-Schönbein et al., 1982) that sits on the viscoelastic (i.e. strain- and strain rate-dependent) cytoplasm of the main cell body. Active pseudopod projection can be
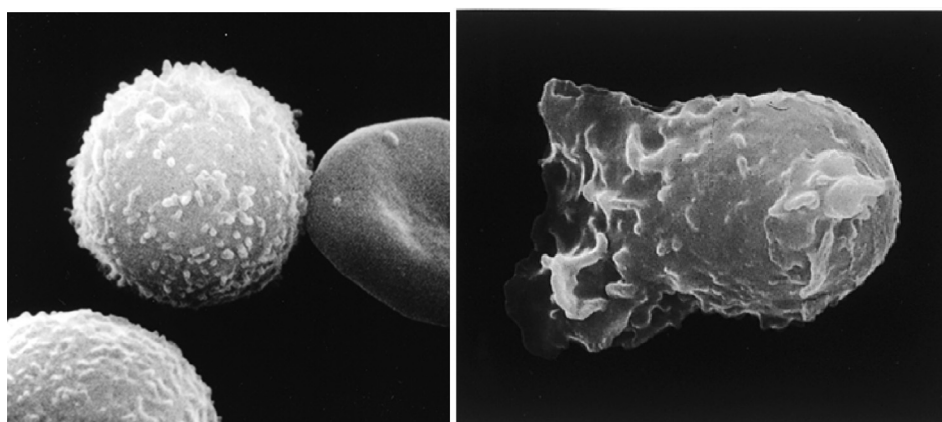

Fig. 1. Scanning electron microscopic image of human neutrophil without (left) and with pseudopod (right).

stimulated by soluble inflammatory agents, such as small formyl peptides (*N*-Formyl-methionine-leucyl-phenylalanine, f-Met-Leu-Phe or fMLP), lipid products (platelet activating factor, PAF) and numerous others (Fukuda et al., 2000; Zhelev et al., 2004). Pseudopod retraction occurs when the cell breaks down the elastic F-actin structure in the pseudopod (Zhu et al., 1989).

Fluid shear stress applied to a leukocyte of a healthy individual causes retraction of pseudopods in different in-vivo and in-vitro experiments (Fig. 2a). In addition, fluid shear stress elicits other deactivating reactions (Table 1). The list of these responses needs to be regarded at the time of this review as preliminary - more remain to be uncovered. The ability of fluid flow to promote pseu-
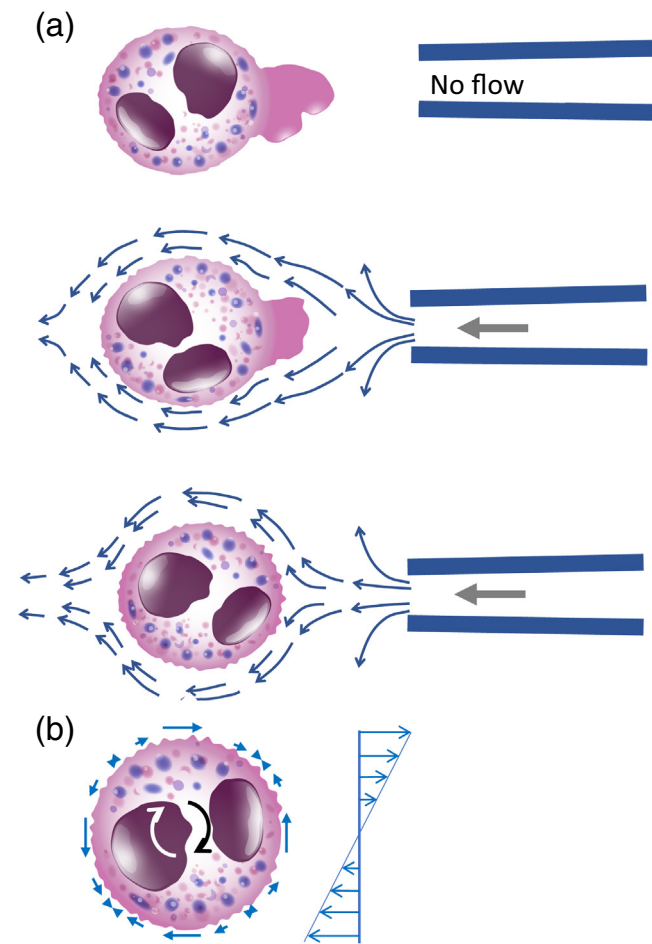

Fig. 2. Fluid flow setup and shear stresses on a leukocyte with pseudopod in (a) micropipette design and (b) rotating in free suspension in a velocity field with constant strain rate, γ. The cell rotates with angular velocity that is equal to $\frac{1}{2}\gamma$ and points on the membrane are subject to transient positive and negative shear stress with intermediate zero shear stresses (Sugihara-Seki and Schmid-Schönbein, 2003).

Table 1
Shear Stress Responses of Leukocytes.

Cellular Reactions	Reference(s)
retract pseudopods	Moazzam et al., 1997
depolymerize F-actin cytoskeleton	Shive et al., 2000
reduce phagocytotic activity	Shive et al., 2000
apoptosis	Shive et al., 2000
cleave CD18 integrins (LFA1 / Mac1)	Fukuda et al., 2003; Zhang et al., 2013
internalize FPR	Makino et al., 2006; Chen et al., 2010
shed L-selectin	Lee et al., 2007

dopod retraction is consistent with normal fluid shear stress serving as an anti-inflammatory mediator. But at increasing shear stresses other reactions are observed; neutrophils degranulate, assume different cell shapes, and even become damaged (Dewitz et al., 1977; Dewitz et al., 1979). Neutrophils adherent to a foreign surface and exposed to fluid stress may release microparticles and undergo apoptosis (Radley et al., 2019; Shive et al., 2000). The reaction of leukocytes to fluid shear stress is multi-faceted.

3. Shear stress regulation of pseudopods in the presence of inflammatory mediators

The response to fluid shear depends on inflammatory mediators and their concentrations (Fukuda et al., 2004b) and shear stress may increase, rather than only decrease, leukocyte activation. Low levels of inflammatory mediators ((e.g.(fMLP at <1 μM, or PAF at <10 μM)), mildly stimulate neutrophils and promote pseudopod formation (Zhelev et al., 2004), but they do not abolish the pseudopod retraction under shear stress (Fukuda et al., 2000; Makino et al., 2007; Moazzam et al., 1997; Zhang et al., 2011). In contrast, these same agonists at higher concentrations cause leukocytes to lose their ability to retract pseudopods under shear (Fukuda et al., 2000; Mitchell et al., 2014).

4. Membrane integrin distribution under fluid shear stress

A similar relationship exists between fMLP concentrations and shear-induced CD18 cleavage (Akenhead et al., 2017). CD18 integrins, required for neutrophil adhesion and migration, are the beta subunits of four integrin heterodimeric complexes: lymphocyte function antigen-1 (CD11a/CD18), macrophage-1 antigen (CD11b/CD18), integrin $\alpha_X\beta_2$ (CD11c/CD18), and integrin $\alpha_D\beta_2$ (CD11d/CD18) (Mazzone and Ricevuti, 1995). Among these, CD11a/CD18 and CD11b/CD18 are critical for neutrophil-substrate interactions (Fukuda and Schmid-Schönbein, 2003; Root, 1990; Zhang et al., 2013) and play key roles in neutrophil adhesion to microvessels (Diacovo et al., 1996; Hentzen et al., 2000). CD18 expression on the membrane is reduced in the presence of ~ 10 dyns/cm^2 fluid stress (Fig. 3). Their shear-induced cleavage is consistent with cell deactivation to keep cells rounded and non-adhesive. Shear stress also influences L-selectin distribution and capping on leukocytes (e.g. while settling during centrifugation (Lee and King, 2007)). Shear stress transduces outside-in signals at focal sites of
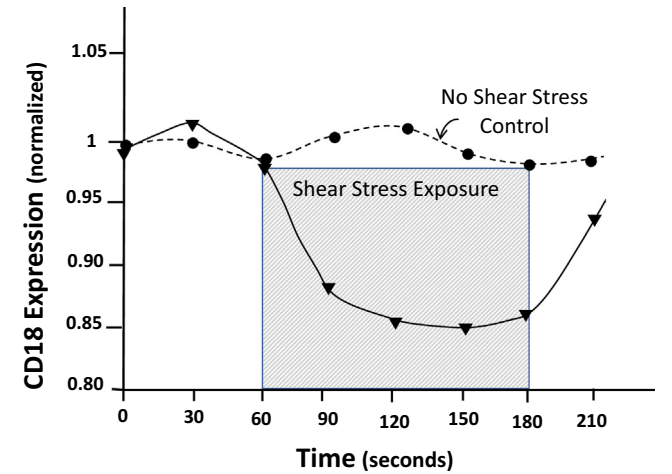

Fig. 3. Time course of the average CD18 expression level on neutrophils after exposure to fluid shear (for two minutes) with no shear control (adapted from Shin et al., 2008).

high-affinity CD11a/CD18 that provides contact-mediated direction for migration (Dixit et al., 2011).

Fluid shear stress exposure of suspended neutrophils has been reported to produce cell–cell ("homotypic") aggregates. In the contact region between neutrophils actin polymerizes with increase in intracellular Ca_2^{++}. This shear stress-induced actin polymerization is not observed when neutrophils are treated with anti-CD11a/CD18 or anti-ICAM-3 antibodies. In contrast, in fMLP-stimulated neutrophils actin polymerization in pseudopods is not mediated by a rise of cytosolic intracellular Ca^{2++} (Okuyama et al., 1996).

5. Fluid shear response under control of nitric oxide, cGMP and Rho GTPases

Fluid shear regulation of leukocytes is controlled by cGMP and NO. When cGMP is depleted, pseudopods no longer retract under shear stress. However, leukocytes that no longer retract pseudopods by shear stress after stimulation by PAF or fMLP, recover pseudopod retraction by administration of cGMP analogs or NO donors (Fukuda et al., 2000). cGMP and inflammatory stimulators have the opposite effect of the shear stress on pseudopod formation during inflammation. The balance between these two mechanisms may serve to control pseudopod formation and cell spreading.

Since NO in the circulation is mainly produced by endothelial cells, they may regulate the reaction of leukocytes under shear stress. In eNOS$^{-/-}$ mice without NO production, leukocytes exhibit a suppressed pseudopod retraction. eNOS inhibition by N^G–methyl-L-arginine enhances the ability of PAF to attenuate the shear stress response of leukocytes in venules. Endothelial cells generate eNOS-derived NO under fluid shear stress. The enhancement of the shear stress response of leukocytes by the endothelium-derived NO suppresses pseudopod projection and spreading on microvascular endothelium and consequently the level of the inflammatory reaction (Fukuda et al., 2000). Steady state physiologic shear stress enhances nitric oxide formation while irregular shear stresses promotes superoxide in endothelial cells (Hsieh et al., 2014).

The ability of shear stress to mediate retraction of pseudopods depends on the Rho family small GTPases. Specifically, disruption of Rac1,2 activity makes leukocytes nonresponsive to fluid shear stress (Makino et al., 2005).

6. Reversal of the reaction to fluid shear stress

A reversed reaction to fluid stress in form of pseudopod projection under shear and retraction when the shear is stopped, has multiple physiological effects.

6.1. Reversal of shear reaction by integrins

When neutrophils are adhered to a substrate via β_1-integrins, as compared to β_2-integrins, they spread under fluid stress (Marschel and Schmid-Schönbein, 2002), an effect that is enhanced by proteolytic cleavage of the glycocalyx (Coughlin and Schmid-Schönbein, 2004).

6.2. Fluid shear reaction in the presence of glucocorticoids

Glucocorticoids directly affect the fluid shear response. Normal leukocytes, exposed to fluid shear in-vitro during migration, retract pseudopods followed by decreasing intracellular calcium ions. In contrast, dexamethasone-treated leukocytes project pseudopods after shear exposure with a rise in intracellular calcium (Fukuda et al., 2004a). At the same time glucocorticoids reduce leukocyte

adhesion to the endothelium (Cronstein et al., 1992; Filep et al., 1997). Dexamethasone also reverses the leukocyte shear stress response in-vivo and suppresses their ability to transmigrate the endothelium. Leukocytes in postcapillary venules of dexamethasone-treated rats may continue to project pseudopods after flow restoration but are less adhesive and washed away in the venular/venous blood stream even though they still have pseudopods (Fay et al., 2016; Fukuda et al., 2004a). Thus, the reversal of the leukocyte shear response by glucocorticoids may contribute to an enhanced incidence of circulating leukocytes with pseudopods (Fay et al., 2016; Fukuda et al., 2004a). It is interesting to note that dexamethasone in healthy subjects reduces actin polymerization in leukocytes. Such softer cells have a reduced passage time through narrow channels in-vitro (Fay et al., 2016). Their reaction to shear stress remains to be determined.

6.3. Reversal of the reaction to fluid shear stress in hypertension

Genetic hypertensives, such as the spontaneously hypertensive rat (SHR), have elevated numbers of circulating neutrophils displaying a suppressed expression of adhesion molecules and an activated phenotype (Arndt et al., 1993; Schmid-Schönbein et al., 1991; Suematsu et al., 2002; Suzuki et al., 1994). When subjected to fluid shear stress in-vitro the fraction of SHR leukocytes with pseudopods increases, in contrast to a reduction in normotensive control rats (Fig. 4). Consequently, SHR neutrophils exhibit an abnormal pattern of pseudopod projection as well as a reduced retraction under fluid shear compared with neutrophils of control Wistar-Kyoto (WKY) rats (Fukuda et al., 2004b). The combination of reduced adhesion and increased number of leukocytes with pseudopods has a direct hemorheological effect (Eppihimer and Lipowsky, 1996; Mazzoni and Schmid-Schönbein, 1996; Worthen et al., 1989) and raises the peripheral vascular resistance. Circulating leukocytes have in spite of their low numbers, compared to red cells or platelets, an unproportionally large effect on microvascular resistance in organs like skeletal muscle due to their interaction with red cells in capillaries (Sutton and Schmid-Schönbein, 1992; Helmke et al., 1998). It is further amplified by pseudopods in the SHR (Fukuda et al., 2004b) (Fig. 5). The reversal of leukocyte shapes under shear stress and the normal regulation of adhesion
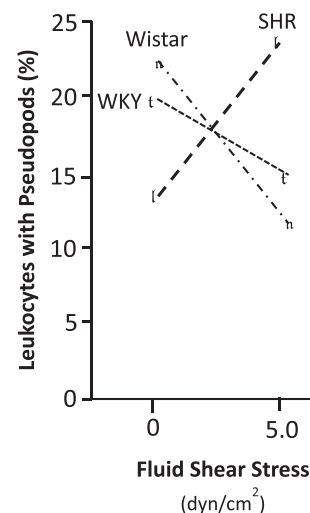
Fig. 4. Average fraction of leukocyte (neutrophils, monocytes) in fresh whole blood before and after exposure to fluid shear stress in a cone-and-plate shear field for 10 min. Leukocytes from the spontaneously hypertensive rats (SHR) increase the number of cells with pseudopods whereas control rat strains (low blood pressure control, Wistar-Kyoto rat (WKY); Wistar rat, a control strain) reduce pseudopod formation (adopted from Fukuda et al., 2004b).
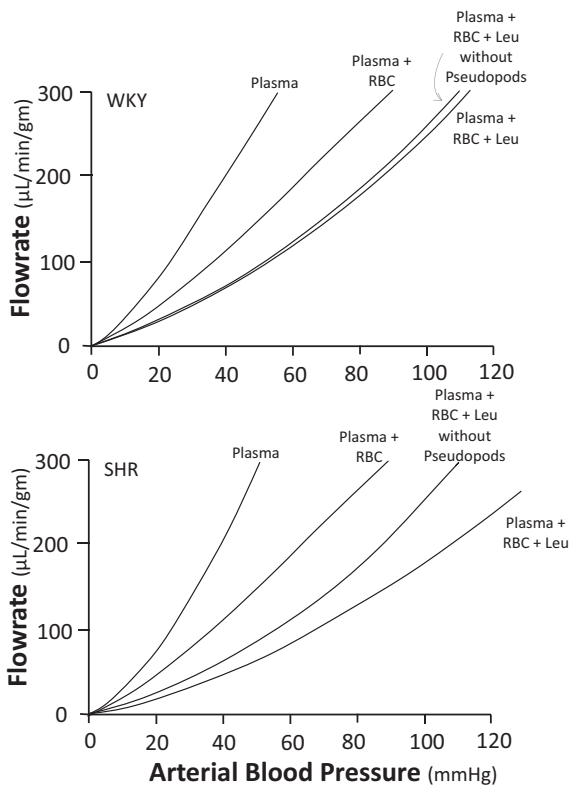
Fig. 5. Microvascular flowrate as a function of the arterial pressure in WKY and SHR rat gracilis muscle and perfusion media with plasma only, plasma and red blood cells (RBC), whole blood (plasma with RBC and leukocytes (Leu)), and whole blood without pseudopods on leukocytes (by depolymerization of actin with cytochalasin B). Note the increase of perfusion pressure due to the relatively few leukocytes (<1% cell volume) in both rat strains, which is further enhanced in the presence of pseudopods in the SHR but not present in the normotensive WKY strain (adopted from Fukuda et al., 2004b).

molecules on leukocytes in SHR are key requirements for the mechanism of hypertension in SHR.

The SHR has a form of hypertension which depends on glucocorticoids (DeLano and Schmid-Schönbein, 2004). Adult SHRs have more circulating leukocytes with pseudopods, slower cell passage times through capillaries with elevated hemodynamic resistance (the ratio of pressure drop and flow rate) under normal physiological blood flow, and as a result reduced flow in the microcirculation and enhanced capillary plugging. The resulting elevation in the capillary hemodynamic resistance mediated by abnormal leukocytes disturbs the passage of erythrocytes in capillaries and reduces their velocities and their positioning in capillaries (Helmke et al., 1998), an effect which contributes to the SHR's elevated arterial blood pressure. This mechanism is supported by the observation that infusion of SHR leukocytes into the circulation of the control WKY rats causes an elevation of arterial blood pressure which depends on pseudopod formation (Fukuda et al., 2004b). It remains to be investigated in human hypertensives.

6.4. Reversal of the fluid shear stress response by centrifugation

A reversal of the reaction to fluid shear stress can also be triggered by exposure to high gravitational forces. As a regular practice for collection cell subtypes, leukocytes are subject to centrifugation. The leukocyte's reaction to fluid shear is reversed by centrifugation, depending on duration and magnitude of the acceleration and time of centrifugation (Fukuda and Schmid-Schönbein, 2002). For example, after centrifugation at 900g, application of

fluid shear induces pseudopods projection instead of retraction in a calcium-dependent manner. Pseudopod retraction under shear stress is a key requirement for normal passage of leukocytes through the microcirculation. Therefore, loss of a normal fluid shear response due to centrifugation leads to a shift in microcirculatory insufficiency; centrifuged leukocytes have an enhanced tendency to migrate into tissue when reinfused into the circulation. This issue has received little consideration particularly as it relates to procedures used for the preparation and handling of leukocyte suspensions intended for therapeutic purposes.

7. The fluid shear stress magnitude on a leukocyte surface

Hemodynamic shear stresses in the microcirculation are in a range that can go from as low as 1 dyn/cm^2 to as high as –50 dyn/cm^2 with shear rates of up to ~10,000 sec^{-1}. These are shear stresses that are exerted by the flow of blood over leukocytes adherent and migrating on the endothelium. Non-adherent leukocytes flowing in the bloodstream experience lower shear stresses and shear rates although they are enhanced by the interaction with surrounding red blood cells.

The fluid shear stresses that modulate the leukocyte activities are in the same order of magnitude as those in the circulation. These shear stress values may be compared with the magnitude of fluid pressure in the circulation which is orders of magnitude higher (note for comparison 1333 dyn/cm^2 = 1 mmHg). It is similar to shear stresses required in other cell types, even though it evokes cell reactions that are different from those of leukocytes (Lee et al., 2002; Lorenzen-Schmidt et al., 2006; Song et al., 2017; Thi et al., 2003; Tian et al., 2019). The fluid shear stress magnitude is too low to generate a passive mechanical deformation of the cell or its pseudopod that can be detected with current light microscopy (>100 dyn/cm^2). Instead, the reaction of the cell is mediated by mechanosensors that are embedded in the cell membrane and have not been detected with current techniques other than by molecular methods, e.g. fluorescence resonance energy transfer (FRET).

Understanding the action of fluid shear stress requires a detailed picture of its actual magnitude on the cell membrane. This is determined by the shape of leukocytes, their rotation in a fluid shear field, and by the interaction with neighboring cells. For example, the shear stresses, τ_θ and τ_φ in spherical coordinates (r, θ, φ), on an isolated sphere in plasma with viscosity μ in a linear shear gradient γ with velocity field (γy, 0, 0) away from the cell is

$$\tau_\theta = 5(\mu\gamma/2)\cos2\theta\cos\varphi.$$

$$\tau_\varphi = -5(\mu\gamma/2)\cos\theta\sin\varphi.$$

and the cell's angular velocity is -γ/2 (Fig. 2b). Since the cell rotates, a single point on the membrane is subject to a positive shear stress and within one rotation to a negative shear stress, i.e. the cell is subject to *temporal* stress gradients (even though the external flow field is at steady state) (see e.g. Fig. 4 in (Sugihara-Seki and Schmid-Schönbein, 2003)).

In a parabolic velocity profile, the time period of shear oscillations is lowest if the cell is in the vessel center with low shear rate and assumes maximum value close to the vessel wall with the highest shear rate. As a leukocyte passes through narrow arterioles or venules in the microcirculation (e.g. ~10 μm in diameter), while freely suspended, the shear stress on their membranes will be several times larger compared to the estimated shear stress that would be exerted on the vessel wall by an undisturbed, cell-free, Poiseuille flow field. The shear stress on the leukocyte membrane region exposed to fluid flow increases (up to an order of magni-

tude) when the leukocyte makes firm attachment to the endothelium (Sugihara-Seki and Schmid-Schönbein, 2003).

The magnitude and direction of the shear stress on the membrane depends on details of the cell morphology. The membrane folds tend to have the highest shear stress at their tips and lower shear stresses in the troughs between folds (also designated as microvilli, ruffles) (Fig. 1). Reconstruction of leukocyte shapes with serial confocal microscopy during attachment and migration shows that the membrane regions protruding into the blood flow have the highest shear stress (of the order of 10 dyn/cm^2) while membranes in the contact region are subject to the lowest fluid shear stress (near zero dyn/cm^2); those membrane regions are subject to shear and normal stresses carried by membrane adhesion molecules (selectins and integrins) (Su and Schmid-Schönbein, 2008).

In the circulation the shear stress on the membrane depends also on the hydrodynamic interaction with the erythrocytes. The shear stress distribution on the surface of such suspended leukocytes becomes stochastic since each red cell passing a leukocyte influences it. A more complex picture arises that depends on the mechanical stress and also biochemical interaction with the red cells. Whereas erythrocytes facilitate retraction of neutrophil pseudopods in whole blood suspensions, replacement of erythrocytes with rigid microspheres abolishes the pseudopod retraction. This response in red cell suspensions requires scavenging of superoxide levels (Komai and Schmid-Schönbein, 2005). The fraction of leukocytes with pseudopods in a blood suspension decreases as the fluid shear increases (Komai and Schmid-Schönbein, 2005). At constant hematocrit the shear stress (adjusted to different magnitudes by addition of macromolecules to plasma), rather than shear rate, correlates with neutrophil de-activation, suggesting that the mechanotransduction is a force-dependent (as compared to a transport-dependent) mechanism by the mechanosensors.

It is interesting to note here, that other cell morphological structures control the details of the fluid shear stress. The glycocalyx on the plasma membrane of leukocytes reduces the magnitude of the fluid shear stress at the lipid membrane to low values (Secomb et al., 2001). The glycocalyx per se has been proposed to serve as mechanotransducer by bending of glycans under fluid shear which promote signaling the cytoplasm (Weinbaum et al., 2007).

Furthermore, endothelial cells and several tissue cells, but not leukocytes, have membrane caveolae. These flask-shaped invaginations, filled with membrane receptors, are ideal membrane structures to lower the shear stress inside the caveolae and serve as "fluid shears stress shelters" for receptors residing inside the caveolae (Shin et al., 2019) (see Section 11).

8. Fluid shear mechanosensors

A key issue is how fluid shear stresses are sensed by leukocytes. Mechanotransduction is likely initiated at the interface between the extracellular fluid and the interior cell signaling machinery. Moreover, the biological molecules on, or spanning, the cell membrane (bi-lipid layer, glycocalyx, cell surface receptors, ion channels, structural proteins, cell–cell adhesion molecules, etc.) that exhibit structure-dependent activity would have the ability to transduce fluid shears on cell surface into an intracellular activity, like actin polymerization. In effect, transmembrane proteins, while serving as receptors for autocrine and paracrine signaling molecules, also appear to "moonlight" as shear stress mechanosensors.

Leukocytes retract pseudopods under fluid shear whether they are adherent or freely suspended and regardless of the spatial distribution of shear stresses imposed on their membrane, as demonstrated by experiments with micropipettes (Moazzam et al., 1997),

cone-plate viscometer (Fukuda et al., 2000; Makino et al., 2007; Moazzam et al., 1997; Zhang et al., 2011; Zhelev et al., 2004), and parallel plate flow chamber techniques (Su and Schmid-Schönbein, 2008). Thus, the cell membrane, is likely the site at which local fluid shear stresses in the local milieu are transduced into cellular signaling. The possibility that the cell membrane is the site of shear stress mechanotransduction is consistent with its key roles in orchestrating cyclical projection and retraction of pseudopods (Bodin and Welch, 2005). Interestingly, neutrophil phagocytosis (Berlin and Fera, 1977; Wiles et al., 1994) and migration is under the influence of cell membrane fluidity by its impact on chemokine receptor number and affinity (Tomonaga et al., 1983; Yuli et al., 1982). Thus, shear stress may modulate neutrophil activity via membrane mechanotransduction.

A number of membrane molecules and structures have been proposed as mechanical sensors for stresses and in particular fluid shear stress (Baeyens and Schwartz, 2016; Bao et al., 2010; Yamamoto and Ando, 2018; Zeng et al., 2018; Zhang et al., 2010). Fluid shear stress induces acute changes in the fluidity of the lipid portion of a cell membrane (Butler et al., 2001; White and Frangos, 2007). Shear stress mechanotransduction may occur through lipid rafts (Ferraro et al., 2004; Rizzo et al., 1998a; Rizzo et al., 1998b; Simons and Toomre, 2000), which regulate neutrophil signaling (calcium flux, integrin expression, actin dynamics, etc.), morphology (Niggli et al., 2004; Seely et al., 2003; Tuluc et al., 2003), and cell adhesion (Marwali et al., 2003; Solomkin et al., 2007). But the lipid portion of the cell membrane lacks the phenotypic specificity that would explain differences in responses of specific cell-types to shear stress (neutrophils vs. endothelium vs. smooth muscle cells vs. bone cells, etc.). Instead, such differences in the cell reactions to fluid shear may be explained by cell specific expression profiles of transmembrane receptors and signaling molecules.

9. Membrane receptors as mechanosensors for leukocytes

Among transmembrane proteins, G protein-coupled receptors (GPCRs) (Cattaruzza et al., 2000; Chachisvilis et al., 2006; Makino et al., 2006; Yasuda et al., 2008; Zhang et al., 2009; Zou et al., 2004), receptor tyrosine kinases (Chen et al., 1999; Iwasaki et al., 2000; Jin et al., 2003; Lee and Koh, 2003; Milkiewicz et al., 2007; Palumbo et al., 2000; Shay-Salit et al., 2002), ion channels (Gu et al., 2001; Maingret et al., 1999; Tarbell et al., 2005), integrins (Haugh et al., 2015; Kamm and Kaazempur-Mofrad, 2004; Lee and King, 2007; Teravainen et al., 2013; Watabe et al., 2011), and signaling proteins (such as G-proteins (Gudi et al., 1998; Gudi et al., 1996)) have been reported as mechanosensors of fluid flow on endothelium, bone cells and others. Two cell surface receptors have been reported to be mechanosensitive on neutrophils during shear stress exposure: formyl peptide receptors (FPRs) and CD18 integrins.

9.1. Formyl peptide receptors

FPRs are a family of GPCRs that regulate neutrophil chemotaxis (Migeotte et al., 2006). In the specific case of pseudopod formation, FPR1 exhibits high activity during pseudopod formation (Makino et al., 2006). This receptor mediates pseudopod projection in response to small formyl peptides (e.g. f-MLP) and supports the ability of neutrophils to mount an antibacterial response (Gao et al., 1999). fMLP elevates FPR activity triggering cyclic actin polymerization/depolymerization and projection/retraction of pseudopods during neutrophil adhesion and migration (Gerisch and Keller, 1981; Welch et al., 1997).

Makino et al. were the first to link fluid shear stress-induced pseudopod retraction and FPR activity (Makino et al., 2005;

Makino et al., 2006). Fluid shear stress decreases the constitutive activity of the receptor as detected by binding of G_i subunits ($G_{\alpha\zeta}$ and $G_{\beta\gamma}$) with FRET. FPR is critical for neutrophils to project pseudopods since undifferentiated HL60 cells, which do not express FPR, lack the ability to form pseudopods (Makino et al., 2006). Promyeloid HL60 cells that can be differentiated into morphologically mature myeloid cells with many of the markers and capabilities of neutrophils also exhibit a robust fluid shear cell reaction. If the expression of FPR is blocked by transfection of the HL-60 cells with small interfering RNA (siRNA) before differentiation into neutrophil-like cells, the differentiated cells exhibit little reaction to fluid shear stress. Notably, transfecting HL-60 cells with siRNA to silence FPR expression in HL-60-differentiated neutrophils, inhibited shear-induced pseudopod retraction even though these cells were still able to project pseudopods, likely due to expression of other cytokine receptors (Makino et al., 2006). This result supports FPR as a shear stress mechanosensor.

While the precise mechanism of how shear stress reduces FPR activity on the cell surface remains unclear, it does not require matrix metalloproteinases (MMPs) (Mitchell and King, 2012), which are capable of cleaving FPR (Chen et al., 2010). In fact, exposure of adherent neutrophils to shear stress leads to redistribution of the FPR on the cell membrane followed by internalization and aggregation in an intracellular endosome (Mitchell and King, 2012; Su and Schmid-Schönbein, 2010). The FPR copies are derived from cell membrane regions with both high and low fluid shear stresses and result in a reduction of FPR copies on the membrane. The membrane density and internalization of the FPR receptor depends on shear stress magnitude (Mitchell and King, 2012). Reduction of pseudopod formation by freely suspended neutrophils goes hand in hand with shedding of L-selectin and activation of $\alpha_M \beta_2$ integrin, a process that is produced by fMLP itself but enhanced in the presence of fluid shear stress via protease (ADAM 17) and p38MAP kinase-dependent mechanisms (Mitchell et al., 2014).

9.2. CD18 integrins

Exposure of a neutrophil to fluid shear stress reduces surface expression of CD18 integrins, similar to FPR. But unlike the case of FPRs, shear-induced reductions in CD18 integrins involves the enzymatic activity of cellular protease(s) that appears to be released from lysosomal stores and targets a site on the extracellular domains of CD18 integrins, particularly CD11a/CD18 for lymphocytes and CD11b/CD18 for neutrophils (Zhang et al., 2013). Fluid shear stress reduces CD18-associated immunofluorescence of extracellular epitopes, especially in membrane regions exposed to elevated shear levels. CD18 is also translocated over the leukocyte surface from regions of higher shear to regions of lower shear and into the membrane contact areas with substrates (Akenhead et al., 2017; Fukuda and Schmid-Schönbein, 2003). Chelation of extracellular Ca^{2+} abolishes CD18 downregulation (Akenhead et al., 2017; Fukuda and Schmid-Schönbein, 2003). The protease for cleaving CD11b/CD18 integrin subunits appear to be the cysteine protease cathepsin B (Akenhead et al., 2017; Fukuda and Schmid-Schönbein, 2003).

CD18 cleavage requires a conformational shift in the extracellular domain which appears to be triggered by shear stress (Shin et al., 2008). The shift in the CD18 ectodomain exposes cysteine repeats and provides access for cathepsin B to cleavage site. It is conceivable that such a conformational shift triggers outside-in signaling, although such a molecular event has not been shown to date. Ligand binding induces conformational perturbations in the cytosolic domains of CD18 that initiates intracellular signaling. The current evidence points, however, to shear-induced CD18 cleavage reducing the number of adhesion molecules on the surface of neutrophils with the consequence of preventing their adhesion and migration to other cells (e.g., platelets) and/or substrates (Akenhead et al., 2017; Zhang et al., 2013). This is consistent with the important role of cathepsin B in restricting neutrophil pseudopod activity and Mac-1-dependent cell adhesion in the presence of fluid flow.

10. Attenuation of the fluid shear stress response in hypertension and hypercholesterolemia

The cellular reaction to fluid shear stress is critical to the ability of leukocytes to remain in an inactivated state. Considering that fluid flow mechanotransduction is predicated on the ability of shear stress to induce changes in the conformational activity of proteins, the response of a cell depends on the

1) number and distribution of mechanosensors on the cell surface,
2) ability of these mechanosensors to undergo conformational activity under shear stresses, and
3) the mechanical properties of the mechanosensors themselves and the properties of the support medium in which they reside.

Changes in the "deformability" of mechanosensors themselves can occur as a result of genetic mutations in the amino acid sequence that governs the conformational activity of mechanosensory proteins. Alteration in the protein-coding genetic sequences of receptors in form of point mutations to proteins such as CD11b (McCleverty and Liddington, 2003) or glycoprotein IIb/IIIa (Luo et al., 2009) has been shown to enhance binding affinity to ligands. Moreover, genetic mutations can enhance or attenuate the opening/closing of various mechanosensitive channels under physical stresses (Brown et al., 2007; Grillet et al., 2009). But to date no specific contribution of genetic mutations to impaired shear stress regulation of leukocyte activity has been reported.

There are other mechanisms that can lead to a loss of shear stress response and dysregulated leukocyte activity and thereby contribute to progression of a disease. Two cases we discuss here are hypertension and hypercholesterolemia, both of which are associated with sustained elevations in the numbers of activated leukocytes in the circulation long before the onset of overt vascular diseases. Leukocytes from hypertensive and hypercholesterolemia animals display an impaired and even reversed reaction to fluid shear.

10.1. Proteolytic receptor cleavage and impaired shear stress response in hypertension

In the SHR the impaired reactions of neutrophils to fluid shear have been attributed to an increase in proteolytic activity in the blood that cleaves FPR receptors on the cell surface. Specifically, unchecked activity of matrix metalloproteinases (MMPs) in SHR causes proteolytic cleavage of several cell membrane receptors, including the FPR and CD18. Cleavage of the FPR from the surfaces of neutrophils is seen by measurement of the immunohistochemical label density using antibodies against the extracellular domain of the receptor. SHR neutrophils or neutrophils treated with MMP-9 have a reduce number of extracellular domains and impaired pseudopod retraction under fluid shear compared to control cells (Chen et al., 2010). Cells even *extend* cellular projections under shear stress which contributes to the elevated peripheral hemodynamic resistance in the SHR (Fukuda et al., 2004b) (Fig. 4). The pseudopods amplify the substantial effect of relatively small number of leukocytes on the hemodynamics in organs like skeletal

muscle (Fig. 5). CD18 on SHR neutrophils is also proteolytically cleaved and leads to a reduced neutrophil adhesion to endothelial cells, resulting in a form of immune suppression in this animal (Arndt et al., 1993; DeLano and Schmid-Schönbein, 2008). Treatment of the SHR with MMP inhibitors restores the reaction to fluid shear stress and neutrophil adhesion to the endothelium (Chen et al., 2010). It is interesting to note that multiple other receptors in the SHR are also proteolytically cleaved causing a multitude of cell dysfunctions in this animal strain (Mazor and Schmid-Schönbein, 2015).

10.2. Membrane cholesterol enrichment and impaired shear stress response

Cholesterol enrichment decreases lipid bilayer fluidity (i.e. reduces membrane microviscosity), while membrane cholesterol depletion increases it (Chabanel et al., 1983; Cooper, 1978). Cholesterol modification of the cell membrane has been shown to alter neutrophil membrane ruffling, cell polarization, and F-actin polymerization during activation with fMLP (Khatibzadeh et al., 2013; Pierini et al., 2003). The underlying mechanism may be how the fluidity of the membrane influences conformational activity of transmembrane proteins.

In a similar fashion, cholesterol governs cell reactions to shear stresses. Membrane cholesterol depletion inhibits shear-dependent activation of extracellular signal-related kinase (ERK) in endothelial cells (Park et al., 1998) and influences osteoblast mechano-signaling (Ferraro et al., 2004). The neutrophil shear stress response depends on cholesterol-related membrane fluidity with an optimal membrane cholesterol/fluidity range permissive for shear-induced pseudopod retraction (Zhang et al., 2011). Thus, the cholesterol-related fluidity of the cell membrane may control the neutrophil's reaction under fluid flow by its influence on either mechanosensor (e.g. FPR, CD18), protein dynamics (e.g., conformational activity), or cathepsin B transport across the cell surface. Endothelial membranes directly respond to shear stress by rapidly decreasing their lipid phase and increasing their fluidity, which has been associated with shear stress mechanisms in endothelial cells (Yamamoto and Ando, 2015).

The link between membrane cholesterol and its potential biophysical impact on leukocyte shear stress responses has implications that may provide an explanation for cholesterol-dependent leukocyte activity levels associated with hypercholesterolemia. Leukocytes of mice placed on high fat diet (over a 12-week period) are unresponsive to fluid shear stress with a deficient pseudopod retraction (Fig. 6). The degree of impairment in neutrophil shear

responses is time-dependent and tracks with the gradual elevations in blood cholesterol levels over the duration of the high fat diet (Zhang et al., 2011). Conceivably, as blood cholesterol concentrations increase, cholesterol accumulates in the neutrophil membrane (Chabanel et al., 1983; Cooper, 1978; Day et al., 1997). The increases in membrane cholesterol likely reduce membrane fluidity, which contributes to the impaired mechano-response (Zhang et al., 2011).

11. Differential control of receptor activity: shear stress versus agonist binding

The ability of membrane receptors to respond with a unique conformational transformation to two different stimuli, biochemical agonists and mechanical shear stress, raises the interesting question of how and to what degree biophysical and biochemical stimuli can be differentiated in-vivo. We proposed that caveolae, which are membrane invaginations filled with numerous receptors (Patel et al., 2008), facilitate exposure of these receptors to a chemical agonist in the plasma by providing short diffusion distances into the caveolae but also serve as fluid shear stress shelters (Shin et al., 2019) (see Section 7). In contrast, plasma membrane receptors on the outside of the caveolae are exposed to chemical agonists in the blood and to the fluid shear stress. Thus, the caveolae are an effective mechanism to differentiate biochemical and biophysical mechanisms for stimulation of receptors that can exhibit mechanosensitive activation.

Although without caveolae, leukocytes have membrane folds (microvilli, ruffles), which also cause a nonuniform shear stress on the membrane. Receptors preferentially located at the tip or in the troughs between the membrane folds are exposed to elevated or reduced fluid shear stresses, respectively (Su and Schmid-Schönbein, 2008). The reaction of a receptor may be controlled by their location on a membrane. For example, immunolabeling evidence suggests that integrins and selectins, two families of membrane-associated leukocyte adhesion proteins, are preferentially located in the troughs and at the tips, respectively, on resting cells. Integrins redistribute over the entire membrane after activation (Bruehl et al., 1996; Erlandsen et al., 1993; Kubo et al., 1999).

12. Conclusion and outlook

The evidence presented here suggests that membrane receptors on circulating leukocytes serve as mechanosensors for the relatively weak shear stresses in the circulation. Fluid shear can stimulate or inhibit specific activities carried out by these receptors and control cell fates. The ability of a receptor to undergo conformational shifts due to altered lipid bilayer microviscosity or proteolytic cleavage of its extracellular domain compromises mechanotransduction by fluid shear of leukocytes and also endothelial cells (Altshuler et al., 2012).

The actions of fluid shear stress on various circulating leukocyte types, i.e. B- and T-lymphocytes, basophils, eosinophils, etc. remain to be determined. Other cells in the circulation, such as stem cells, known to respond to fluid shear stress (Ahsan and Nerem, 2010; O'Cearbhaill et al., 2008), remain to be investigated under circumstances when they are still in free circulation. Their "targeting" to different organs is controlled by membrane adhesion molecule expression and cell cytoplasmic properties, which may also be under control of the specific fluid shear stresses. A similar issue arises with circulating tumor cells (Cognart et al., 2020). Fluid stress may control their properties and determine in which part of the circulation they become trapped and initiate metastasis.
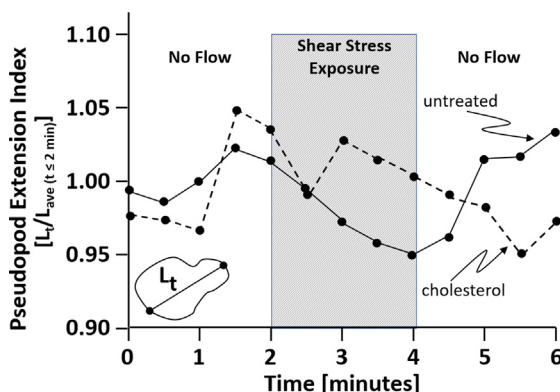

Fig. 6. Tracings of typical pseudopod retraction responses of leukocytes exposed to up to 2 dyn/cm² fluid shear stress from a micropipette setup. Cells treated with cholesterol exhibit cyclical extension and retraction of pseudopods independent of shear stress exposure (adapted from Zhang et al., 2013).

Receptor cleavage and loss of the shear stress response is a special aspect of a broader phenomenon when degrading enzymes, e.g. digestive proteases, enter into the circulation. Multiple cell functions may become compromised, beside the fluid shear stress response (Mazor and Schmid-Schönbein, 2015). Uncontrolled receptor cleavage may cause insulin resistance by proteolytic cleavage of the insulin receptor. A proteolytic process generates a multitude of cell dysfunctions at the root of the co-morbidities that accompany disease such as high blood pressure, hypercholesteremia and others. The loss of shear stress response may be part of a broader phenomenon which we refer to as autodigestion (Schmid-Schönbein and Chang, 2014).

13. Disclaimer

This article reflects the views of the authors and should not be construed to represent FDA's views or policies.

Declaration of Competing Interest

GWSS is scientific advisor to Leading Biosciences, San Diego Cailfornia, and owns founder's stock. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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