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Platelet-Activating Factor (PAF) and its effects on human neutrophil CD43

by

Diane Marie Teoh

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ABSTRACT

The overall aim of this thesis was to characterize the effect of the proinflammatory agent, Platelet-Activating Factor (PAF) on human neutrophil CD43. CD43, a cell surface molecule expressed abundantly on neutrophils, has been proposed to act as an anti-adhesive molecule and inhibit cell-cell interactions. Previous research has shown that human neutrophil CD43 is downregulated upon activation of neutrophils, however, the effects of PAF on CD43 expression have not been thoroughly investigated. In my studies, I was able to demonstrate that soluble PAF was able to downregulate CD43 expression on neutrophils. The role of membrane-bound PAF on CD43 shedding was not clearly demonstrated and requires further study. CD43 shedding occurred through proteolytic mechanisms and was dependent on protease(s) located within neutrophil granules. PAF's action was inhibitable by the specific PAF receptor antagonist, WEB 2086 as well as DIDS, a non-specific ion channel blocker. Functional studies *in vitro* with E-selectin transfectants revealed that neutrophils with partial CD43 expression had enhanced rolling interactions. These results suggest the potential role of CD43 as a functional barrier and that CD43 downregulation by endothelial membrane-bound PAF may serve to enhance cell-cell interactions.

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This thesis is also dedicated to Frisky.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

ARDS	Adult Respiratory Distress Syndrome
BSA	Bovine Serum Albumin
CGD	Chronic Granulomatous Disease
DIDS	4,4' Diisothiocyanato-stilbene 2,2'-disulfonic acid
DHCB	dihydrocytochalasin B
EDTA	Ethylene-diaminetetra-acetic acid
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
fMLP	formylmethionyl-leucyl-phenylalanine
Fura-2-am	fluorescent calcium chelator
GlyCAM-1	Glycoprotein Cell Adhesion Molecule-1
HBSS	Hanks Balanced Salt Solution
HNE	Human Neutrophil Elastase
HSA	Human Serum Albumin
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Cell Adhesion Molecule
IL-8	Interleukin-8
MFI	Mean Fluoresence Intensity
PAF	Platelet-Activating Factor
PBS	Phosphate Buffered Solution
PECAM-1	Platelet/endothelial cell adhesion molecule-1
PKC	Protein Kinase C

PLA_2	Phospholipase A_2
PMA	Phorbol myristyl acetate
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethyl-sulfonyl fluoride
PSGL-1	P-selectin glycoprotein ligand -1
RBC	Red blood cells
Rpm	revolutions per minute
S'ase	Neuraminidase/ Sialidase
(sLe^x)	sialyl Lewis X
TNF-α	Tumour Necrosis Factor α
VLA-4	Very Late Antigen-4
WEB 2086	Platelet Activating Factor receptor antagonist

Chapter One

Introduction and literature review

The ability of our immune system to mount a response to the invasion of foreign pathogens is known as the acute inflammatory response. This response is non-specific, swift and most times, effective. The consequences of this response are seen immediately, within minutes to hours following contact with the offending pathogen. They include the symptoms of redness, swelling, heat, pain, and sometimes loss of organ function. Whether this happens in reaction to a small abrasion or something more serious such as a myocardial infarct, the participants in the inflammatory response are the same. The neutrophil is the first cell type to arrive. The ability of neutrophils to accumulate at sites of tissue injury involves the directed movement of neutrophils extravascularly into tissues and is referred to as infiltration. This has been reviewed extensively [1-4].

The process of infiltration involves a coordinated series of steps that allow neutrophils and other leukocytes to emigrate from the circulation into tissues in response to chemotactic stimuli that includes bacterial products, components of the complement cascade and proinflammatory mediators such as cytokines and growth factors. This is mediated by adhesion molecules that allow the circulating cells to make contact with the vessel wall (tethering and rolling), slow down and halt suddenly (adhesion), before making its way out into the tissues (emigration). This is often referred to as the leukocyte recruitment cascade (Figure 1.1)[1-5]. This recruitment cascade is a common mechanism by which all leukocytes emigrate out of the vasculature. For the purpose of this thesis, references to leukocyte recruitment will be restricted to neutrophil recruitment.

Although neutrophil infiltration is an essential part of natural (or innate) immunity, excessive or uncontrolled neutrophil infiltration often leads to injurious tissue damage and has been implicated in several disease conditions. There is a fine delineation between

healthy neutrophil infiltration and that of excessive neutrophil infiltration into tissues. A slight shift in the balance between both states manifests serious disease states. It is becoming increasingly clear that the inflammatory process is complex and multi-factorial. We now know that diseases such as adult respiratory distress syndrome (ARDS), ischemia-reperfusion injury, inflammatory bowel disease and rheumatoid arthritis are a result of excessive neutrophil infiltration into tissues [2, 6, 7].

There has been considerable focus on the elucidation of leukocyte recruitment in recent years. Movement of neutrophils to extravascular sites is mediated by a series of adhesion molecules and until lately, majority of research in this field has focused on pro-adhesive molecules expressed by neutrophils. Recently, there has been a growing interest in anti-adhesive mechanisms that may impair neutrophil adhesion and thus keep inflammation under control. Although the roles of several pro-adhesive molecules in this cascade are well characterized, little is known about the role of anti-adhesive molecules in the leukocyte recruitment pathway. One such molecule is CD43 (leukosialin, sialophorin), a transmembrane sialylated glycoprotein with expression restricted to hematopoietic cells and circulating leukocytes (neutrophils, T lymphocytes, monocytes, macrophages, NK cells, activated B cells and plasma cells)[8, 9]. The exact function of CD43 is complex, although it has been proposed that CD43 may serve as a "functional barrier" to neutrophil cell-cell and cell-matrix interactions [10-14]. As will be discussed later, there is also evidence to suggest that CD43 has more than one function on different cell types.

1.1 Neutrophil Structure and Function

The neutrophil, also known as a polymorphonuclear granulocyte, is the most abundant (50-70%) circulating leukocyte in humans. Mature neutrophils are non-proliferative (i.e. are terminally differentiated) and have a normal circulating lifespan of about 7 to 10 hours following release from the bone marrow. After this, they enter the tissues and survive for an additional 12-24 hours. Unlike lymphocytes, neutrophils do not recirculate. Upon entering the tissues, they either die by activation and release of cytoplasmic contents or are removed by phagocytic macrophages [2]. Neutrophils arise from pluripotent stem cells and go through various stages of maturation following stimulation by various growth factors and cytokines produced by stromal cells in the bone marrow (eg G-CSF, GM-CSF, and IL-3). It takes approximately 7 to 10 days for a myeloblast to become a segmented neutrophil [15, 16]. CD43 expression appears early in myeloid cell maturation and is often used as a marker for hematopoietic cells [17, 18]. Neutrophils develop their characteristic granules as they mature. The primary granules form in the promyelocyte stage whilst the secondary granules appear in the myelocyte stage of neutrophil development [15, 16].

The primary function of the neutrophil is microbicidal as they help maintain normal host defense against invading microorganisms. Although the microbicidal function of neutrophils is essential, improper stimulation of neutrophils result in the indiscriminate release of these mediators into extravascular space and the neutrophil becomes proinflammatory. Besides microbicidal activities, neutrophils also act as scavengers of damaged and necrotic tissues. There are two mechanisms of microbicidal activity.

The first is oxygen-dependent, also known as the respiratory burst. It is a reversible event that normally requires the ingestion of bacteria or degranulation. The respiratory burst involves the activation of a membrane-bound, multi-component enzyme complex, the NADPH-oxidase, in which molecular oxygen is converted into superoxide anion. Superoxide anion reacts with hydrogen ions to form hydrogen peroxide. Hydrogen peroxide is potent against bacteria and other microorganisms but its potential killing power is increased when catalyzed by the enzyme myeloperoxidase from the primary granules of neutrophils. The result is the formation of hypochlorous acid (the main ingredient in household bleach). Superoxide, together with its potent and toxic derivatives facilitates the killing of microorganisms in an effective manner. All this normally happens in a sequestered space so damage to innocent bystander cells is minimal.

The second general pathway of microbial killing is oxygen- independent, and is mediated by various proteases, polypeptides (defensins) and other antimicrobial proteins (eg. BPI) contained within the various granules of the neutrophil. Table 1.1 outlines the arsenal of neutrophil granule contents. Within these granules are various agents capable of killing bacteria (eg. defensins that act through pore formation on bacterial cell walls) as well as enzymes that are capable of attacking the extracellular matrix, allowing neutrophils to leave the bloodstream and migrate towards foreign particles that are to be ingested.

Neutrophils are also capable of recognizing, attaching and engulfing these particles, where they discharge the contents of their cytoplasmic granules into vacuoles, and release a burst of oxidants. During the process of phagocytosis, neutrophil cytoplasmic granule membranes fuse with membranes of the phagocytic vacuole, discharging the granule contents into the newly formed phagolysosome. Phagolysosome formation is important in

neutrophil function, as it is the organelle where ingested microbes are killed and digested [19]. Although there is tremendous redundancy in neutrophil killing, the importance of neutrophil microbicidal function is highlighted in certain inherited human disorders. For example, Chronic Granulomatous Disease (CGD) in which there is a defect in the NADPH-oxidase system. Patients with CGD have an increased incidence of severe recurrent, often life-threatening, pyogenic infections.

1.2 Neutrophil-Endothelial Interactions

The process of neutrophil infiltration is outlined in Figure 1.1, which summarizes the mechanisms by which neutrophils leave the vasculature and enter tissues. This process primarily involves adhesion molecules expressed on the surface of neutrophils interacting with their ligands expressed on the endothelium.

1.2.1 The role of endothelium and adhesion molecules

The endothelium, specialized cells that line the blood vessels were once thought to be static and inert. Today, we know that the endothelium plays a very active and dynamic role in inflammation. Not only does it form a physical barrier between the circulatory system, and the extravascular space, it is also capable of upregulating various adhesion molecules and proinflammatory mediators (cytokines, vasoactive amines) that are crucial to the acute inflammatory process [20]. Endothelium is activated by various stimuli such as thrombin, hydrogen peroxide and cytokines such as Tumour Necrosis Factor -α (TNF-α) and Interleukin-1 [20, 21]. Activated endothelial cells express adhesion molecules such as E-selectin and P-selectin. Endothelium is also capable of producing nitric oxide and other

agents that modulate the inflammatory process. In essence, the endothelium possesses the functions of a "*gatekeeper*" [20].

Adhesion molecules are molecules that exist to regulate cell movement from one compartment to another. The importance of adhesion molecules is underscored by their presence on cancer cells. Table 1.2 outlines some of the adhesion molecules that play an important role in neutrophil infiltration into tissues. These molecules mediate the directed movement of neutrophils to sites of injury.

The importance of adhesion molecules is highlighted in the disease Leukocyte Adhesion Deficiency I (LAD). The absence of β_2-integrins on neutrophils cause individuals to be extremely susceptible to various life-threatening infections [2]. Adhesion molecules include selectins, integrins and various members of the immunoglobulin gene superfamily.

1.2.2 Selectins

Selectins are a family of Ca^{2+} dependent lectins that share a common molecular structure. They contain an NH_2 terminal, carbohydrate-binding lectin domain, a single epidermal growth factor domain and various short consensus repeats that determine the length of the molecules [22]. The short consensus repeats serve to elevate the interacting ends of the selectins above the glycocalyx and it is thought that the epidermal growth factor domain may allow selectins to interact with their ligands [22]. They are three known members of the selectin family; L-selectin, P-selectin and E-selectin. They differ from each other by the number of consensus repeats and it is thought that the cytoplasmic tail functions in signaling during cell activation [22, 23]. Selectins are ideal mediators of tethering and initial contact for several reasons. First, they are long molecules that extend

out of the glycocalyx, suitable to make initial contact with passing leukocytes. Also, L-selectin localizes to the microvilli, again allowing for maximal contact with the endothelium [24]. Selectins also mediate activation- independent adhesion. This means, that selectin interactions can take place before cellular activation. Finally, selectins mediate transient and fairly weak adhesion. If activation is not triggered, the selectins can disengage [2].

1.2.3 Integrins

Integrins are a large, structurally and functionally complex family of heterodimeric cell adhesion molecules that recognize a broad spectrum of extracellular surface proteins. The presence of divalent cations, Mg^{2+}, Mn^{2+} and Ca^{2+} is required for ligand binding [25, 26]. Research in this field has focussed mainly on the β_2 integrins. There are several members to the β_2 family of which three are most studied: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1) and CD11c/CD18. They exist as heterodimers of unique α subunits non-covalently bound to a common β subunit (β_2) [27];[3, 28, 29]. CD11a and CD11b are both capable of interacting with immunoglobulin superfamily members ICAM-1 and ICAM-2, although the latter is also capable of interacting with ligands such as complement fragments, denatured albumin and fibrinogen [30]. A characteristic property of integrins is the requirement for conformational activation before optimal ligand binding can occur [28]. Interaction of CD11/CD18 with its ligand results in the activation of the integrin: the integrin undergoes a conformational change that is associated with an increase in avidity of the molecule.

1.2.4 Immunoglobulin Gene Superfamily

Members in this family share the immunoglobulin domain, composed of 90-100aa arranged in a sandwich of two sheets of anti-parallel β strands [4]. Among the members of this family are molecules important in leukocyte recruitment. They include the ligands Intercellular Cell-Adhesion Molecules; ICAM-1, ICAM-2, and Vascular Cell Adhesion Molecules; VCAM-1 and VCAM-2 expressed on endothelium as well as Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1), expressed on specialized endothelial cells known as high endothelial cells in lymph nodes and important in lymphocyte recirculation [29]. Other molecules that are also members of this family, PECAM-1 (CD31) on neutrophils and endothelial cells and CD47 which has a wide distribution on endothelial cells, hematopoietic cells and epithelial cells.

1.3 Neutrophil Recruitment

The initial neutrophil-endothelial interactions are mediated by selectins that specifically interact with carbohydrate moieties bearing the tetrasaccharide;sialyl-Lewis x (sLe^x) linked to mucin-like molecules expressed by leukocytes and activated endothelium. Tethering is described as the first contact made by the neutrophil to the endothelium. Cells briefly tether then release in a transient manner with quick on and off rates. Adhesion molecules such as L-selectin, P-selectin and E-selectin mediate tethering by binding to their respective ligands [1-5]. P- and E-selectin are expressed on the endothelium whilst L-selectin is expressed on leukocytes [1-5]. One of the molecules that helps mediate the initial tethering and rolling on leukocytes is L-selectin [29]. Although L-selectin was initially described in lymphocyte trafficking, it is constitutively expressed on all leukocytes including, but not restricted to, circulating neutrophils. On unstimulated neutrophils, L-selectin is located on structures known as the microvilli; small, finger-like projections that

extend from the tips of the villi that surround the cell [24]. It's primary ligand, sialyl Lewis X (sLe^x) is present on the surface of endothelium, however, other ligands for L-selectin have been identified, including GlyCAM-1 (in the mouse), CD34 and MAdCAM-1 [22]. Experiments with function-blocking L-selectin antibodies and L-selectin deficient animals have defined a role for L-selectin in leukocyte recruitment. L-selectin is proteolytically shed from activated neutrophils and the current concept is that L-selectin makes way for the interaction of other molecules that further activate the cell [22].

After the initial contact with the endothelium, neutrophils often move in a continuous manner, and seem to "roll" on the endothelium of post capillary venules. This rolling is important because it allows the neutrophil to slow down and come in contact with other cell surface molecules that will further activate the cell.

Tethering and rolling are also mediated by E- and P-selectin (expressed on the endothelium) which interact with their counterligand, PSGL-1, and other sLe^x molecules present on the surface of leukocytes [31]. Both E- and P- selectin are upregulated upon endothelial stimulation by oxidants, cytokines and other chemokines released upon tissue injury [21, 32-37]. P-selectin is typically upregulated within minutes and is thought to mediate neutrophil recruitment in acute inflammation. Stimuli such as histamine, thrombin, leukotriene C_4 or free radicals are capable of upregulating P-selectin on endothelium almost instantly but disappear by one hour following stimulation [38]. The efficiency of P-selectin upregulation is a result of P-selectin being pre-stored in Weibel-Palade bodies within endothelial cells. E-selectin, on the other hand, is synthesized *de novo* upon stimulation by cytokines such as TNF-α and Interleukin 1 and is thought to play a role in chronic inflammatory states. Classically, it was thought that P-selectin mediated acute

inflammation while E-selectin mediated chronic inflammation. New research has shown that P-selectin may also be found in the so-called chronic inflammatory states where P-selectin upregulation has been found several hours after initial stimulation [39].

Rolling not only allows the neutrophil to slow down and prepare to leave the vessel but also allows neutrophils to come into close contact with endothelial-bound proinflammatory molecules that are capable of neutrophil activation. Within minutes of stimulation of the endothelium, the activators described above are capable of expressing newly synthesized membrane phospholipid Platelet-Activating Factor (PAF) and pre-stored P-selectin [28, 33]. This process of co-expression of selectin and proinflammatory mediators interacting with neutrophils and mediating their activation is an example of juxtacrine activation [28].

Although rolling is a prerequisite for neutrophil spreading and adhesion to endothelium under flow conditions, firm adhesion and transmigration requires another set of obligatory adhesion molecules. In neutrophils, firm adhesion (defined as the cessation of neutrophil movement along the length of the endothelium) is dependent upon the upregulation and activation of the β_2 integrins (CD11/CD18) [21, 28, 30, 33]. Neutrophils that come into contact with agonists on activated endothelium become activated. This results in degranulation, whereby the integrins are upregulated on the surface of neutrophils. Functional upregulation of integrins also takes place and they interact with their ligands of the ICAM family expressed on endothelium.

After the neutrophils adhere, they undergo a physical shape change. They flatten out and spread, in preparation for emigration into tissues [8]. Recent studies have shown that neutrophil emigration across the endothelial barrier may be mediated by several

molecules including CD11/CD18, CD31 (Platelet-Endothelial Cell Adhesion Molecule-1; PECAM-1) [40] as well as CD47 (Integrin Associated Protein; IAP) [41]. It has been established that firm adhesion is also a prerequisite for emigration, therefore, impairment in any of the neutrophil-endothelial interactions will attenuate neutrophil infiltration. There exists cooperation between the leukocyte and endothelium as the former squeezes in between tightly opposed endothelial cells by inserting the pseudopod from the leading edge into the junction between two endothelial cells [42]. PECAM-1 has also been proposed to play an important role as it is found on both the leukocyte and the endothelium and can interact homotypically with endothelial glycosaminoglycans [43].

There are potential therapeutic benefits in understanding the molecular mechanisms of each of these interactions; earlier animal studies have demonstrated that antibodies to cell adhesion molecules attenuate disease pathogenesis through reduction of inflammation.

Until recently, it was believed that neutrophils roll, adhere and emigrate via the mechanism mentioned above. Researchers have now shown that neutrophils are capable of migrating into tissues via other forms of adhesion molecules such as the Very Late Antigen -4 (VLA-4). Reinhardt *et al.* have demonstrated that activated neutrophils are capable of expressing VLA-4 when migrating into heart tissues [44]. VLA-4 appears to allow the neutrophils to adhere and emigrate in a CD11/CD18independent manner. VLA-4 interacts with its ligand, VCAM-1 expressed on endothelium.

1.4 CD43

CD43, also known as leukosialin and sialophorin, is a sialylated, transmembrane glycoprotein expressed on the surface of all circulating human leukocytes. Characterization

of the cDNA of human CD43 has localized it to a single gene located on Chromosome 16 [45]. Further characterization of CD43 indicates that CD43 has a 23 amino acid residue hydrophobic transmembrane region and a 123 residue cytoplasmic region [45, 46]. The 239 amino acid extracellular domain of CD43 is a membrane-associated mucin with a high content of serine, threonine and proline residues that have 70-85 O-linked oligosaccharides and abundant sialic acid residues such as sLe^x [45]. CD43, like other mucins (e.g.GlyCAM-1, MAdCAM-1, CD34 and PSGL-1) contain a high local density of O-linked sugar moieties [47]. More than 50 % of its molecular weight is derived from the addition of O-linked oligosaccharides as the precursor protein molecule has a molecular weight of approximately 54kDa [48]. There exists two isoforms of CD43. One isoform of CD43 (115kDa) is found on circulating T lymphocytes, thymocytes and monocytes, while the second isoform (135kDa) is expressed by neutrophils and platelets [49, 50].

There has been considerable speculation as to the precise function that CD43 may serve on hematopoietic cells. There are several reasons why CD43 is a likely candidate to act as an anti-adhesive barrier. These are summarized in Table 1.3.

Although the exact function of CD43 on human neutrophils still remains unknown, it has been postulated that CD43 may serve as a "functional barrier" impeding neutrophil cell-cell and cell-matrix interactions [10 -14]. The evidence for this is as follows. First, neutrophils exhibit a net negative surface charge, which has been attributed to the sialic acid residues present on membrane glycoproteins [51]. A high level of glycosylation, coupled with a net negative charge retards cell-cell contact. It is estimated there are more than 10^5 CD43 molecules present on the surface of neutrophils making CD43 the most abundant sialoprotein on neutrophils [52]. Therefore, it is likely that CD43 is a significant

contributor of neutrophil net negative surface charge. In the unstimulated state, this net negative charge critically retards cell-cell contact *in vitro*. Removal of sialic acid residues from neutrophils has been associated with a reduction in cell surface charge and changes in cell-cell interactions such as increased adhesiveness, homotypic aggregation and cell spreading [8, 53 , 54]. Second, structurally, mucins are important in that they extend well beyond the glycocalyx that surrounds the surface of cells. The extracellular domain of CD43 is an extraordinarily long rod-like structure protruding 45 nm from the plasma membrane, and may therefore, impede receptor ligand interactions through steric hindrance [45, 52]. It is also possible that in view of the abundance and the extraordinary length of CD43, a "blanketing" effect of other cell adhesion molecules by CD43 occurs, preventing receptor-ligand interactions.

The concept of proteolytic modulation of neutrophil membrane proteins is hardly a new one. However, there is increasing evidence that this is playing a larger role on neutrophil adhesion molecules than first thought. Proteolytic downregulation has been shown to effect several surface molecules, including L-selectin, FcγRII, ICAM-3, CD53 and CD44 [55,56]. Like these surface proteins, CD43 expression is rapidly downregulated by proteolytic shedding following cellular activation [8, 53, 57-59]. Agonists capable of degranulating primary and secondary granules of neutrophils have been shown to cause CD43 downregulation as well as activate neutrophils [14]. Proteolytic shedding of cell surface molecules is important for cell-cell interactions as it provides a mechanism for rapid and exquisite regulation of receptor-ligand interactions between the endothelial cell and neutrophil.

In support of the above, there is evidence for proteolytic shedding of CD43 *in vivo*. A plasma galactoglycoprotein identical to the N-terminus of CD43 has been found in normal human plasma, and since lymphocytes do not shed CD43 and there is only a single gene for CD43 with one exon, this suggests that CD43 is indeed shed from neutrophils [60]. Furthermore, consistent with the proposed "barrier" function of CD43, differences in CD43 surface expression between circulating and emigrated leukocytes have been demonstrated previously. In one study, synovial fluid neutrophils had decreased CD43 expression compared to circulating neutrophils [57]. In a separate study, a decrease in CD43 was also seen with rat peritoneal macrophages compared to blood monocytes [61].

In light of this, it is plausible that partial desialation of neutrophils would result in unmasking of cryptic molecules, permitting interaction of previously "hidden" molecules with their respective counterligands.

Recently, a paradigm for CD43 has been proposed [62]. In this paradigm, CD43 is described as a non-specific molecule that inhibits cell-cell interactions, yet CD43 may also be pro-adhesive. There is mounting evidence that CD43 plays different roles on different cell types. It is interesting that CD43 is not downregulated on T cells, while on neutrophils and macrophages, downregulation is seen on cells that enter tissues [57, 61]. It is important we do not ascribe a generalized function for CD43, as it is evident that CD43 plays multiple roles in leukocyte-endothelial interactions.

In addition to the biochemical properties of CD43 discussed above, there is more evidence supporting the anti-adhesive function of CD43. This includes reports that CD43 expression decreases cell adhesion in various cell types. The development of the CD43

deficient mouse has increased the scope of our knowledge on CD43 [63]. In their *in vivo* model, CD43 is anti-adhesive as T cells from CD43 knockout animals markedly increased their proliferative responses to T cell activators. These T cells were also prone to homotypic adhesion. Further evidence demonstrated that disruption of CD43 expression resulted in increased cell adhesion [11]. The existence of a mechanism by which CD43 is removed (proteolytic shedding) also supports the theory that CD43 is anti-adhesive [64, 65].

There is also some compelling evidence that CD43 may be pro-adhesive. It is thought that CD43 may bind counterligands on neighbouring cells to stabilize cell-cell interactions and many putative counterligands have been proposed for CD43, including ICAM-1 [66], C1q[67], and E-selectin [68]. It must be pointed out that the evidence above is limited to *in vitro* studies and, to date, this has not yet been proven *in vivo.* The ability of anti-CD43 monoclonal antibodies to inhibit cell interactions also supports the view that CD43 is pro-adhesive [69, 70]. In their study, McEvoy *et al.* demonstrated the ability of an anti-CD43 antibody to block T cell binding to lymph nodes and Peyer's patch high endothelial venules, as well as inhibit T cell extravasation into secondary lymphoid organs. A follow-up study utilizing the same monoclonal antibody (L11) found that the development of diabetes in non-obese diabetic mice was prevented through the blocking of T cell migration into inflamed pancreatic islets [71]. However, the use of antibodies to inhibit leukocyte adhesion and emigration is hardly an infallible argument for CD43 being proadhesive. The addition of anti- CD43 antibodies may simply enhance steric hindrance and actually be promoting anti-adhesive effects. Finally, CD43 has been considered proadhesive because of a study reporting that ICAM-1 was a ligand for CD43 in vitro [67].

However, experiments with neutrophils from LAD Type I patients (deficient in β_2 integrin expression) do not adhere to endothelium despite normal CD43 expression. This certainly provides irrefutable evidence thatICAM-1 is not a ligand for CD43 [74].

To date, the majority of research on CD43 has involved T cells and other cell types. CD43 has been proposed as an important regulator of immune cell function, particularly during T cell activation. Ligation of CD43 by monoclonal antibodies has been shown to augment the proliferation of activated T cells. A more thorough study of *in vivo* and *in vitro* T cell responses mediated by CD43 indicates that negative regulation of the aforementioned responses may involve intracellular signaling pathways, rather than just as a physical barrier [72]. In both *in vivo* and *in vitro* responses to stimulation, T cells from CD43-deficient mice were hyper-responsive. This hyper-responsive behavior was also observed following receptor- independent activation.

Recent experiments from our research group using intravital microscopy examined the effects of CD43 deficiency *in vivo*. Our results indicated that leukocytes in CD43-deficient animals demonstrated significantly increased rolling and adhesion interactions in post-capillary venules compared to leukocytes from wild-type [73]. This is in agreement with the proposed anti-adhesive function of CD43. Interestingly, although CD43-deficient cells interacted more avidly with the endothelium, there was an impairment in emigration with leukocytes from CD43-deficient animals in response to PAF superfusion over the cremaster preparation.Other experiments examining leukocyte emigration (monocytes and neutrophils) into the peritoneum also illustrated the inability of CD43-deficient leukocytes to emigrate. Together, these results support the idea that CD43 may also function to promote leukocyte emigration.

It is important to emphasize that in this model, there is a complete lack of CD43 on the surface of the leukocytes. With the exception of individuals suffering from Wiscott-Aldrich Syndrome, this may not be representative of what is happening physiologically during inflammation. Others have described a population of protease-insensitive CD43 that is residual on neutrophils [13]. It is plausible that this residual CD43 is necessary for the emigration process. Several researchers have described the mobilization of CD43 to the uropod during cell spreading [74-76] . Others have also shown that CD43 is associated with F-actin, important in the mobilization of the cell [74].

1.5 Platelet-Activating Factor (PAF)

Platelet-Activating Factor (PAF) is a physiological agonist known to activate neutrophils at the time of neutrophil-endothelial contact. A bioactive phospholipid with diverse potent physiological effects, PAF is capable of activating not only platelets but also neutrophils and monocytes at nanomolar concentrations [21, 33, 77, 78]. Most cells involved in the inflammatory response produce PAF, strongly suggesting that it may be a mediator in this response. Its role in the pathogenesis of asthma, ischemia-reperfusion injury, ARDS, arthritis, allergy and shock have made it the focus of intense research in the area of inflammation [77-79].

PAF, composed of a glycerol backbone with alkyl, acetyl and phosphocholine side groups is also known as 1-O-alkyl-2 acetyl-*sn* -glycero-3-phosphocholine (see Figure 1.2) [79] [78]. PAF exerts its actions via a specific high-affinity receptor [80] and thus the stereochemisty of the molecule is critical for receptor interactions. The ether linkage at the *sn*-1 position of the glycerol backbone is crucial as molecules with similar structure to PAF

but with a fatty acid at the sn-1 position, possess less than 1% of PAF's potency in platelet aggregation assays. The fatty alcohol at this position is usually hexadecanol. The length of the alkyl chains often corresponds to the potency of the molecule [78]. Another important structural feature is the presence of acetate at the *sn*-2 position. Biological activity is abolished upon removal of acetate from this position by the enzyme acetylhydrolase. A change in the polar head group at the *sn*-3 position also corresponds with a reduction in the biological potency of PAF [78].

Unlike many other proinflammatory molecules, PAF is not stored. Rather, it is synthesized by many cell types (monocytes, eosinophils, endothelial cells) upon cellular activation. PAF is synthesized by one of two pathways; the first involves remodeling of cell membrane phospholipids, while the second requires *de novo* synthesis [79]. The remodeling pathway is the only mechanism of PAF synthesis in neutrophils, endothelial cells and monocytes. The first step to the remodeling pathway requires the participation of phospholipase A_2 (PLA_2) to catalyze hydrolysis of the *sn*-2 fatty acid from alkyl-phosphoglycerides to yield an intermediate, 1-O- alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) and a free fatty acid (Figure 1.3). PLA_2 prefers a phospholipid that has an arachidonic acid at the 2-position, thus synthesis of PAF is often coupled with production of arachidonic acid metabolites. Lyso-PAF is then converted to PAF by the addition of an acetate, catalyzed by a specific acetyltranferase. In the second route of PAF synthesis, the *de novo* pathway, a separate series of enzymes mediate PAF synthesis. PAF degradation occurs through the removal of the acetyl group by acetylhydrolases abundant in tissues and plasma. Their importance is evident in light of the potency of PAF as a mediator for a wide range of immune and allergic reactions [81,82].

Although CD43 shedding can be mediated *in vitro* without cellular activation by exogenous proteases, *in vivo* it is likely that CD43 downregulation occurs following cellular activation. PAF is one candidate that is likely to mediate CD43 shedding for several reasons. First, PAF is co-expressed with P-selectin by activated endothelial cells [21] and it is the former that appears to be responsible for neutrophil activation. Second, because PAF is membrane-bound, it is capable of neutrophil activation at the time of neutrophil-endothelial cell contact [83]. The effects of PAF on neutrophils are numerous. Not only does it cause fluxes in intracellular calcium levels, it is also capable of mediating changes in membrane potential and intracellular pH, shape change and extracellular release of granule enzymes along with the upregulation of several surface molecules [84]. PAF upregulates CD11b expression whilst downregulating L-selectin on neutrophils [85]. Finally, PAF is a potential mediator of CD43 shedding as it is capable of inducing neutrophil exocytosis and protease translocation to the plasma membrane [34].

PAF-induced shedding of CD43 has not yet been investigated. If PAF is capable of shedding CD43 *in vivo*, we could, for the first time, demonstrate an endogenous method of modifying neutrophil-endothelial interactions. This would further support the concept that CD43 is anti-adhesive. PAF would likely mediate CD43 shedding after the neutrophil has adhered to the endothelium. CD43 shedding would decrease the surface charge, and possibly promote further the adhesive interactions between the neutrophil and the endothelium.

1.6 Statement of Hypothesis and Objectives

Primary Objective

To characterize the effects of the pro-inflammatory agent, Platelet-Activating Factor (PAF) on human neutrophil CD43 expression *in vitro*.

Hypothesis

Endothelium-associated PAF mediates CD43 shedding on human neutrophils and consequently promotes enhanced neutrophil-endothelial cell-cell interactions.

Specific Objective 1

To characterize PAF-mediated CD43 shedding of human neutrophils *in vitro*.

Specific Objective 2

To evaluate the ability of membrane-bound PAF upregulated on thrombin-stimulated endothelial cells, to shed CD43 on human neutrophils, to allow adherence to endothelium in a static manner.

Specific objective 3

To evaluate the ability of the non-specific inhibitor, DIDS and the specific PAF receptor antagonist, WEB 2086 in the inhibition of PAF-mediated CD43 shedding.

Specific objective 4

To examine selected functional consequences of CD43 shedding from human neutrophils.

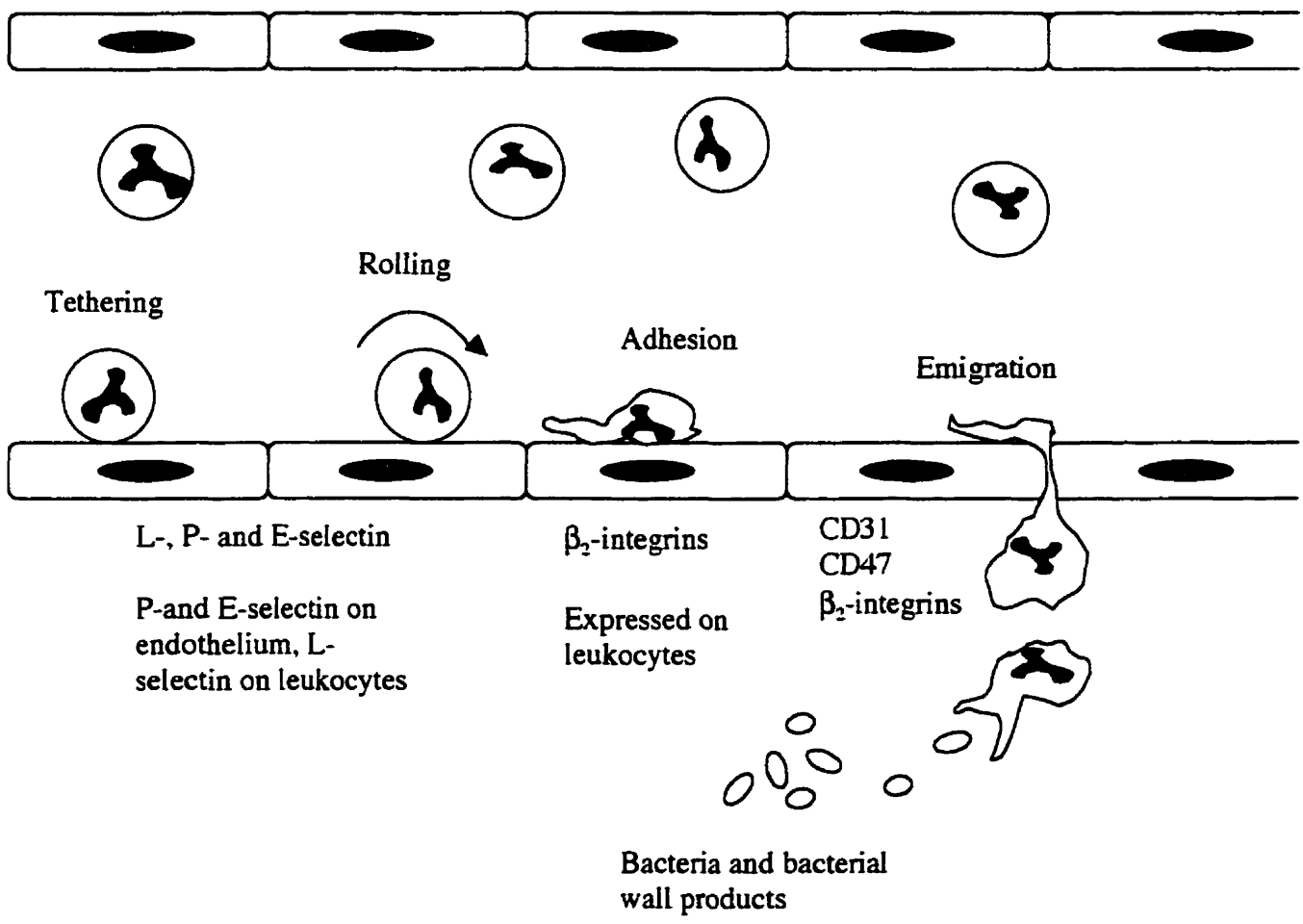

Figure 1.1 The leukocyte recruitment cascade. Leukocytes in a fast flowing blood stream "sample" the endothelium. Activation of endothelial cells lining the blood vessels results in the upregulation of selectin molecules, which can interact with ligands expressed on the leukocytes. This mediates leukocyte tethering and rolling. Other molecules upregulated on the surface of endothelial cells activate the leukocytes, resulting in the upregulation of integrins that mediate leukocyte firm adhesion. The leukocytes then migrate out to sites of tissue injury, mediated by various integrins and other adhesion molecules [1, 4, 5].

Table 1.1 Granule contents of neutrophils (adapted from [15, 16])

Primary granules	**Secondary granules**	**Tertiary granules**
β-glucuronidase	lysozyme	gelatinase
lysozyme	alkaline phosphatase	
myeloperoxidase	lactoferrin	
acid phosphatases	Vit B_{12} binding protein	
arginase	Cytochrome b_{558}	
glucosidase	complement receptor 3	
defensins	gelatinase	
cathepsin G	collagenase	
azurocidin	fMLP receptors	
BPI	CD11b/CD18	
elastase	CD11c/CD18	
proteinase-3	histaminase	
alkaline phosphatase	plasminogen activator	
hydrolases		

Table 1.2 Table of the various adhesion molecules that are involved in the leukocyte recruitment cascade.

Adhesion Molecule	Function	Ligand(s)
L-selectin	Tethering	sLe^x, PSGL-1
P-selectin	Rolling	sLe^x , PSGL-1
E-selectin	Rolling	sLe^x , PSGL-1
$\beta_2$integrins	Adhesion, Emigration	ICAM-1, ICAM-2
VLA-4	Adhesion, Emigration	VCAM-1
CD31 (PECAM-1)	Emigration	PECAM-1
CD47(IAP)	Emigration	$\beta_3$integrins

Table 1.3 Evidence supporting CD43 as an anti-adhesive molecule.

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1) Steric Hindrance

- Length [45, 52]
- Net negative surface charge [51]
- "Blanketing effect"

2) Proteolytic shedding of CD43 occurs rapidly [13, 14];[55, 64]

3) CD43 differences seen between circulating and emigrated cells[57, 61]

4) Soluble CD43 found in plasma [60]

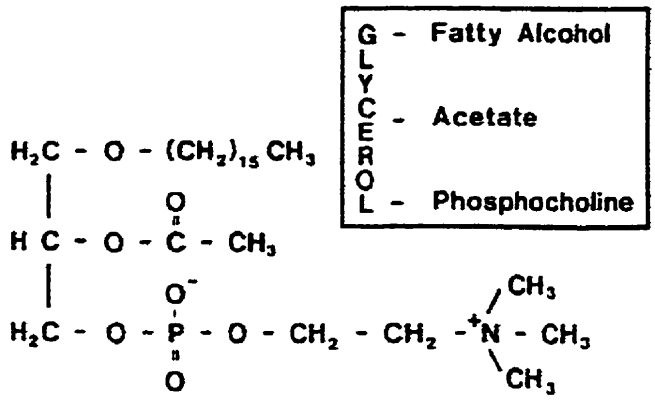


Figure 1.2 Structure of PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) [78]

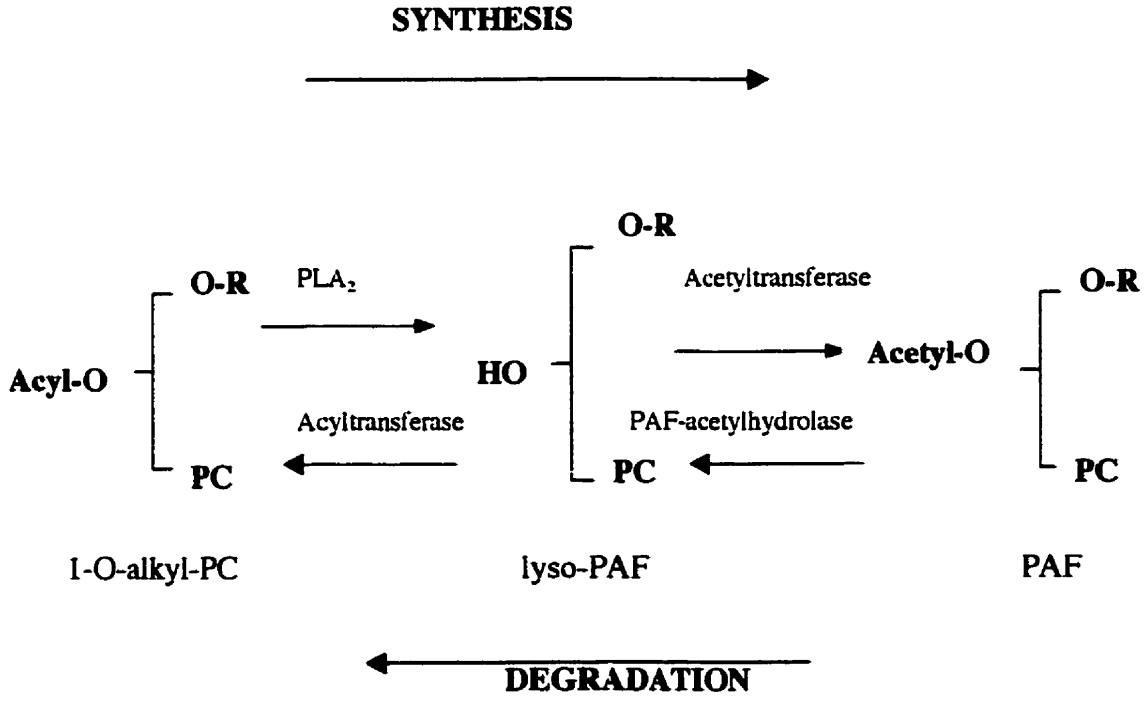


Figure 1.3 Remodeling pathway of PAF synthesis and degradation [78].

Chapter Two

Methods and Materials

2.1 Neutrophil Isolation

Isolation procedures were designed to minimize cellular activation and limit spontaneous shedding of sialoproteins including CD43. There were two isolation methods used. The first method (Isolation I) was used for biochemical characterization of PAF-induced CD43 shedding. Although minimal cellular activation occurs with this method (determined by FACS analysis of L-selectin expression), it has previously been shown that some L-selectin is shed during this isolation procedure. A second method (Isolation II) of neutrophil isolation (which minimized L-selectin shedding) was used to examine leukocyte-endothelial interactions . The presence of red blood cells (RBC) within the preparation of the second method (Isolation II) mimics that of the postcapillary venules, where RBCs help push the neutrophils along the length of the venule. Enough RBCs were removed from the preparation to allow for visualization of the interacting cells.

Isolation I

Briefly, neutrophils were harvested from citrate anti-coagulated blood collected from healthy adult donors and isolated as described [12]. 6% dextran (MW 69000) from *Leuconostoc mesenteroides* (Sigma, St. Louis, MO) was made up in saline and added to equal 1/2 the total volume of blood plus anti-coagulant. This mixture was allowed to stand undisturbed in polypropylene graduated cylinders for an hour to allow sedimentation of the red blood cells. This was carried out at room temperature. The following steps were carried out at 4°C. The supernatant was removed and centrifuged (1150 rpm, 6 min to obtain a pellet containing the leukocytes and some residual contaminating RBCs. Hypotonic lysis was performed with cold double-distilled water to effectively lyse red blood cells and the reaction brought back to isotonicity with potassium chloride (KCl). In between lyses, the

leukocyte rich pellet was washed with Phosphate Buffered Saline (PBS). The leukocyte-rich pellet was then resuspended in a small amount of PBS and layered over Histopaque 1077 (Sigma, St. Louis, MO) and centrifuged at 1500rpm for 30 min at 4°C to separate mononuclear leukocytes from neutrophils. The resultant neutrophil-rich pellet was washed and resuspended in PBS at a concentration of $1x10^7$ cells/ml. Neutrophils were >99% pure and >95% viable.

Isolation II

Human neutrophils were harvested from whole blood in citrate anti-coagulant that had been collected from healthy adult volunteers. Like the previous isolation, neutrophils were purified by dextran sedimentation for an hour, followed by centrifugation on a density gradient of 6% Ficoll Type 400 (Sigma, St Louis, MO) with 10% Hypaque Sodium (Sterling-Winthorp Inc.,Markham, ON). The differences between Isolation Methods I and II are as follows: 1) a heavier dextran was used (MW 250 000; Spectrum Quality products, New Brunswick,NJ) ; 2) the isolation was done at room temperature and 3) there were no hypotonic lysis steps in this isolation. Cells were washed and resuspended in Hanks Balanced Salt Solution (HBSS) at a density of $1x10^6$ cells/ml. Neutrophils represented about 97% of all leukocytes and were 95% viable. All steps were performed at room temperature to minimize L-selectin shedding. This method of isolation was utilized in the parallel plate flow chamber assays, as the presence of red blood cells (RBC) within this preparation mimics that of the postcapillary venules, where RBCs help push the neutrophils along the length of the venule.

2.2 Cytoplast Preparation

Cytoplasts were prepared using a previously published method [85]. Neutrophils were isolated (Isolation I) and suspended ($1x10^8$cells/gradient) in preheated (37°C) 12.5% (w/v) Ficoll-70 with 5µg/ml cytochalasin B. This was then over-layered on a preheated (37°C) discontinuous gradient of 16% and 25% Ficoll-70 and centrifuged for 30 min at 81,000 x g in a pre-warmed (37°C) rotor (Figure 2.1). Cytoplasts were then harvested from between the 12.5% and 16% Ficoll layer, washed in PBS containing 0.5% human serum albumin (HSA) and 10% fetal calf serum (FCS). Surface CD43 expression was measured by FACS analysis, using a panel of anti-CD43 antibodies after treatment with various agonists.

2.3 Preparation of Biological Substrates

Human Umbilical Vein Endothelial cells (HUVEC) were isolated under sterile conditions from human umbilical veins using a standard isolation technique involving collagenase digestion [86]. Briefly, umbilical cord veins were rinsed with 100ml sterile pre-warmed PBS to remove blood clots and subsequently filled with 25ml pre-warmed collagenase (Type 2, Worthington Biochemical Corp., Lakewood, NJ) solution (0.25% in PBS) for 20 min to facilitate detachment of endothelial cells. After incubation, the cords were massaged gently to ensure detachment of endothelial cells. The contents of the umbilical vein were emptied into 20% heat-inactivated FCS (Hyclone, Logan, UT) to deactivate collagenase and the vein was then rinsed with primary HUVEC media M199 (Life Technologies, Grand Island, NY) supplemented with, sodium bicarbonate, Thymidine (Sigma, St. Louis, MO), Hepalean (Organon Teknika, Toronto, ON), Antibiotic-Antimycotic cocktail and Glutamine (both from Life Technologies, Grand Island, NY)

before being washed and plated in T75 flasks pretreated with bovine fibronectin (Biomedical Technologies Inc, Stoughton,MA). HUVEC cells were cultured at 37°C in 5% CO_2 and 100% humidity.

Using this method, the identity of the endothelial cells was confirmed by the presence of human Factor VIII and uptake of acetylated low-density lipoprotein using standard methods. Because HUVEC have been shown to lose their ability to upregulate P-selectin normally [87] only freshly isolated HUVEC (termed primary HUVEC) were used in the static adhesion experiments.

For functional rolling experiments, HUVEC cells were passaged (1-5 times) using Trypsin (0.05%)/EDTA (0.53nM)(Life Technologies, Grand Island, NY) to detach cells from flask. Secondary HUVEC were cultured in media containing reduced levels of Fetal Calf Serum (10%) which was supplemented with endothelial mitogen (Biomedical Technologies Inc, Stoughton,MA). Experiments were also carried out with P- and E-selectin transfectants, generously provided by Dr. Kubes. Glass coverslips were prepared as follows: coverslips were soaked overnight in 0.1% sodium carbonate anhydrous (Na_2CO) and rinsed in double-distilled water at least six times to ensure removal of all traces of anhydrous sodium carbonate. The coverslips were laid out on a tray and placed in a drying oven (350°C) overnight. Carefully, the coverslips were placed between layers of gauze in a metal container and autoclaved to ensure sterility. The day before experiments, HUVEC, P- and E- selectin cells were detached from the flasks and carefully layered on fibronectin-treated coverslips at a density of 1×10^6 cells/ml (HUVEC) and 0.5×10^6 cells/ml for P- and E-selectin transfectants. The cells were allowed to adhere for a couple of hours before appropriate media was added, and then allowed to reach confluence overnight. For

experiments involving HUVEC, varying concentrations of TNF-αwas added to the media and layered over the coverslips for four hours at 37°C to upregulate E-selectin expression.

2.4 Flow Cytometry and FACS analysis

CD43 expression was measured by FACScan analysis (Becton and Dickinson with Cell Quest) using antibodies recognizing a sialyl-dependent epitope(s) of CD43 (IgG_1, DFT1 clone from Serotec). Nonspecific binding was controlled by using matched isotype-specific controls (purified mouse IgG_1, Becton Dickinson). Neutrophils were labeled with a secondary antibody (Goat-anti Mouse-FITC, Becton Dickinson) and CD43 surface expression was determined by using the channel number (log scale) representing the mean fluorescence intensity (MFI) of 10,000 cells. Results were expressed as % shedding and calculated using the following formula:

$$\% \text{ CD43 Shedding} = \frac{\text{MFI control (37°C)} - \text{MFI sample}}{\text{MFI control (37°C)}} \times 100$$

CD43 binding sites were also quantitated with Quantum Simply Cellular Microbeads as previously described from our lab. Temperature-dependent shedding of CD43 was controlled for in all experiments by incubation of cells at 4°C and 37°C.

2.5 Parallel Plate Laminar Flow Assay

A previously published laminar flow chamber system was used to examine neutrophil-endothelial interactions *in vitro* [23] [86]. Monolayers of cells (HUVEC, E-selectin transfectants, and P-selectin transfectants) were seeded at a density of $0.5\text{-}1 \times 10^6$,

and grown to confluence on coverslips pretreated with 0.1% anhydrous sodium carbonate. The coverslips were then assembled onto a polycarbonate parallel-plate laminar flow chamber and the entire chamber mounted on an inverted phase-contrast microscope. The flow chamber was enclosed in a thermostatted (37°C) cabinet. A syringe pump drew the isolated human neutrophils (after shedding of CD43 with HNE, or after control incubations) through the flow chamber at 2 dynes/ cm^2, which is at the lower end of physiological shear for postcapillary venules. See Figure 2.2 for further clarification. A fresh coverslip was used for each experiment. Rolling and adhesive interactions between leukocytes and the monolayer were recorded for subsequent manual analysis via a CCD camera (Hitachi, Japan) and a video cassette recorder (Panasonic, Syracuse, New Jersey). Rolling cells were visualized as bright cells that moved across the monolayer, while adherent cells were cells that remained stationary for more then 10 secs. Neutrophil-endothelial interactions were recorded the last 10 secs of every minute for a total of 5 mins.

2.6 Neutrophil Static Adhesion Assay

Experiments were conducted as previously described [12] with the following modifications. Primary HUVEC ($2.5x10^5$ cells/ml) were seeded onto a fibronectin-coated 3.5 cm sterile petri dish and grown to confluence, then stimulated with 2U/ml thrombin (Sigma, St. Louis, MO) for 10 min at 37°C. Thrombin, a serine protease, is part of the coagulation cascade and is known to upregulate PAF expression on endothelial cells [34, 36, 83]. This concentration of thrombin has been used previously in similar assays to upgregulate PAF expression on endothelium. Also, previous studies from our lab have shown that a wide range of thrombin concentrations (0.01 to 100U/ml) does not shed

neutrophil CD43. Neutrophils ($5.5x10^6$ cells/ml) were carefully layered onto thrombin-stimulated monolayer of HUVEC. Neutrophils were allowed to adhere for 10 min then non-adherent neutrophils were removed. Adherent neutrophils were removed by incubation for 2 min with 2mM EDTA (BDH Chemicals, Toronto, ON). Both adherent and non-adherent neutrophils were enumerated by manual counting on a hemocytometer and assessed for CD43 expression via FACS. In some experiments, adherent cells were also visualized through a microscope and counted as fields/view.

2.7 Calcium Flux Assay

Experiments were conducted as previously described [88]. Neutrophils ($1x10^7$ cells/ml) were loaded with the intracellular fluorescent dye Fura-2-am (Molecular Probes) (1mM) for 45 min at 37°C by gentle agitation, then washed 3 times in HBSS and recounted. Neutrophils were then saturated with calcium ($CaCl_2$, 100mM) and samples run on a SPF500 spectrofluorometer to establish a baseline. Settings for the flourometer were as follows: excitation 339nm, emission 510nm and bandpass at 5 and 10 nm, respectively, which were determined in Slow Time-Based Acquisition. Neutrophils were activated with various concentrations of PAF (10^{-6}-10^{-9}M). Fluorescence readings were obtained at this step (F). The cells were then lysed with 1% Triton X to release intracellular calcium and flourescence readings were again measured at this step (F_{max}) A third fluorescence measurement was taken after the cells were quenched with Tris / EGTA (3M) (F_{min}). Calculations were done using the following formula:

$$[Ca^{2+}]_i = 224nM\ (F-F_{min})/(F_{max}-F)$$

2.8 PAF Measurement Assay

Experiments were conducted as previously described [77, 89]. Monolayers of primary HUVEC were grown to confluence on 35mm TC petri dishes. Media was removed from the monolayer and replaced with 1ml HBSS/10mM HEPES (pH7.4) containing 25 μCi carrier- free ^{3}H Acetate and Thrombin (2U/ml) for 10 min at room temperature. The interactions were stopped with 0.5ml of 50 mM acetic acid in methanol. Cells in acidified medium were scraped off, and the dishes rinsed twice with 1ml methanol. Carrier PAF (10 nmol) and 1.25 ml chloroform were added to all samples and vortexed to form a monophase. To split the phases, 1.25 ml chloroform and 1.25 ml of 0.1M sodium acetate in water was added. The samples were vortexed, then centrifuged at 800rpm for 10 min. The lower organic phase was collected and washed three times with 4.75ml methanol/0.1M sodium acetate/water (2.5/1.25/1,v/v) and evaporated under N_2, then resuspended in chloroform/ methanol (9:1). An aliquot of the recovered lipid was then used to determine total amount of radioactivity present, the remainder fractionated by Thin Layer Chromatography (silica gel G, heat-inactivated) and developed in chloroform /methanol/ glacial acetic acid/ water (50:25: 8:4). The silica was scraped in fractions corresponding to authentic standards from the entire lane of the TLC plate and radioactivity in each fraction was determined by liquid scintillation spectroscopy.

2.9 Statistics

Data results are expressed as the arithmetic mean $\pm$ standard error of the mean. Comparisons between data groups were done by the Student-T test. Statistical significance was defined as $P < 0.05$.

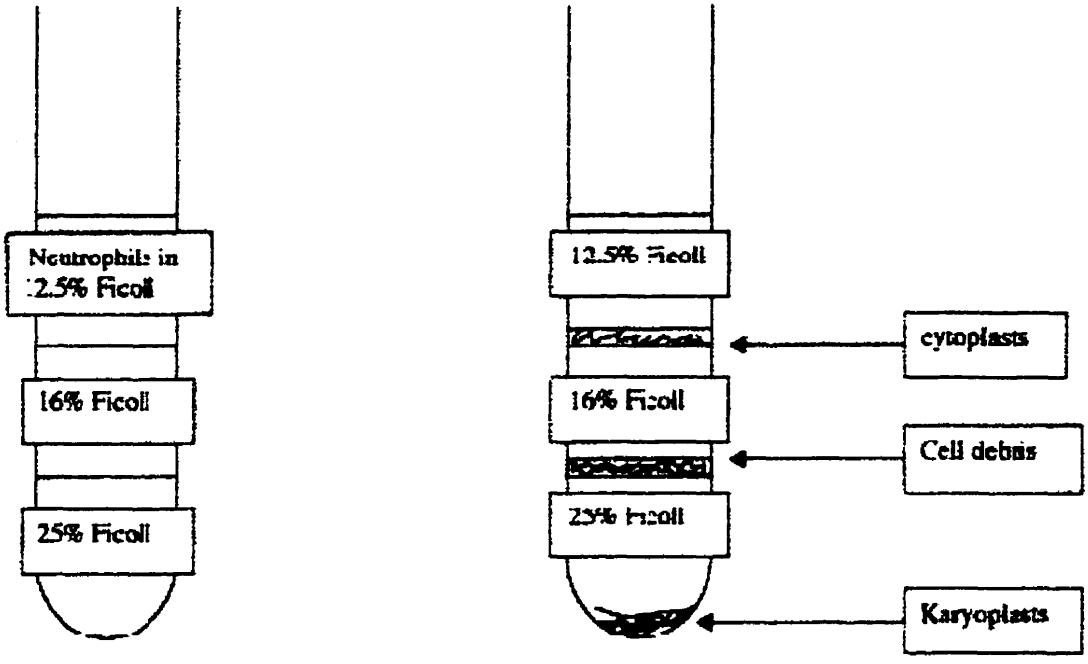
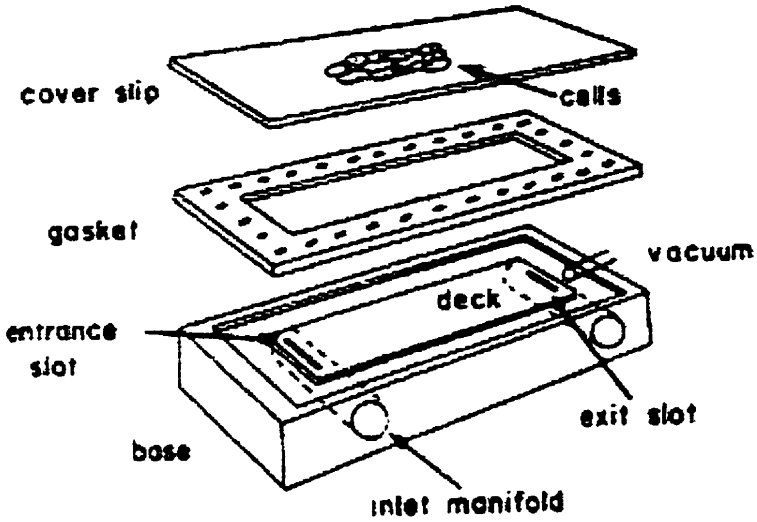


Figure 2.1 Neutrophil cytoplast formation. Neutrophils were layered on discontinuous gradients and centrifuged for 30 min at 81,000 x g. Neutrophils travel up and down the gradients. Heavier material (nuclei) stay on the bottom and because the membranes are fluid, the granules exit from within the neutrophils and with the consequence being formation of cytoplasts, agranular, anucleate structures, basically, plasma membrane surrounding neutrophil cytosol [85].

A



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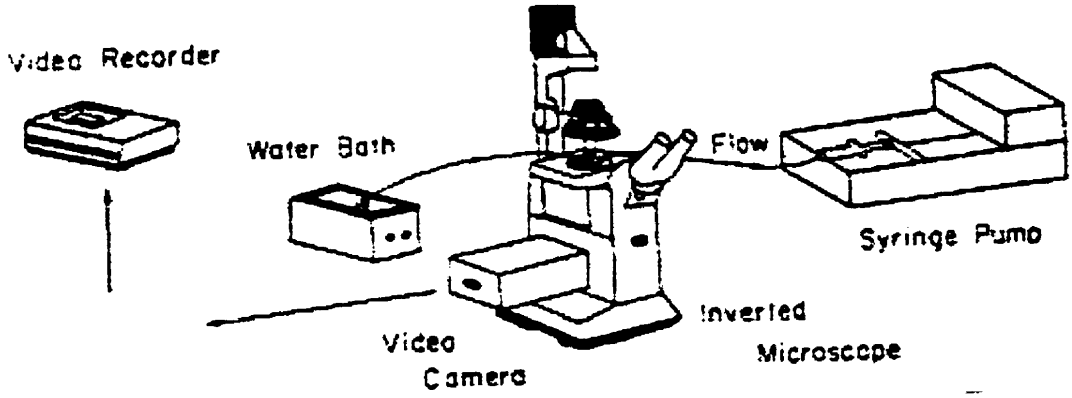


Figure 2.2 Panel A illustrates the polycarbonate parallel plate laminar flow chamber in detail. Confluent monolayers of cells are grown on coverslips and assembled onto the chamber. Panel B illustrates the assembly setup for the parallel plate laminar flow assay. Pretreated neutrophils are drawn through the flow chamber by a syringe pump. Interactions taking place at the monolayer are recorded to a video cassette recorder via a CCD camera attached to the inverted phase contrast microscope for manual analysis at a later time [23].

Chapter Three

The effects of exogenous, soluble PAF on human neutrophil CD43 expression *in vitro*

The first published report of CD43 shedding on neutrophils was by Campanero *et al.* in 1991, utilizing TNF-α as an agonist [65]. In their experiments, they illustrated that TNF-α decreased CD43 and CD44 expression, as well as L-selectin while increasing the expression and avidity of CD11b/CD18 on neutrophils. They also showed that various protease inhibitors such as PMSF and aprotinin were capable of blocking CD43 downregulation by TNF-α. Subsequent reports indicated that CD43 could be downregulated by a variety of agonists including PMA, fMLP and LPS [53].

Generally, the agonists used to shed CD43 were soluble as opposed to membrane-bound and some were even non-physiological (e.g. PMA). Furthermore, the effects of these agonists on CD43 expression were only investigated using cells in suspension. Because we propose that one function of CD43 is anti-adhesive, we surmised that it would be important to examine the mechanism by which CD43 shedding affects neutrophils interacting at the endothelial interface and what is the potential role of membrane-bound physiological agonists that are expressed at this interface.

We postulated that there might be membrane-bound physiological agonists capable of downregulating surface CD43 at the neutrophil-endothelial surface. Several potential physiological agonists may fulfill this role but there are two in particular that are immediately obvious. Both PAF and Interleukin-8 (IL-8) are chemoattractants upregulated on the surface of activated endothelial cells that are capable of neutrophil activation [84, 90]. Therefore, it is quite likely that both might affect CD43 expression and, if CD43 is anti-adhesive, shedding by these agonists could be very important in promoting neutrophil infiltration into tissues.

One proposed sequence of events taking place in the recruitment of neutrophils into tissues would be as follows. Neutrophils tether and roll on the activated endothelium. In the process, they encounter membrane-bound agonists, become activated, and as a result, degranulate. Degranulation causes CD43 shedding from neutrophils, which become firmly adherent to the endothelium. These cells then further emigrate out of the post- capillary venules. It has been well established that activation of endothelium results in juxtacrine endothelial expression of P-selectin and PAF [21, 83, 91]. The role of CD43 may be to act as a non-specific, anti-adhesive molecule impeding activation of the cell. When neutrophils are activated, degranulation occurs, resulting in granule translocation to the surface. This releases proteases that are capable of CD43 shedding. This in turn results in a charge reduction and removal of steric hindrance. Such a charge reduction might limit the barrier effect on cell adhesion imparted by CD43, thus allowing increased interactions of activated leukocytes with endothelium. At the time these studies were performed, there had been no published reports regarding the ability of PAF to downregulate CD43 on neutrophils.

3.1 Results

3.1.1 Evaluating the ability of soluble PAF to affect CD43 expression

To address the question, whether exogenous PAF is capable of downregulating CD43 on human neutrophils, a stepwise approach was taken. An initial dose-response study of the effects of exogenous soluble PAF (10^{-5} to 10^{-9} M) on neutrophil CD43 expression was undertaken. The purpose was to evaluate the ability of PAF to mediate CD43 shedding on human neutrophils. Figure 3.1 illustrates the results of the PAF dose response on human neutrophil CD43 expression. Neutrophils (1×10^6 cells) were pre-incubated for 30 min at 37°C with the following neutrophil agonists: PMA (0.1μM), fMLP (10μM) in the presence or absence of DHCB (5.2μM) and PAF (10^{-8} to 10^{-5}M). Following incubation, the agonists were removed with excess PBS and the neutrophils stained for the presence of CD43 by FACS analysis (as outlined in Chapter 2). Temperature-dependent shedding was examined in each experiment, whereby neutrophils incubated at 37°C shed CD43 minimally (approximately 10-20% decrease). This was taken as a baseline to exclude temperature-dependent shedding as a variable in the experiments.

PAF was capable of mediating shedding CD43 at various doses. There was minimal CD43 shedding at 10^{-8} M ($28 \pm 4\%$) and 10^{-7} M ($20 \pm 3\%$), with significant shedding seen at the higher concentrations of PAF. PAF (1μM)($60 \pm 2\%$ shedding) was chosen as the concentration for all subsequent studies and was comparable to

previously reported studies examining the effects of PAF on various neutrophil effector functions [56, 92].

The phorbol ester PMA, a potent agonist of secondary granule translocation [59, 92] was capable of shedding 87 ± 1% of surface CD43. This is consistent with other reports examining CD43 shedding on neutrophils. Although a potent activator of neutrophils, PMA is limited in that it is a non-physiological agonist often utilized to study maximal neutrophil responses.

fMLP, a bacterial cell wall oligopeptide capable of activating neutrophils, shed CD43 from neutrophils (29 ± 4%). Unlike PMA, fMLP is a physiological agonist. The presence of DHCB, a microfilament inhibitor, significantly increased CD43 shedding from neutrophil surface (79 ± 4%). This suggested that neutrophil granules were essential in the shedding of CD43. This result is anticipated as inhibition of microfilaments prevent fMLP receptor recycling at the surface of the cell, and results in a continual stimulation of the fMLP receptor [93]. The results we obtained with respect to other agonists (PMA, fMLP / DHCB) are comparable to that previously reported [53].

A time course of CD43 shedding was also done to determine the optimal duration of incubations with various agonists. In Figure 3.2, neutrophils were incubated in the presence of various agonists for 5, 10, 30 and 60 min at 37°C. PAF at $1x10^{-6}$ M concentrations was utilized for these studies. In all conditions except for the temperature-dependent condition, maximal shedding occurred by 10 min and persisted for 30 min.

To confirm that PAF-mediated CD43 shedding occurred in association with cellular activation, the effects of various concentrations of PAF (10^{-6} to 10^{-9} M) on intracellular Ca^{2+} signaling was undertaken as outlined in Chapter 2. The cytosolic free calcium concentration $[Ca^{2+}]_i$ is an important determinant of cellular activity and plays a central role in receptor mediated intracellular signaling events[84]. Various concentrations of PAF (10^{-9} to 10^{-6} M) and fMLP (10μM) induced a rise in intracellular calcium uptake within neutrophils, over and above resting neutrophils (Figure 3.3) consistent with cellular activation.

In these experiments, PAF (10^{-9} to 10^{-6} M) was capable of activating the neutrophils, indicating that PAF-mediated CD43 shedding occurred in association with cellular activation. The results obtained here are in agreement with those previously published by other researchers as the acceptable range for free intracellular calcium concentrations in resting cells (50-60nM) with a maximum of 400nM after stimulation. [94].

3.1.2 The importance of neutrophil granules in the downregulation of CD43 on neutrophils

To confirm the importance of neutrophil granules in PAF-mediated CD43 shedding on neutrophils, CD43 shedding on neutrophil cytoplasts was examined. Cytoplasts are agranular, anucleate neutrophils that we have demonstrated express CD43 on their surface (Figure3.4). As cytoplasts are agranular, they are ideal to confirm the importance of neutrophil granules on CD43 downregulation. In these experiments PMA (0.1μM), which elicits secondary granule translocation had no effect on cytoplast CD43 expression (Figure 3.5). This was also seen with PAF ($1x10^{-6}$ M) in

which CD43 shedding (63 ± 6%) was observed on intact neutrophils, in comparison to shedding of CD43 on cytoplasts (24 ± 8%).

However, exogenous proteases such as HNE and neuraminidase (sialidase) are still capable of shedding CD43 from cytoplasts. Previous research has shown that HNE (1μg/ml) is capable of shedding CD43 without any cellular activation [12]. HNE cleaves CD43 at the protein core, close to the surface of the plasma membrane while sialidase (0.02U/ml) is capable of removing sialic acid residues from the surface of neutrophils [95]. As would be predicted with these two exogenous proteases, there is comparable CD43 shedding between intact neutrophils and cytoplasts. These experiments outline the importance of neutrophil granules in the downregulation of CD43. They also confirm that cytoplast CD43 is still capable of being proteolytically shed and has not become resistant to proteolytic shedding due to cytoplast preparation. Furthermore, these results demonstrate that CD43 shedding is not mediated by proteases present within the plasma membrane of neutrophils [96].

3.1.3 Examining the ability of IL-8 to modulate CD43 expression on neutrophils

To examine the effects of IL-8 on CD43 downregulation, a dose-response study of IL-8 on neutrophil CD43 was undertaken. Neutrophils ($1x10^6$ cells) were pre-incubated with various concentrations of IL-8 (10^{-8} to 10^{-11} M) (Figure 3.5). At these concentrations of IL-8, upregulation of CD11b was seen, indicating that neutrophils were capable of being activated and that the IL-8 used was functional (data not shown). These results corroborate an earlier report, that IL-8 does not have any significant effects on CD43 downregulation [95].

3.2 Discussion

To date, there have been no studies reported regarding the effects of endothelial membrane-associated physiological agonists on neutrophil CD43 expression. The compelling reason to investigate these agonists is that endothelial-bound molecules such as IL-8 and PAF are upregulated following activation of the endothelium [21, 32, 33, 37] and are more relevant *in vivo* than soluble agonists. This upregulation provides a mechanism whereby leukocytes interacting with the endothelium are capable of being activated. Activation of the neutrophil results in granule translocation to the plasma membrane where CD43 is proteolytically shed and, if anti-adhesive, potentially facilitates closer interaction between neutrophils surface adhesion molecules and their respective endothelial ligands. These studies demonstrated for the first time that soluble exogenous PAF is capable of CD43 downregulation whilst exogenous IL-8 had no effect on CD43 downregulation.

In these sets of experiments, we have evidence illustrating that PAF is capable of downregulating CD43 from the surface of neutrophils in a rapid manner and in association with cellular activation. These results are similar to what has been reported for fMLP [53]. These experiments also indicate that neutrophil granules are crucial in CD43 downregulation.

The importance of protease(s) within neutrophil granules in CD43 shedding is further underscored by experiments with cytoplasts. These results also suggest that the protease(s) shedding CD43 are not located within the plasma membrane. Previous studies utilizing protease inhibitors to block CD43 shedding have been somewhat successful at blocking CD43 shedding [95, 97]. Experiments using the serine protease

inhibitor, PMSF blocked downregulation of CD43 by various agonists [53, 65] whereas others have suggested that metalloproteases are responsible for CD43 shedding[97]. It remains unknown which protease(s) are responsible for CD43 downregulation and it is possible that more than one enzyme may be responsible for CD43 shedding. Recent work from our lab suggest that the proteases responsible for CD43 downregulation are localized to the secondary granules [98].

PAF, a mediator expressed on activated endothelium is an ideal candidate to downregulate CD43 on the surface of neutrophils. Its action is swift; it activates neutrophils, causes granules to be translocated to the surface, which in turn release proteases that are capable of downregulating CD43. Reduced CD43 expression results in decreased negative charge, rendering the cell more adhesive and possibly revealing cryptic molecules that would now be free to interact with ligands on the endothelial cells, theoretically promoting increased cell-cell interactions. *In vivo* studies performed on mice with CD43-deficient leukocytes demonstrated increased rolling and adhesion compared to leukocytes from wild-type animals [73]. This is consistent with a potential anti-adhesive role for CD43 on leukocytes during neutrophil-endothelial interactions. However, it must be emphasized that with CD43-deficient mice in which there is complete absence of CD43, this may not be physiological. In these experiments the effects of partial or incomplete shedding of CD43 were examined. It is likely that partial shedding is the more relevant physiologic setting because there is a population of residual CD43 that is insensitive to downregulation [13].

There is also evidence that residual CD43 is required for the emigration process. Further experiments with CD43-deficient animals indicated that leukocytes adhered at a

higher rate, although they failed to emigrate optimally into tissues [73]. It is possible that following partial shedding of CD43, this residual CD43 functions to promote neutrophil emigration. Perhaps even 30% CD43 shedding is sufficient to decrease the negative surface charge and in turn, enhance adhesion and promote increased neutrophil endothelial interactions. We observed this at 10^{-8} M PAF. Although not statistically significant, this level of CD43 shedding is consistent with CD43 shedding by other physiologically relevant agonists (eg fMLP). It is also plausible that the area of plasma membrane from which CD43 shedding had occurred influences neutrophil-endothelial interactions. Studies with immunogold labeling have shown that there is a marked decrease of CD43 at points of cellular contact [99] as well as the phenomenon of CD43 capping, the redistribution of CD43 molecules to the uropod of the cell [100, 101].

3.3 Limitations

Although the micromolar concentrations of PAF utilized in our experiments to downregulate CD43 are high compared to that observed for the other physiological effects of PAF on neutrophils, utilization of micromolar concentrations of PAF has been required by some investigators to study PAF effects [56, 92]. One explanation to account for this difference may be the underestimation of PAF concentrations in our experiments. PAF is a very polar lipid and it binds avidly to certain surfaces, in particular, glass and polypropylene [102]. To remove PAF from these surfaces, it may be necessary to use a protein carrier such as human serum albumin [102]. This option was not available to us as it had been previously demonstrated by us (data not shown)

and others [8] that albumin inhibits CD43 downregulation on neutrophils. Instead, siliconized glass tubes were used to perform these experiments. Although it has been previously reported that PAF exerts its effects at sub-nanomolar concentrations, the actual concentration of endothelial-bound PAF at the site of cell-cell contact has not been measured. Therefore, it is possible that the concentrations used in my experiments (10^{-9} M) may in fact be within the physiological range.

3.4 Summary

The results obtained in this set of experiments indicate that PAF as a soluble agonist is capable of rapidly downregulating CD43 on human neutrophils. Treatment of the neutrophils with PAF not only results in the downregulation of CD43, but also in cellular activation. It is therefore reasonable to hypothesize that endothelial-bound PAF may have a similar effect on neutrophil CD43 expression.

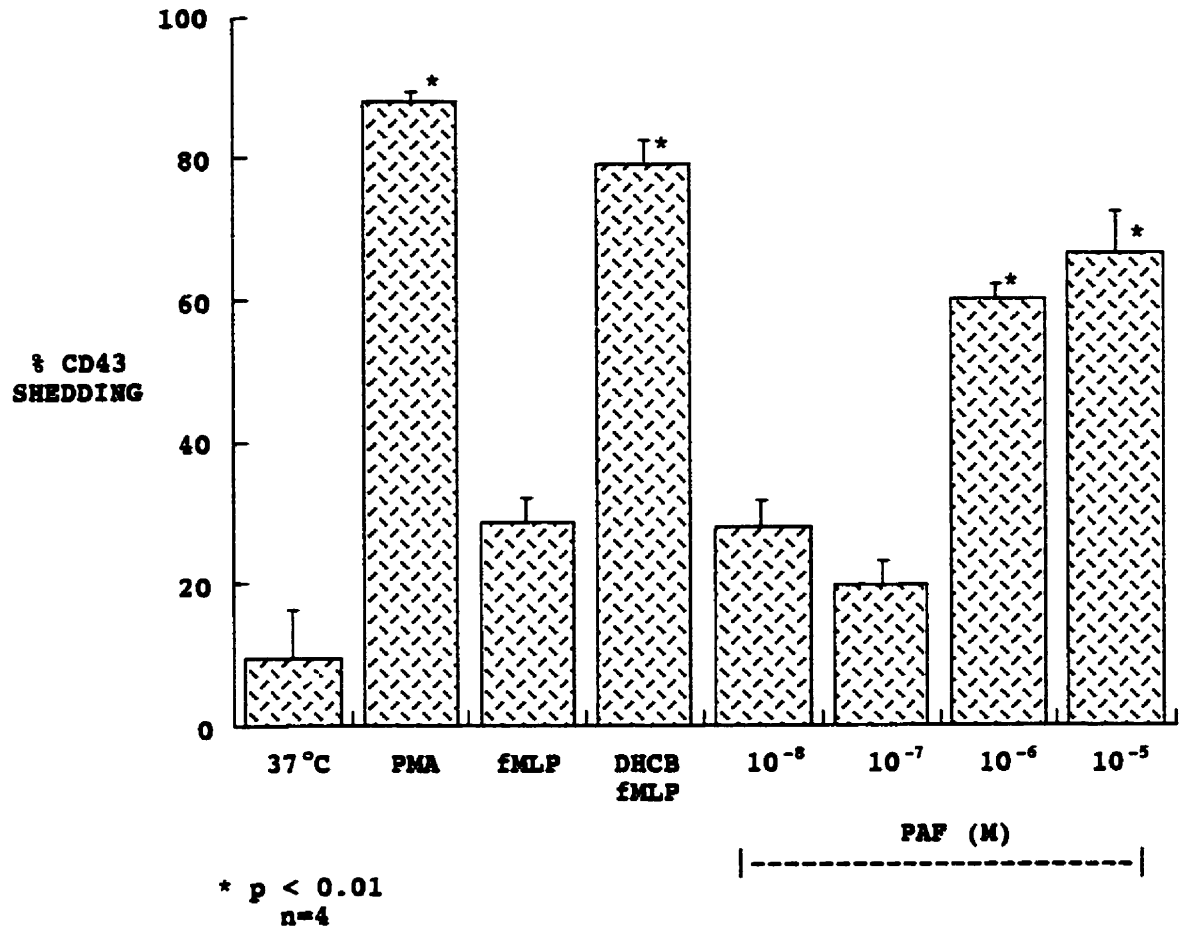


Figure 3.1. The effects of PAF on neutrophil CD43 shedding. Neutrophils were incubated with PAF, and other agonists, for 30 min at 37°C to determine CD43 downregulation. PAF at a concentration greater than $1x10^{-6}$M induces significant CD43 shedding from human neutrophils.* comparing PAF and other agonists to temperature-dependent shedding, $P < 0.01$

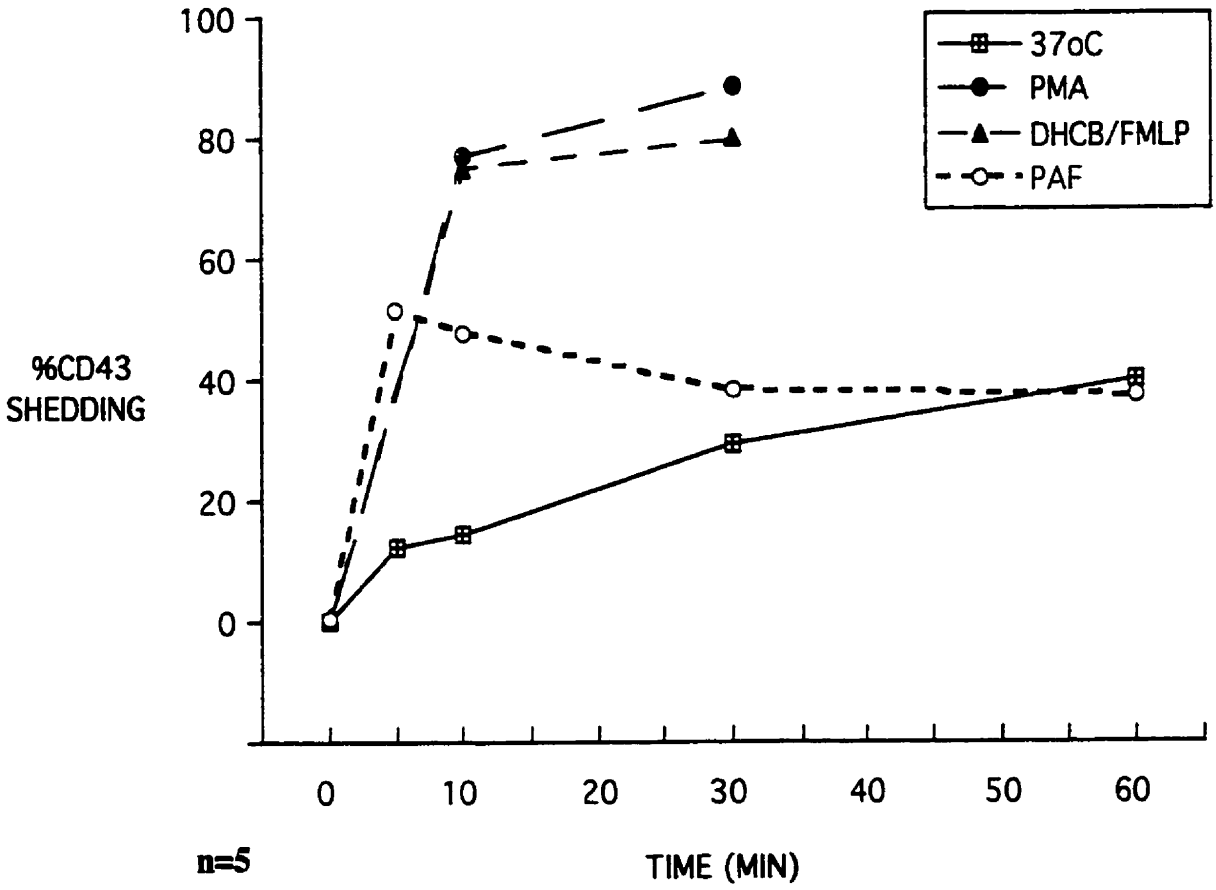


Figure 3.2. Time course of CD43 shedding from human neutrophils. Neutrophils were assessed for the presence of CD43 after pre-incubation with various agonists at several time points. Experiments for PMA and DHCB/fMLP were not carried out beyond 30 min. From this experiment, 30 min was chosen as the optimal incubation time for neutrophils with PAF.

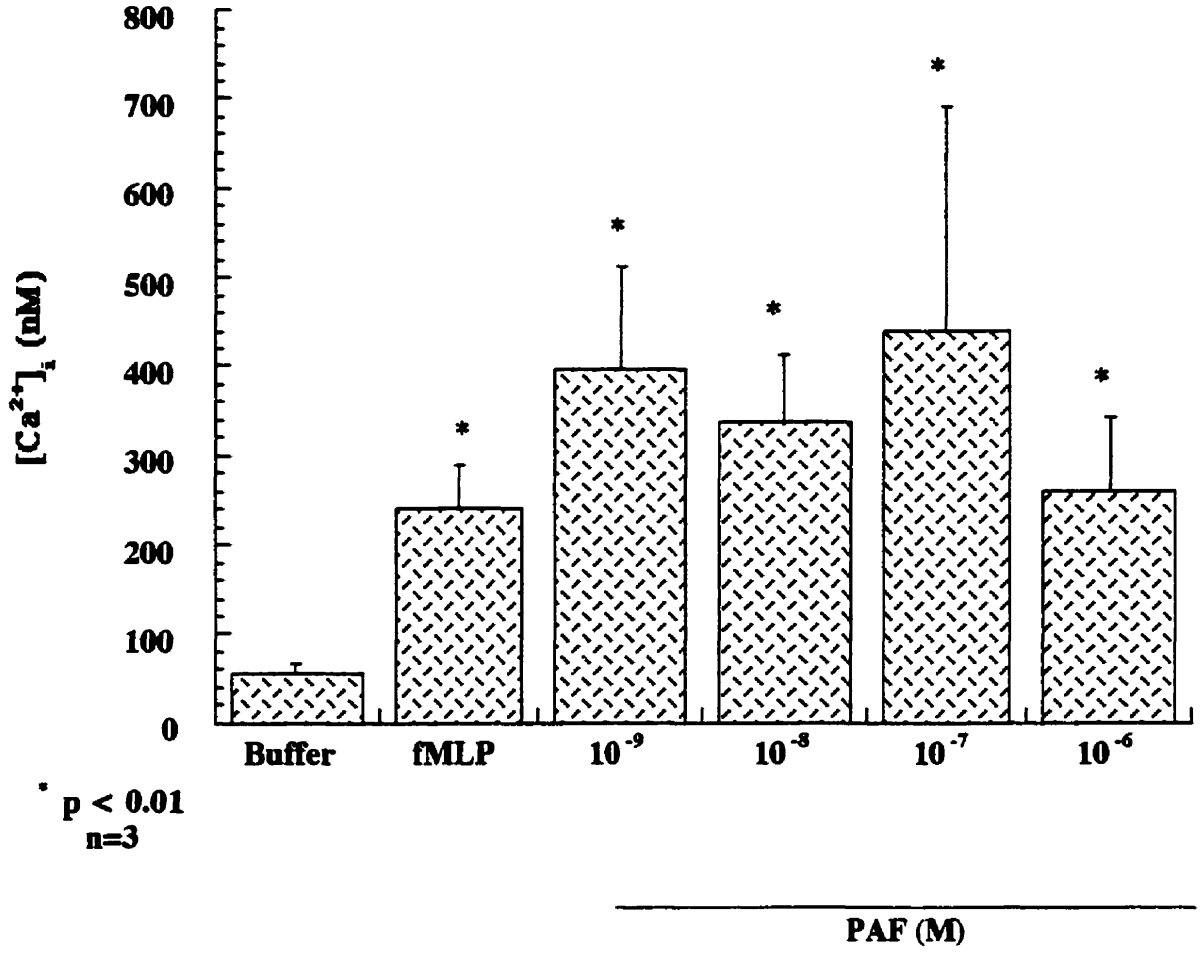


Figure 3.3 Intracellular neutrophil calcium flux in response to bacterial oligopeptide, fMLP(10μM) and to a range of PAF concentrations (10^{-6} to 10^{-9} M). * comparing various conditions to buffer, $P < 0.01$

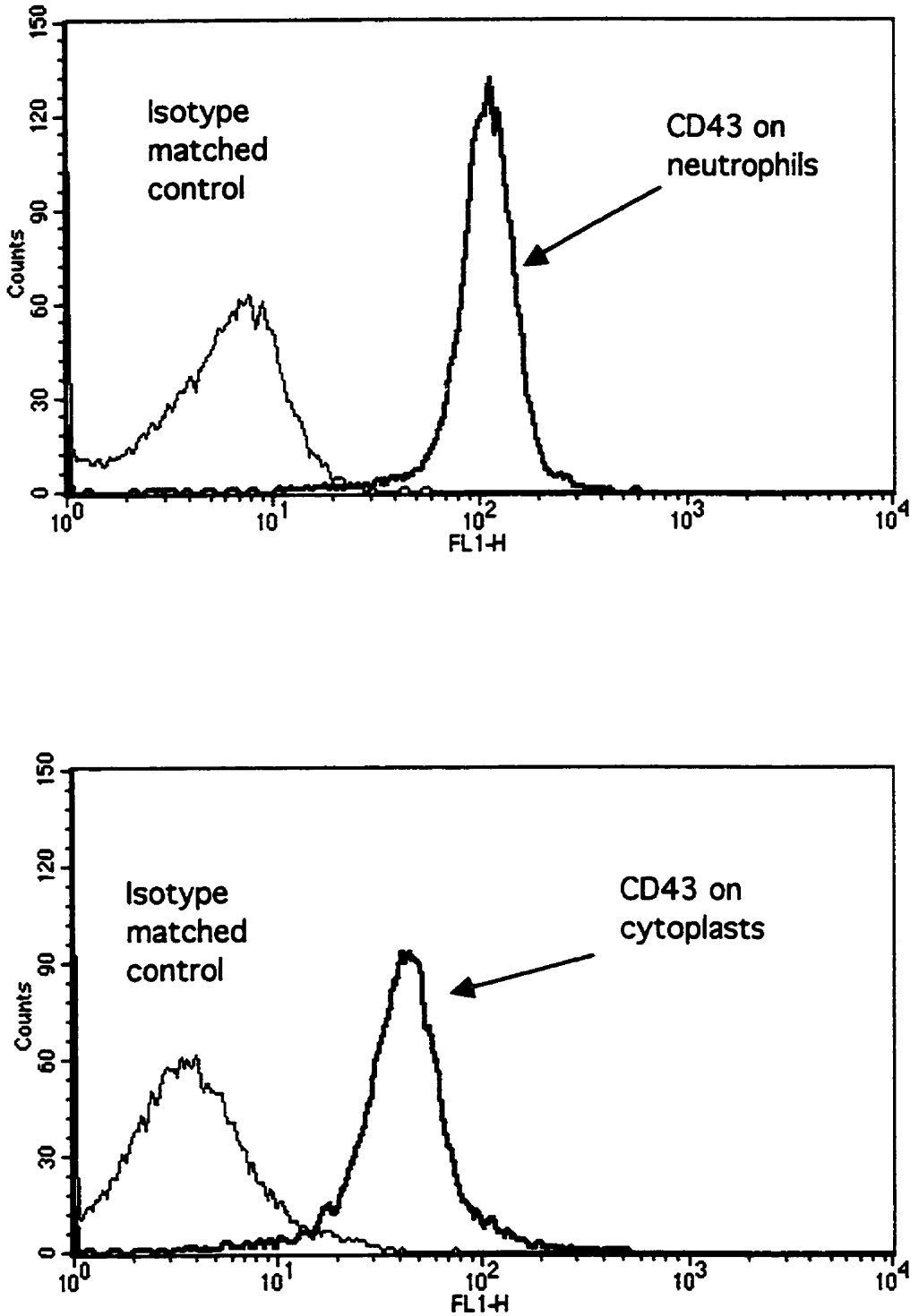


Figure 3.4 A representative FACS histogram demonstrating the expression of CD43 on neutrophils and cytoplasts.

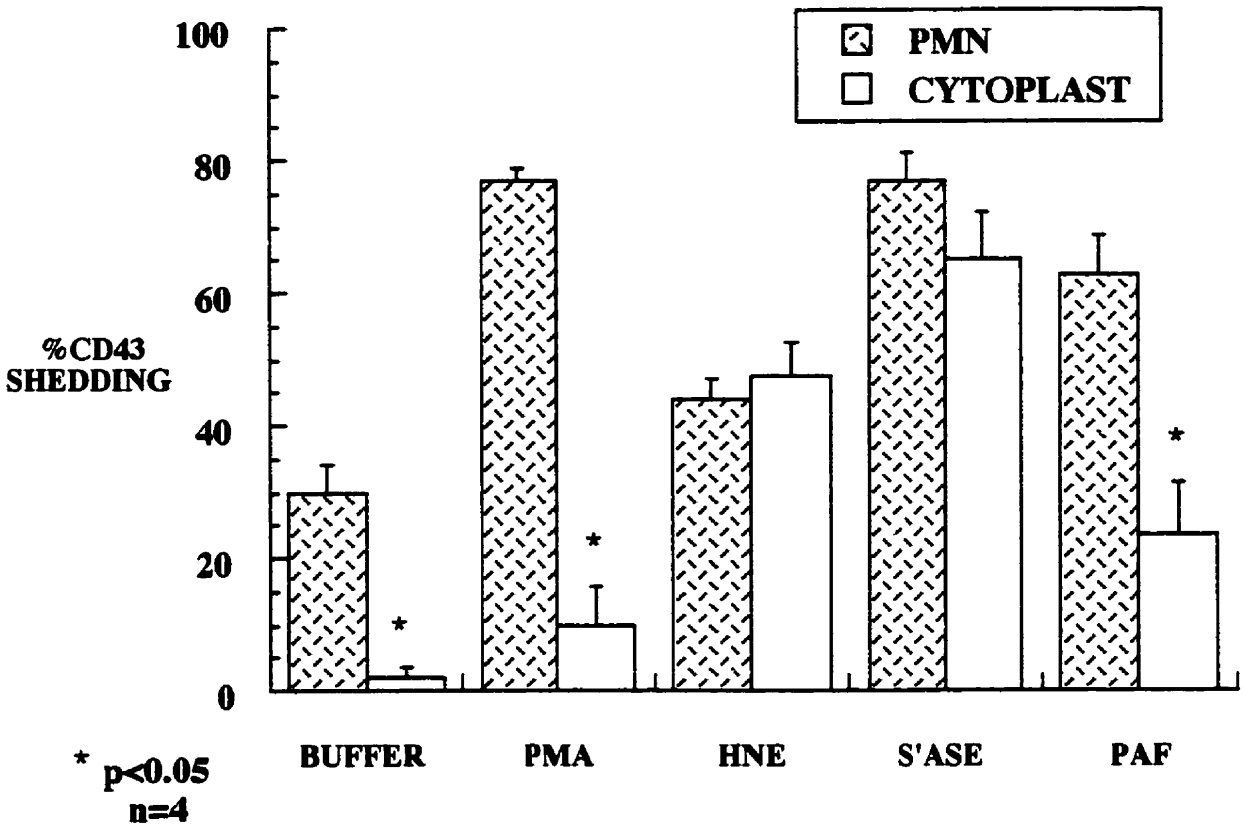


Figure 3.5 CD43 downregulation on neutrophils and cytoplasts. PMA (0.1μM) and PAF (1μM) had no effects on cytoplast CD43 shedding whilst exogenous proteases such as sialidase (S'ase, 0.02U/ml) and HNE(1μg/ml) were capable of downregulating CD43 from intact neutrophils and cytoplasts. *comparing neutrophils and cytoplasts, $P < 0.05$

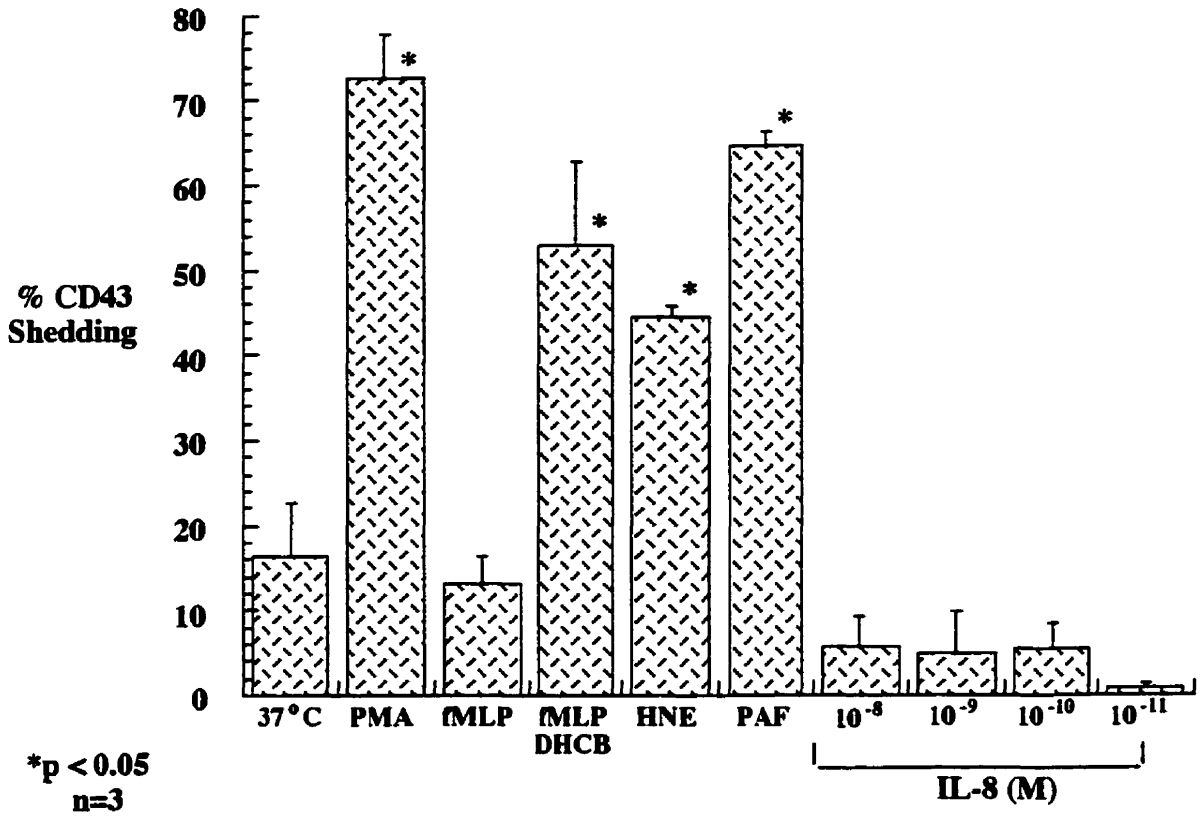


Figure 3.6 Experiments examining the effects of various concentrations of IL-8 on CD43 downregulation. PMA (0.1μM) , fMLP (10μM) / DHCB (5.2μM) , HNE (1μg/ml) and PAF (1μM) are all capable of modulating CD43 on neutrophils. A range of IL-8 (10^{-8} M to 10^{-11} M) did not induce CD43 shedding. * comparing the various agonists to temperature-dependent control, $P < 0.05$

Chapter Four

The effects of endothelial-derived, membrane-bound PAF on human neutrophil CD43 expression

In the previous chapter, we demonstrated that soluble, exogenous PAF is capable of shedding CD43 from human neutrophils in suspension. To determine the physiological potential of PAF-mediated CD43 shedding, the next step was to evaluate the relevance of endothelial-derived, membrane-bound PAF on CD43 shedding from neutrophils interacting at the endothelial interface. If PAF-mediated shedding of neutrophil CD43 is to be relevant, it would occur in this form.

Previous research has demonstrated that PAF and P-selectin are upregulated on endothelium exposed to proinflammatory stimuli such as thrombin, histamine or hydrogen peroxide [21, 33]. This is a model for juxtacrine activation, where P-selectin mediates rolling interactions whilst PAF activates rolling leukocytes, causing cells to halt and firmly adhere to the endothelium. To date, the effect of endothelial-derived PAF on CD43 shedding has not been investigated. In this chapter, we aim to demonstrate that thrombin-stimulated endothelial -bound PAF is capable of downregulating CD43 on neutrophils interacting with activated-endothelium.

4.1 Results

To ensure that we were able to upregulate membrane-bound PAF on activated HUVEC, we reproduced experiments that had been done by others ([21, 33, 91]. Primary HUVEC were grown in six well plates and stimulated with thrombin (2U/ml) to upregulate membrane-bound PAF (Figure 4.1). Although thrombin is a serine protease, previous studies from our lab have revealed that thrombin (0.01-100U/ml) does not induce CD43 shedding from human neutrophils. Thrombin was washed off and PAF extraction was

carried out as described in Chapter 2. Maximal PAF expression was seen at 5 min where there was a significant increase in the incorporation of ^{3}H-Acetate, as indicated on the vertical axis of Figure 4.1. By 10 min, the incorporation of ^{3}H acetate had fallen from 3500 dpm to 1400 dpm, which was still a significant increase over the background counts of 200 dpm. These experiments confirm that primary HUVEC are capable of expressing membrane-bound PAF.

The next set of experiments tested the effects of EDTA on neutrophils in suspension. The rationale behind these experiments was to ensure that EDTA, which was used to detach the adherent neutrophils from activated endothelium did not influence CD43 expression. EDTA, a divalent cation chelator, can act as a protease inhibitor and may thus interfere with CD43 shedding [55]. Figure 4.2 illustrates the results of this set of experiments. Neutrophils in suspension were incubated with various agonists, either in the presence or absence of EDTA, followed by FACS analysis for CD43 expression. The data clearly indicate that the presence of EDTA at the concentration (2mM) studied, did not influence CD43 shedding by the various agonists. There was no difference in the CD43 shedding on neutrophils incubated with or without EDTA. This also suggests that the neutrophil proteases involved in CD43 shedding are not EDTA-inhibitable.

In the following set of experiments, human neutrophils were allowed to adhere statically to thrombin-activated endothelium. Non-adherent cells were washed off, then the adherent cells were detached from the activated endothelium with 2mM EDTA which from the previous experiment , does not affect CD43 expression. Adherent and non-adherent neutrophils were tallied, and further assessed for CD43 expression via flow cytometry.

Table 4.1 demonstrates that thrombin-activated HUVEC supports increased adhesion of neutrophils. In all of the experiments, we see an increase in the number of neutrophils adherent to activated endothelium (1.5 to 7-fold increase). Figure 4.3 is a representative experiment of results gathered by visualizing adherent neutrophils on HUVEC. There were minimal (<50) adherent cells on unstimulated endothelium. In contrast there were more than 150 adherent cells on thrombin-activated endothelium at 5 min and 200 adherent cells at 10 min. This represents a three-fold increase in adherent cells at 5 min and a four-fold increase at 10 min.

Neutrophils from the adherent and non-adherent populations collected during the above assaywere assessed for surface CD43 expression. Figure 4.4 is a representative FACS histogram depicting CD43 expression on adherent and non-adherent neutrophils following a static adhesion assay. The top panel illustrates results from neutrophils that were allowed to adhere to unactivated endothelium. The middle and bottom panels are histograms following HUVEC activation by thrombin at 5 min and 10 min respectively. There is a significant decrease in the amount of CD43 present on neutrophils that were adherent to the endothelium when compared to cells that had not been adherent. On all three panels, we see the same trend; there is a shift to the left on histograms of adherent neutrophils. This suggests an association between adhesion to the endothelium and decreased CD43 expression on neutrophils.

Table 4.2 summates the results of these experiments. We can see that adherent neutrophils had decreased CD43 on its surface and in all the experiments carried out, those cells that adhered to endothelium shed between 30-59% of CD43,while CD43 expression on non-adherent cells was unchanged (data not shown).

4.2 Discussion

The finding that thrombin-stimulated endothelium upregulates PAF has been reported by others [34, 77, 83]. Our results (Figure 4.1) are consistent with previously published results. Others have shown that activated HUVEC express PAF and P-selectin [21, 33]. To exclude any effects of EDTA used in the detachment of adherent cells from HUVEC, CD43 shedding experiments were carried out in the presence of EDTA and the various agonists under investigation including PAF. Although there was potential that EDTA may influence CD43 downregulation because it is a divalent cation chelator, results from these experiments indicate that EDTA does not attenuate CD43 expression on neutrophils. We also observed that neutrophils adhered to thrombin-stimulated HUVEC in a static adhesion assay. This finding also, has been previously reported. The results we obtained (2-6 fold increase of adhesion seen with stimulation of HUVEC) were similar to previously published reports [34, 83, 91].

It was not possible to distinguish if membrane-bound PAF was capable of mediating CD43 shedding from neutrophils as the very act of adhesion was sufficient to induce CD43 shedding from neutrophils. Table 4.2 demonstrates that with unactivated HUVEC, neutrophil adhesion was sufficient to mediate 41% CD43 shedding, whilst adhesion to thrombin-activated HUVEC mediated 50% CD43 shedding. Although there is no significant difference between CD43 shedding from neutrophils adherent to unactivated and activated HUVEC, a 10% increase in CD43 shedding was observed and it is plausible that this increase is mediated my PAF. PAF, whilst activating neutrophils at the neutrophil-endothelial interface, also causes translocation of granules

to the surface of cells, and may cause the shedding of CD43 by various proteases contained within the granules.

It appears that the effects of PAF on CD43 expression may contribute to neutrophil-endothelial interactions and subsequent neutrophil recruitment into tissues. It is conceivable that the limited shedding of CD43 on neutrophils (maximum of 60% with 10^{-6} M soluble PAF and 30-50% shedding seen with membrane-bound PAF) may be sufficient to promote emigration out of the bloodstream into the extravascular space.

4.3 Limitations

One of the limitations of this study is the method in which we enumerated neutrophils adhering statically to activated endothelium. It is not unusual to enumerate static adhesion by other methods (MPO or radioactive labeling). However, in order to assess CD43 shedding after neutrophils had adhered, it was necessary to remove neutrophils intact for staining with CD43 antibody. Therefore, the traditional methods of evaluating static adhesion was not applicable as these methods required lysing of the neutrophils. Removal of neutrophils by EDTA and then counting them manually was the only option available.

In some experiments, background counts (unstimulated) on endothelium were higher than anticipated. Ideally there should be minimal adhesion (if any) on the unstimulated endothelium. However, these results suggest that the endothelium may have been activated through the manipulations taking place during this assay. It is also possible that the variation in background counts is due to variation of HUVEC samples. Although

activation is seen on unactivated endothelium, it is clear that when HUVEC is activated by thrombin, there is at least a twofold increase in adherent neutrophils.

As we were not able to determine if membrane bound PAF mediated CD43 shedding, further experiments are warranted. There are several methods to separate adhesion from membrane-bound PAF effects on neutrophil CD43 shedding. One would be to prevent adhesion with anti-CD18 antibodies [60]. Another would be to utilize agents that prevent neutrophil shape change such as butadione monooxime [60]. By separating the two events, we mat be able to demonstrate which event is causing CD43 downregulation on neutrophils.

4.4 Summary

These results provide evidence that adhesion results in CD43 shedding from neutrophils. However, it cannot be said with certainty that membrane-bound PAF upregulated on thrombin-stimulated endothelium is capable of downregulating CD43 expression on human neutrophils.

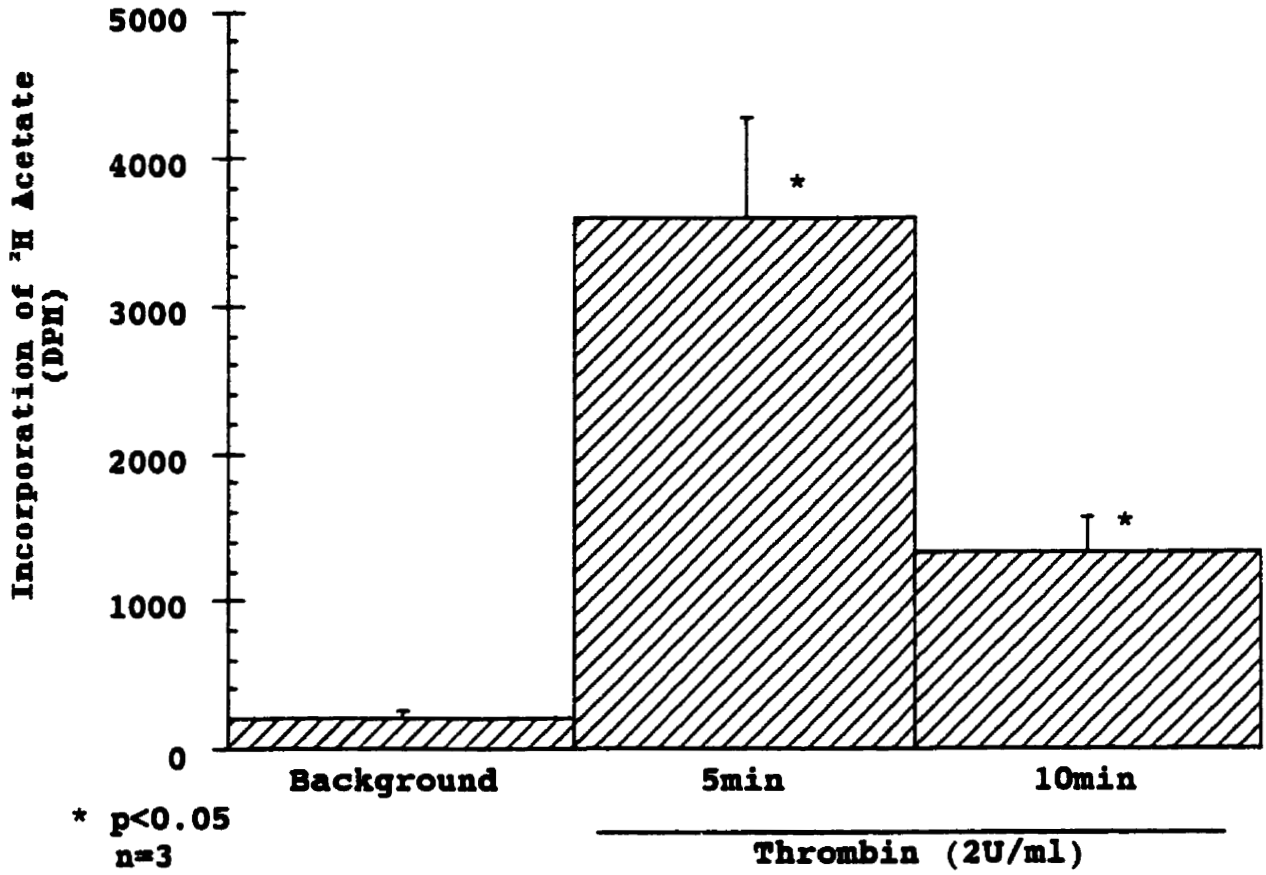


Figure 4.1 Measurement of PAF expression on primary HUVEC after stimulation with thrombin (2 U/ml). Maximal PAF upregulation was observed by 5 min. At 10 min PAF had decreased from 5000 dpm to 1400 dpm. * comparing 5 and 10 min to background, $P < 0.05$

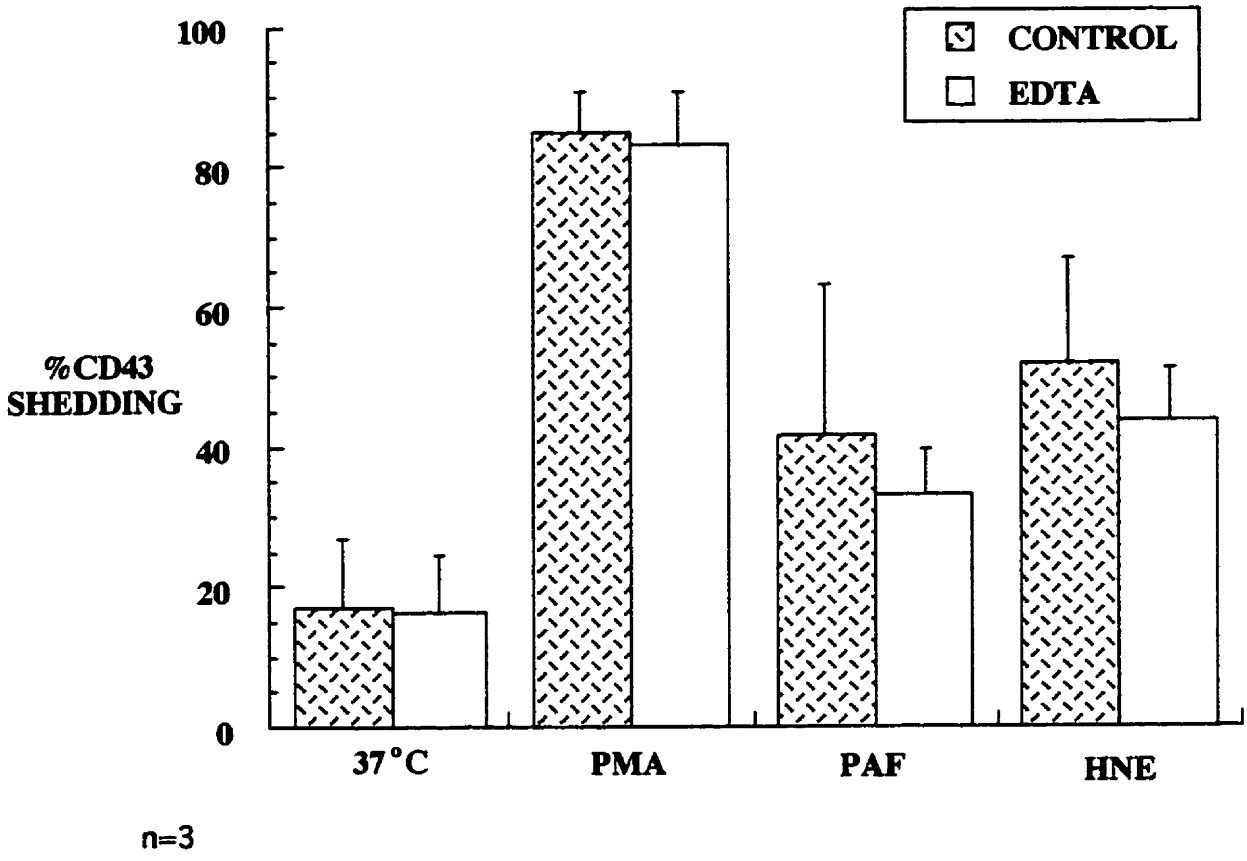


Figure 4.2 CD43 experiments done in the presence of EDTA (2mM) to assess whether EDTA affects CD43 downregulation.

Table 4.1. Results of static adhesion assay done with neutrophils on unactivated and thrombin-activated HUVEC. The numbers are counts of adherent neutrophils detached by EDTA treatment.

Experiment	1	2	3	4
Unstimulated	73,000	1,180,000	730,000	4,49000
Thrombin	490,000	2,220,000	1,040,000	1,000,000
Fold Increase	6.71	1.88	1.42	2.23

Table 4.2 Neutrophils adherent to endothelium and CD43 shedding

	%CD43 shedding		
	Buffer	Thrombin 5min	Thrombin 10min
Experiment #1	41.%	59	42.
Experiment #2	31	44	49.
Experiment #3	52	49	59
Mean	**41%**	**51%**	**50%**

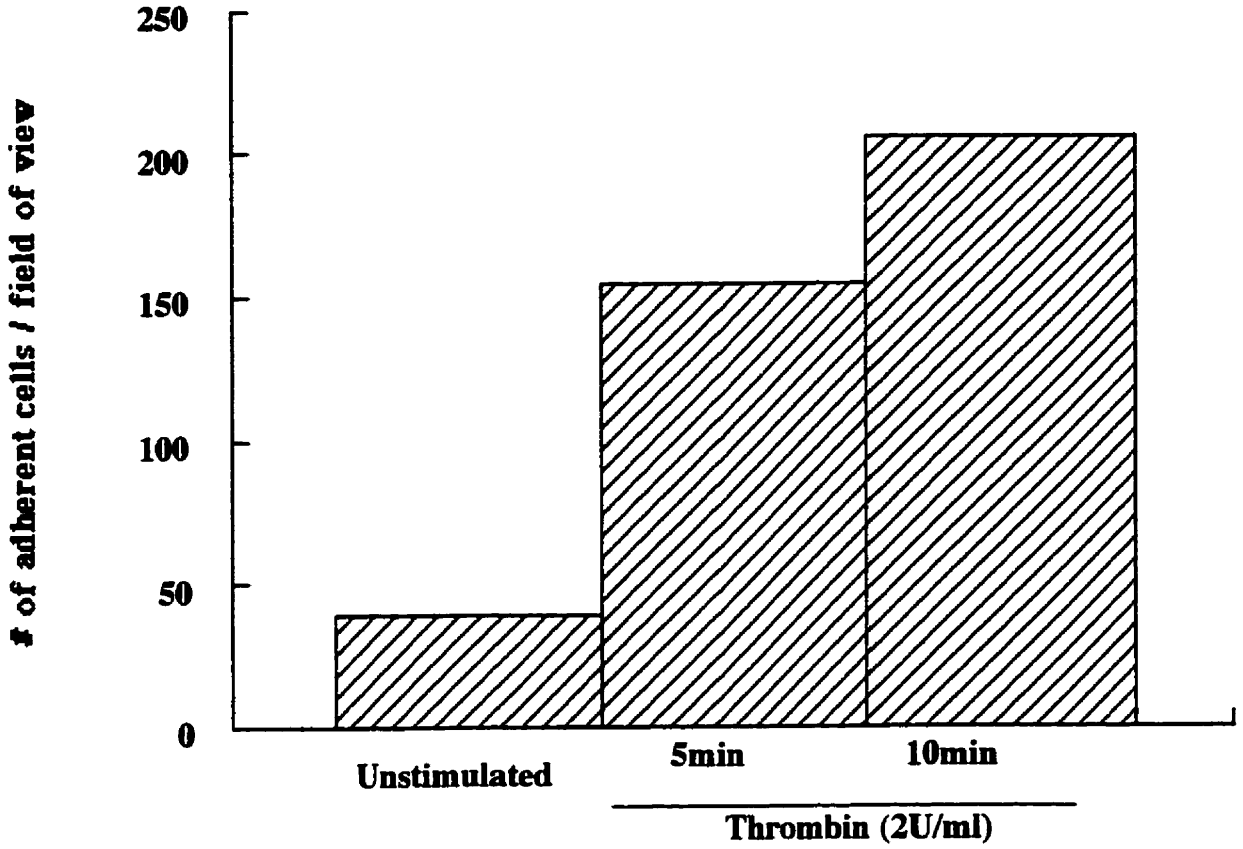


Figure 4.3 Neutrophils were allowed to adhere to activated and unactivated primary HUVEC for 10 min at 37°C. Non adherent cells were washed off with buffer and adherent cells tallied. This is a representative experiment of three experiments.

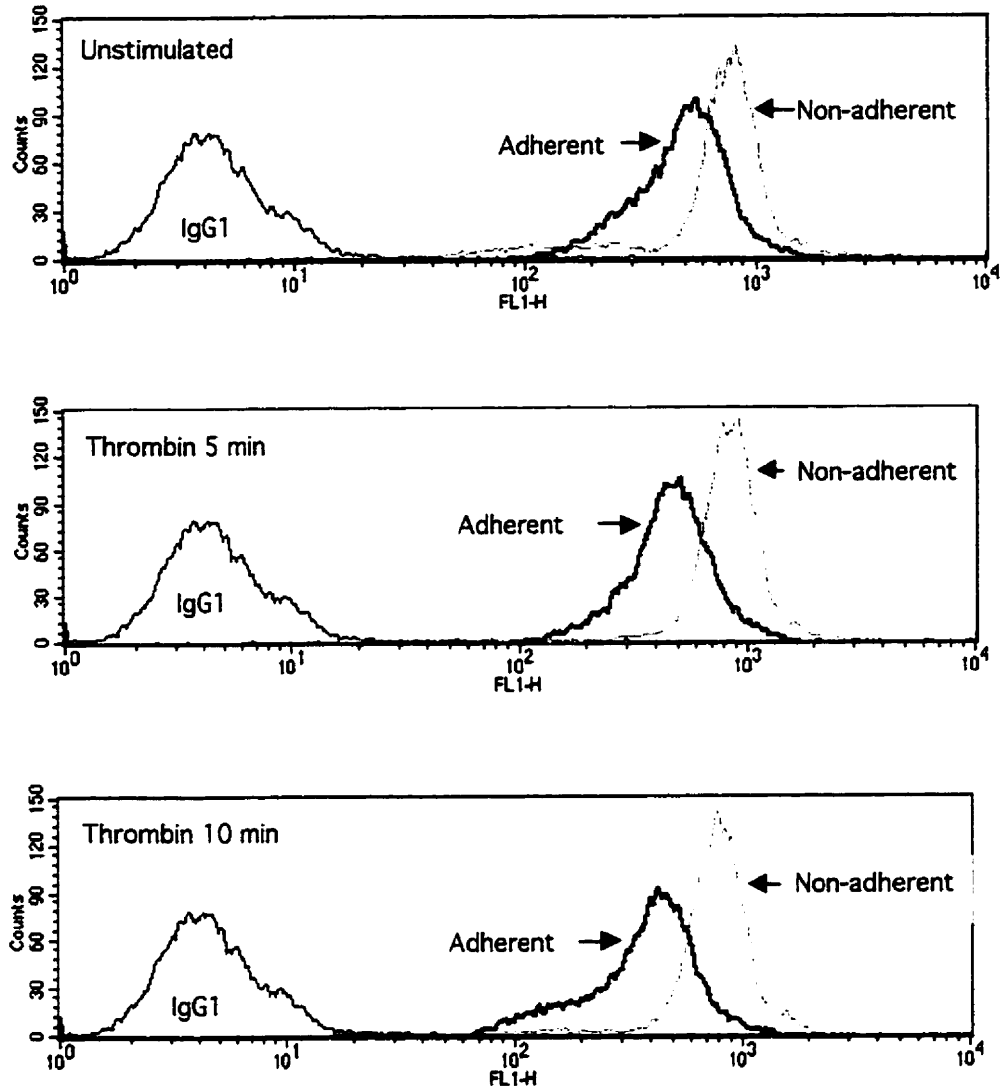


Figure 4.4 A representative histogram depicting CD43 expression on adherent and non-adherent neutrophils. There is a distinct shift in CD43 expression on adherent neutrophils when compared to non-adherent neutrophils.

Chapter Five

Effects of WEB 2086 and DIDS on PAF-mediated CD43 shedding

We have seen in the previous two chapters that PAF, membrane-bound and soluble, is capable of shedding CD43. The aim of this chapter was to determine if blocking PAF could influence CD43 expression.

If PAF mediates CD43 shedding on neutrophils, then it is reasonable to anticipate that a specific PAF receptor antagonist could block the effects of PAF on CD43. To test this, we utilized WEB 2086, which is a specific competitive receptor antagonist of PAF that has been reported to have no effect on neutrophil and platelet responses to other aggregating agents [80]. It has also been demonstrated that WEB 2086 inhibits exocytosis of neutrophil primary and secondary granules upregulated by PAF [103]. Through binding experiments, the activity of WEB 2086 is competitive with an equilibrium dissociation constant (K_D) of 15nM [104].

By blocking granule translocation using the agent, DIDS (4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid;750μM),we were also able to indirectly inhibit PAF effects on CD43 shedding. DIDS, an anion channel blocker, affects microfilaments and blocks translocation of granules (and their proteases within) to the plasma membrane [92, 105]. Previous studies have demonstrated the ability of DIDS (0.1mM) to inhibit β-glucuronidase and lysozyme release from neutrophils, without affecting superoxide generation by neutrophils [92, 105]. They also demonstrated that the effects of DIDS were reversible by washing, suggesting DIDS interacted with neutrophils noncovalently.

5.1 Results

A dose response study of WEB 2086 (0.4-400 μM) was carried out to determine the optimal concentration capable of blocking PAF-induced CD43 shedding. Figure 5.1 illustrates the results of the WEB 2086 dose response. There was no inhibitory effect of WEB 2086 at 0.4μM but at higher concentrations (4-400μM) significant inhibition of the PAF effects were seen (42% to 57%). However, even at the highest concentration of WEB 2086 utilized, we were still not able to completely inhibit PAF-induced CD43 shedding. At the highest concentration, we managed to block PAF induced CD43 shedding by 57%. Although WEB 2086 did not completely inhibit PAF's actions on CD43 shedding, this set of experiments indicated that WEB 2086 was capable of blocking the effects of PAF on granule translocation. 10μM WEB 2086 was chosen for subsequent experiments using WEB 2086.

To study WEB 2086's ability to downregulate endothelial, membrane-bound PAF effects on neutrophil adhesion, a series of static adhesion studies were carried out, in which neutrophils were pre-incubated with WEB 2086 (10μM) prior to adhesion to thrombin-activated HUVEC. The WEB 2086-treated neutrophils were allowed to adhere to the activated endothelium and non-adherent cells were washed off. As with the experiments in the previous chapter, adherent cells were detached with EDTA and enumerated, followed by staining for CD43 and flow cytometry. Table 5.1 shows the counts of neutrophils adherent to endothelium while Table 5.2 is the CD43 MFI for cells following various treatments. In the experiments above, WEB 2086 inhibited neutrophil adhesion (21-73% inhibition) (Table 5.1).

One would predict that if WEB 2086 was inhibiting PAF-mediated CD43 shedding, we would see an increase in neutrophil CD43 MFI (Table 5.2). We see WEB 2086 protection of the experiments done at the 5min time point. Results from 10 min time point was more variable (data not shown). In both experiments, however, WEB 2086 pretreatment was not sufficient to prevent some CD43 shedding from neutrophils. These results are comparable to those obtained from WEB 2086 dose response experiments.

An experiment utilizing DIDS, an anion channel blocker, further emphasizes the importance of neutrophil granules in the downregulation of CD43 from neutrophils. DIDS prevents the translocation of neutrophil granules to the plasma membrane [79, 92, 105]. Figure 5.2 illustrates the results obtained from this set of experiments. Neutrophils were pre-incubated with DIDS (750μM) for 15 min, at room temperature prior to incubating them with the various agonists. For all conditions, pre-incubation with DIDS significantly decreased CD43 downregulation. Not only do these results demonstrate the ability to nonspecifically block PAF mediated CD43 shedding with DIDS, they also highlight the importance of neutrophil granules and their translocation to the plasma membrane in the process of CD43 downregulation.

5.2 Discussion

WEB 2086 was capable of blocking exogenous, soluble PAF effects on CD43 shedding. Previous studies with WEB 2086 have also shown that this agent is highly effective in blocking PAF-mediated degranulation of neutrophils [103]. In this study, we see that WEB blocked CD43 downregulation, although it was not completely

effective in inhibiting PAF's actions. It is possible that the concentration of PAF used for neutrophil activation was excessive, and that PAF being a lipid molecule, is able to nonspecifically diffuse through the plasma membrane and act intracellularly on neutrophils [106].

The results from the static adhesion assays indicated that WEB 2086 inhibited neutrophil adhesion to thrombin-stimulated HUVEC. Although my data is not in agreement with certain previously published studies, there are potential explanations to account for these differences. In similar experiments, it was found that WEB 2086 blocked neutrophil emigration across endothelial cells by 60%, although it did not affect adherence [107]. Hill et al. also found that WEB 2086 at a concentration up to 40μM (we used 10μM) had little effect on adhesion, but blocked the polarization of neutrophils on activated endothelium [108]. A detailed review of these experiments reveals that both groups utilized HUVEC that had been passaged 2-4 times in contrast to my studies, which used primary HUVEC. Passaging HUVEC results in reduced expression of PAF and P-selectin following exposure to agonists [87]. It is likely that in these studies, expression of PAF was decreased and therefore an inability of WEB 2086 to affect adhesion. Although there was no effect seen on adhesion in both papers, WEB 2086 blocked other CD18-dependent events, namely cell spreading and transmigration. Nevertheless, a seminal report by Zimmerman *et al.* indicated that neutrophil adhesion to primary HUVEC was indeed blocked by the PAF receptor antagonist, L652,731 which is in agreement with my results [83].

The results in Table 5.2 are rather variable. We anticipated that WEB 2086 would successfully inhibit PAF's action on CD43 shedding. This would be reflected by

an increase in MFI of CD43 after adhesion to activated endothelium. Although WEB 2086 pretreatment of neutrophils prevents some CD43 shedding, it is not restored to levels of cells that were non-adherent. One potential explanation why WEB 2086 was not as effective with adherent neutrophils may be that adhesion still occurred because of other interacting molecules (eg PSGL-1) on the neutrophils and WEB 2086 did not block these interactions. There are reports that signaling takes place through the selectins [109] and it is plausible that the cells are being activated in that manner.

Another potential explanation for the inability of WEB 2086 to completely block PAF effects is the presence of more than one type of PAF receptor. Based on radioligand binding characteristics from the cloned human PAF receptor, there is evidence that different PAF receptor subtypes exist on human neutrophils [110]. WEB 2086 may be preferentially be binding the low affinity receptors without affecting high affinity receptors. Furthermore, the exact localization of PAF receptor(s) on neutrophil plasma membrane is unknown. It is possible that the PAF receptors are in fact not localized to the microvilli, thus potentially limiting PAF receptor-ligand interactions. Studies examining the distribution of PAF receptors on neutrophils are crucial to determine if the surface topography of PAF receptors are important.

Although the actions of DIDS are non-specific, DIDS was very efficient at downregulating the effects of various agonists, including PAF. By inhibiting microtubule formation, the importance of granules and granule translocation in the downregulation of CD43 from neutrophils is further highlighted. These results are also

consistent with our cytoplast studies (Chapter 3) suggesting that plasma membrane protease(s) is not responsible for CD43 shedding.

5.3 Limitations

In retrospect, there are several other studies that could have complimented these experiments. It is not known what effects WEB 2086 has on CD43 expression. Because WEB 2086 is specific for the PAF receptor, it is expected that WEB 2086 would not influence CD43 expression. Another experiment would be to examine the effects of DIDS pretreated neutrophils on activated HUVEC. It is expected that DIDS might decrease adhesion to activated HUVEC because of its non-specific effects on microfilaments that might prevent neutrophil shape change and subsequent activation.

The results obtained in Table 5.2 needs to be supplemented with more experiments because with such a small number of experiments, we cannot come to any conclusion and therefore further studies are warranted. It may also be useful to see what effects other PAF receptor antagonists have on neutrophil adhesion.

5.4 Summary

The experiments examined the effects of two inhibitors on PAF. WEB 2086, was specific for the PAF receptors while DIDS nonspecifically affected granule translocation. In these experiments, we observed that these agents could successfully block the effect of PAF on CD43 expression.

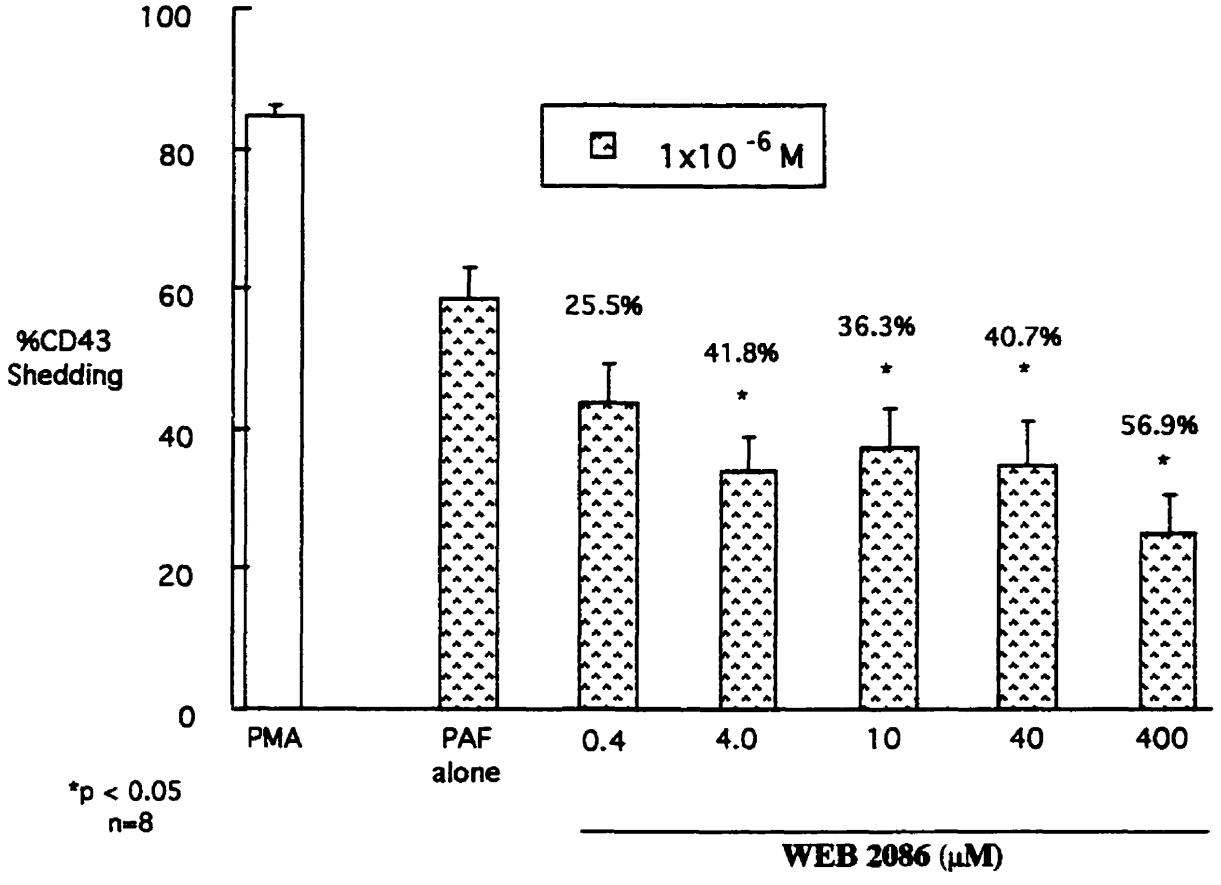


Figure 5.1 WEB 2086 dose response on neutrophil CD43 shedding. Human neutrophils were pre-incubated with various concentrations of PAF receptor antagonist, WEB 2086, followed by PAF ($1x10^{-6}$M). The agonist PMA (0.1μM) was also included as a control for CD43 shedding. 4μM WEB 2086 was sufficient to significantly block PAF induced CD43 shedding. % inhibition of CD43 shedding by WEB 2086 is shown above the bars. *compared to PAF alone, $P < 0.05$

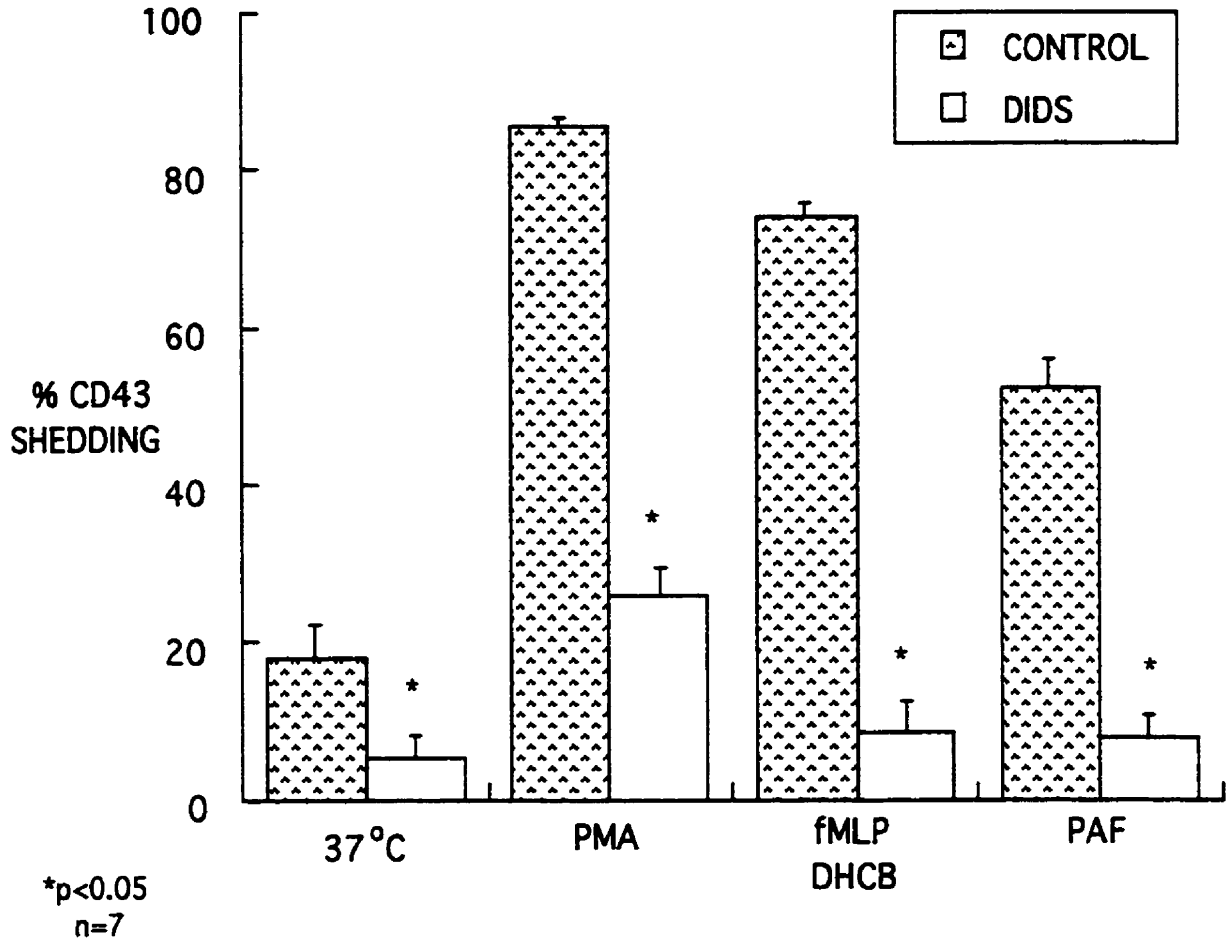


Figure 5.2 CD43 on neutrophils assessed in the presence of DIDS (750μM). DIDS significantly blocked CD43 shedding induced by various agonists. *comparing untreated and DIDS-treated neutrophils, $P < 0.05$

Table 5.1 The effects of WEB 2086 on neutrophils adhesion to thrombin-activated HUVEC

	Thrombin	WEB 2086 + Thrombin	% Inhibition
Experimental #1	490,000	132,000	73
Experimental #2	1,040,000	428,000	58.8
Experimental #3	1,000,000	789,000	21.1

Table 5.2. Neutrophil CD43 expression following 5min adhesion to thrombin-activated HUVEC.

	Unstimulated HUVEC (MFI)		**Thrombin-stimulated HUVEC (MFI)**	
	Non-adherent	Adherent	Adherent	Adherent WEB 2086
Experiment #1				
	648.24	381.66	291.89	342.50 ↑
Experiment #2				
	688.24	333.48	374.2	404.3 ↑

Chapter Six

Some functional in vitro consequences of CD43 shedding from human neutrophils

In the previous chapters, we demonstrated the ability of PAF to downregulate CD43 expression on neutrophils. It is conceivable that CD43 contributes significantly to retard cell-cell interactions through an anti-adhesive role where the net negative surface charge of CD43 on the neutrophil is thought to retard cell-cell interactions[13, 14, 64, 65, 111]. In keeping with the anti-adhesive hypothesis of CD43, removal of CD43 from the surface of neutrophils would therefore result in a decreased surface charge and promotion of neutrophil-endothelial interactions.

To test this hypothesis, parallel plate laminar flow assays were performed using three different cellular substrates (TNF-α stimulated HUVEC, E- and P-selectin transfectants). The advantage of this *in vitro* system is that a pure population of neutrophils can be perfused over a substrate at a fixed rate and cell-cell interactions can be visually examined. By choosing the substrate, we are also able to examine the specific contribution of individual adhesion molecules to neutrophil recruitment. We chose to investigate interactions between neutrophils and P-and E-selectin-transfected cells. These transfectants possess a glycocalyx, similar to the surface of endothelial cells, therefore contributions of the glycocalyx to cell-cell interactions are not excluded. We also utilized HUVEC as this would provide us with a human model to observe neutrophil rolling *in vitro*.

To induce CD43 shedding without causing cellular activation, we elected to induce CD43 shedding with exogenous HNE. It has been demonstrated previously that exogenous HNE is capable of specifically shedding CD43 (30-50%, sialyl-dependent epitopes) without affecting other neutrophil surface sialoproteins (eg. sLe^x or L-selectin) or causing

cellular activation [12]. HNE shedding was chosen as the preferred experimental approach for the following reasons. First, partial shedding of CD43 is probably more physiological than complete shedding of CD43. Second, CD43 is expressed early in hematopoietic cells and there are no known human cell lines that lack CD43. Third, the only known human condition where leukocytes lack CD43 is Wiscott-Aldrich Syndrome (WAS). Since WAS is rare, acquiring cells from WAS patients was not feasible. Therefore, HNE was used as a tool to induce CD43 shedding.

To further extend observations about HNE and to ensure that HNE is specific for CD43, we chose to study the effect of HNE on other surface molecules known to be shed from neutrophils, specifically CD45 and PSGL-1. CD45, like CD43, is a rather long molecule that extends above the glycocalyx [112]. It is found in abundance on all hematopoietic cells and is a tyrosine phosphatase important in T-lymphocyte activation [112, 113]. PSGL-1 was studied because it is the known ligand for all three selectins [22, 114] and like CD43, PSGL-1 is a sialomucin that also extends well above the neutrophil glycocalyx. It is expressed on a variety of cells, including neutrophils as well as endothelial cells at sites of chronic inflammation [114]

CD43-shed neutrophils were perfused over monolayers of confluent E- and P-selectin transfectants as outlined in the methods. Previous research has demonstrated the ability of E-and P-selectin to support neutrophil rolling interactions [35, 86, 115]. The same procedure was followed for HUVEC. Endothelium, activated with TNF-α upregulates expression of E-selectin, IL-8, PAF and various other proinflammatory mediators [20, 21, 32, 37]. A dose response of TNF-α (0.25 -250 ng/ml) on HUVEC was carried out to determine the optimal dose of TNF-α and to determine if rolling and adherent interactions

were dose dependent. If CD43 acts as a nonspecific anti-adhesive barrier, one would expect that removal of CD43 would result in enhanced interactions at the neutrophil-endothelial interface.

6.1 Results

Neutrophils were treated with HNE and stained for CD45 and PSGL-1 expression. Figure 6.1 clearly demonstrates that HNE does not affect CD45. The histogram for both untreated and HNE-treated neutrophils overlapped, demonstrating that CD45 is not susceptible to HNE proteolytic shedding. However, PSGL-1 shedding occurred following treatment with HNE as shown by a shift in PSGL-1 expression compared to untreated cells.

The next step was to examine the role of CD43 on neutrophils interacting with E-selectin transfectants (Figure 6.2). Because these are transfected cells, neutrophils rolled but did not adhere to the cellular monolayer. Neutrophils that had undergone HNE-induced CD43 shedding demonstrated increased rolling interactions compared to control neutrophils (337 ± 31 vs. 233 ± 20 after five min of perfusion at 2 dynes/min).

In the next set of experiments, we investigated the optimal concentration of TNF-α to stimulate HUVEC to upregulate expression of E-selectin. Several doses were examined. No interactions were observed at the 0 and 0.25ng/ml TNF-α(data not shown). At higher concentrations (2.5, 25, and 100 ng/ml) of TNF-α, however there were comparable and significant rolling interactions observed (Figure 6.3). In the same experiments, adhesive interactions between neutrophils and HUVEC were also quantified. Table 6.1 summarizes the rolling and adhesion data after 5 min of neutrophil perfusion in response to various concentrations of TNF-α.

From the dose response described in Figures 6.3, 25ng/ml TNF-α was selected as an optimal concentration to stimulate HUVEC and study neutrophil-endothelial interactions.

Next, we examined the effects of CD43 removal on neutrophils interacting with activated HUVEC (Figures 6.4). HNE-treated and control neutrophils were perfused on 25ng/ml TNF-α stimulated HUVEC. In these experiments, there was a trend for control neutrophils to roll and adhere better than HNE-treated neutrophils. However, this was not statistically significant. This was also in contrast with data seen for the E-selectin transfectants, where CD43-shed neutrophils rolled more than control neutrophils.

The initial experiments with HNE-treated neutrophils indicated that PSGL-1 was shed from neutrophils. This finding was further corroborated by the next set of experiments. We perfused HNE-treated and control neutrophils over P-selectin transfectants to observe interactions taking place (Figure 6.5) and found a significant difference in the two experimental conditions, whereby control neutrophils rolled more than CD43-shed neutrophils after 5 min of perfusion (199 ± 49 vs. 49 ± 13). This data is consistent with HNE-induced shedding of PSGL-1which is reported to be the main ligand for P-selectin. Shedding of PSGL-1 (and CD43) was associated with decreased neutrophil rolling on P-selectin transfectants.

6.2 Discussion

These experiments were designed to examine the functional role of CD43 on human neutrophils. However, it is becoming painfully clear that the role of CD43 on neutrophil-endothelial interactions is more complex than initially hypothesized.

Although PSGL-1 is a ligand for all three selectins, results from the E- and P-selectin transfectants demonstrate that there must be alternate ligand(s) for E-selectin. A putative ligand for E-selectin was described in the mouse, and was later shown to be a variant of a receptor for fibroblast growth factor [116, 117]. The existence of other E-selectin ligands may explain the increased interactions seen with E-selectin transfectants following HNE-induced shedding of CD43. We did not determine if there were differences in E-selectin site densities between the E-selectin transfectants and TNF-α- stimulated HUVEC.

Our experiments do demonstrate that the alternate ligand(s) for E-selectin is insensitive to HNE pretreatment (unlike PSGL-1) as there was a significant increase in interactions between neutrophils and E-selectin transfectants following HNE treatment. This seems to suggest that CD43 removal may be important in E-selectin-mediated neutrophil recruitment into tissues. A recent paper describes the ability of L-selectin to bind to E-selectin [118] and from our lab, HNE does not affect L-selectin expression on neutrophils [12]. Others have shown *in vitro* that L-selectin antibodies can partially inhibit neutrophil attachment to transfectants [91]. Perhaps L-selectin is mediating these interactions with the E-selectin transfectants.

It has previously been demonstrated that TNF-α upregulates expression of E-selectin on HUVEC [37, 74] [32]. It is interesting that with E-selectin transfectants, we observed increased rolling interactions with CD43-shed neutrophils, yet, similar results were not observed on HUVEC. There was no difference seen between CD43-shed neutrophils and control neutrophils, although the trend was for the former to interact less with activated HUVEC. Potential explanations for this may be that TNF-α

upregulates more than just E-selectin. IL-8 is another molecule upregulated by stimulated endothelium [32]. Although we have shown that IL-8 does not affect CD43 expression, IL-8 upregulation will activate neutrophils and promote increased adherence to HUVEC. It has been demonstrated recently that cytokine-activated endothelium release paracrine factors that induce neutrophils to degranulate[119]. This factor is secreted and is not IL-8. They further describe PAF as a weak agonist of neutrophil degranulation. Although endothelial-bound PAF is a weak agonist of neutrophil degranulation, it may be sufficient to induce granule translocation to shed CD43. It is plausible that in our HUVEC experiments, this factor may be involved in activating neutrophils and thereby causing a reduction in rolling and adhesive interactions.

6.3 Limitations

It is evident that HNE used to downregulate CD43 is non-specific and affects other ligands important in neutrophil- endothelial interactions. Another experimental approach to examine the role of CD43 on neutrophil-endothelial interactions is required. The antibody L11, described by McEvoy *et al* ., has been used to successfully block T cell homing [69]. It is plausible that immunoneutralizing antibodies may better define a role for CD43 expression on neutrophils. However, this approach may further enhance steric hindrance as well as induce cellular activation by crosslinking of CD43 which has previously been shown to induce neutrophil aggregation [120].

6.4 Summary

A rather unexpected finding from this set of experiments is that there are other E-selectin ligands that mediate rolling interactions in the event PSGL-1 is removed from the surface of neutrophils. Partial CD43 removal seemed to enhance neutophil interactions on E-selectin transfectants but had no effect on E-selectin expressed by TNF-α activated HUVEC. From these experiments, the nonspecific, anti-adhesive role of CD43 is inconclusive and further studies are warranted.

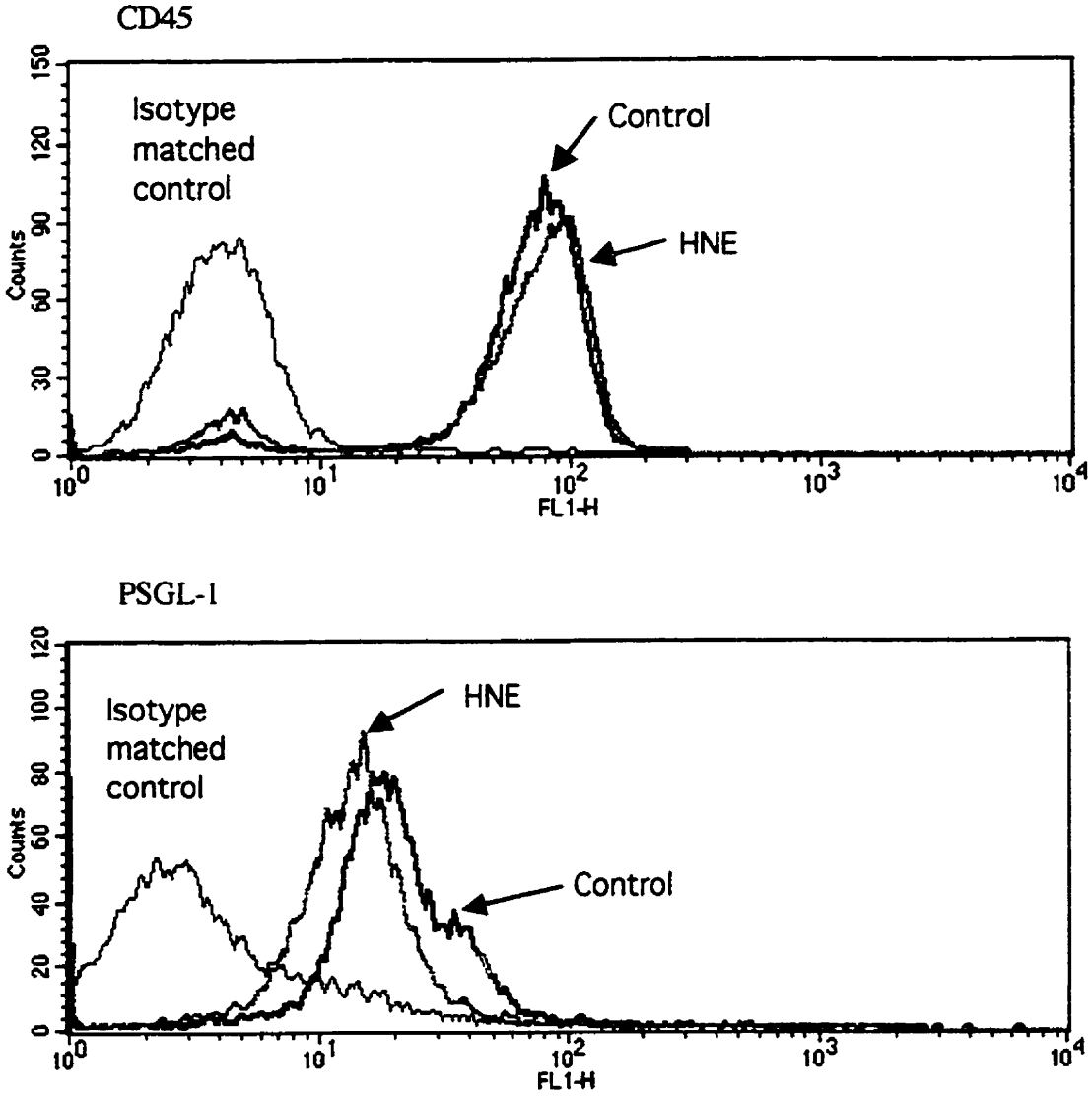


Figure 6.1 Representative FACS histograms demonstrating the effects of HNE on other neutrophil surface molecules. HNE does not affect CD45 (upper panel). However, this is not the case for PSGL-1(lower panel). HNE-treatment of neutrophils resulted in PSGL-1 shedding as demonstrated by a shift in PSGL-1 expression.

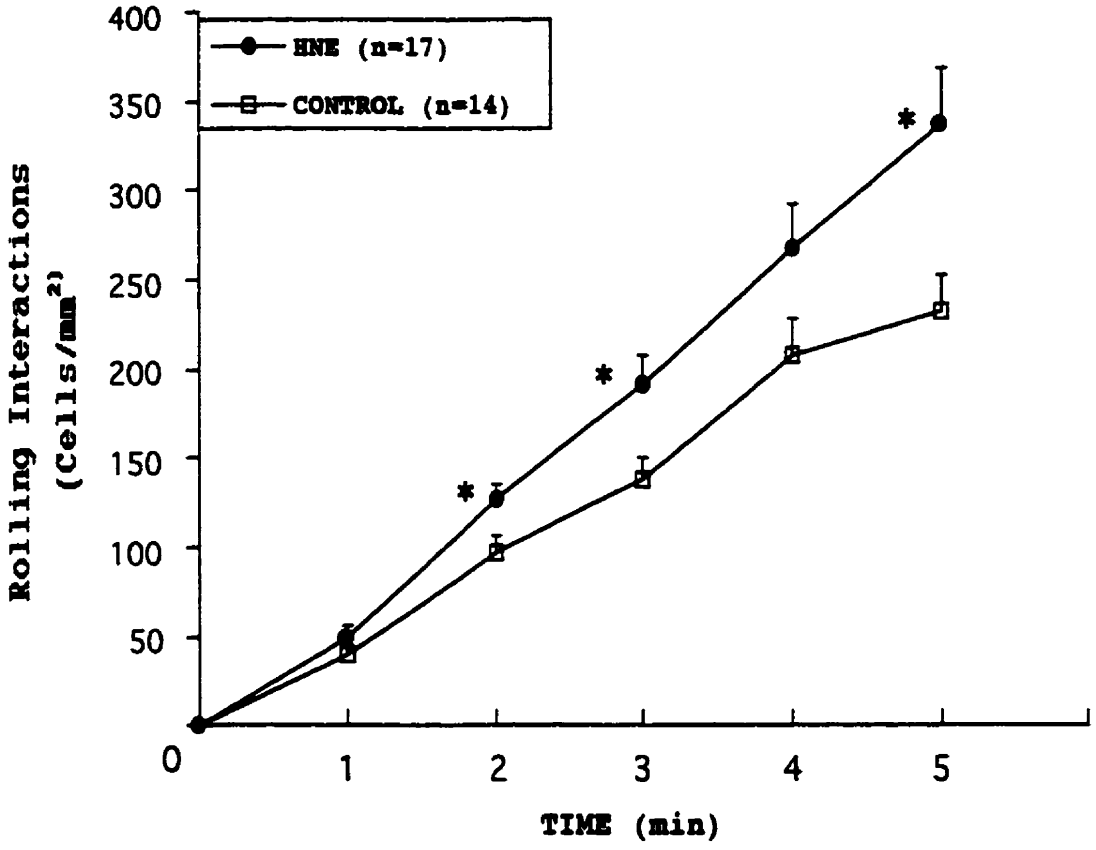
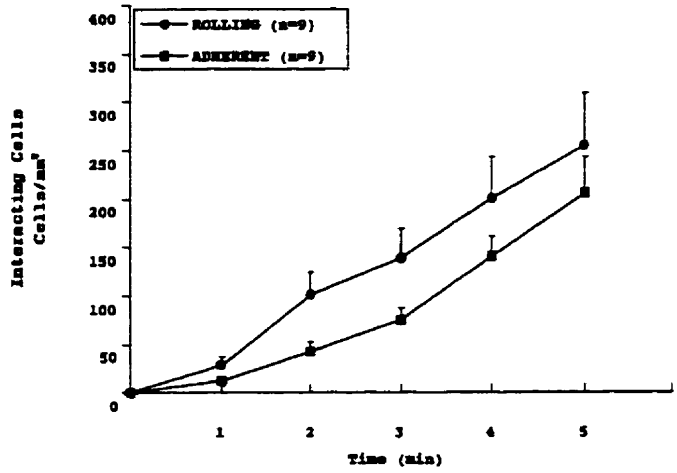
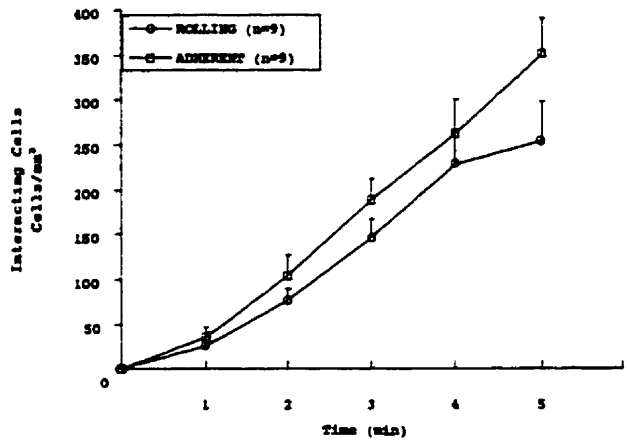


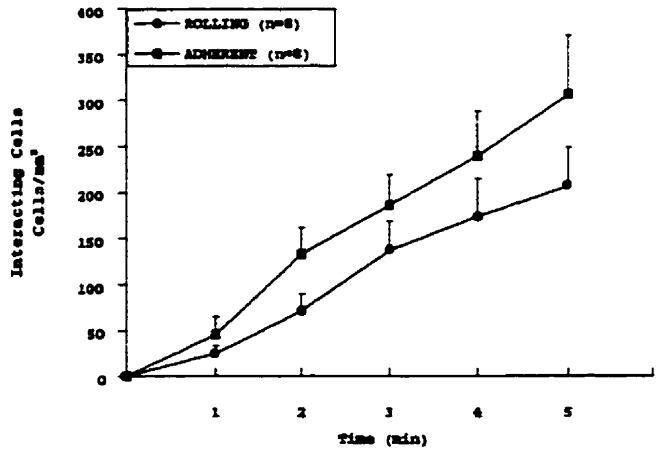
Figure 6.2 Neutrophil rolling interactions using a parallel plate flow chamber assay. Human neutrophils (control or HNE-treated) were perfused over a confluent monolayer of E-selectin transfectants. Neutrophils in which CD43 shedding has been induced by HNE demonstrated more rolling interactions than control neutrophils. * comparing control and HNE-treated neutrophils, $P < 0.05$



2.5ng/ml



25ng/ml



100ng/ml

Figure 6.3 Results from parallel plate laminar flow chamber assays examining neutrophil interactions on HUVEC stimulated for 4 hours with various concentrations of TNF-α.

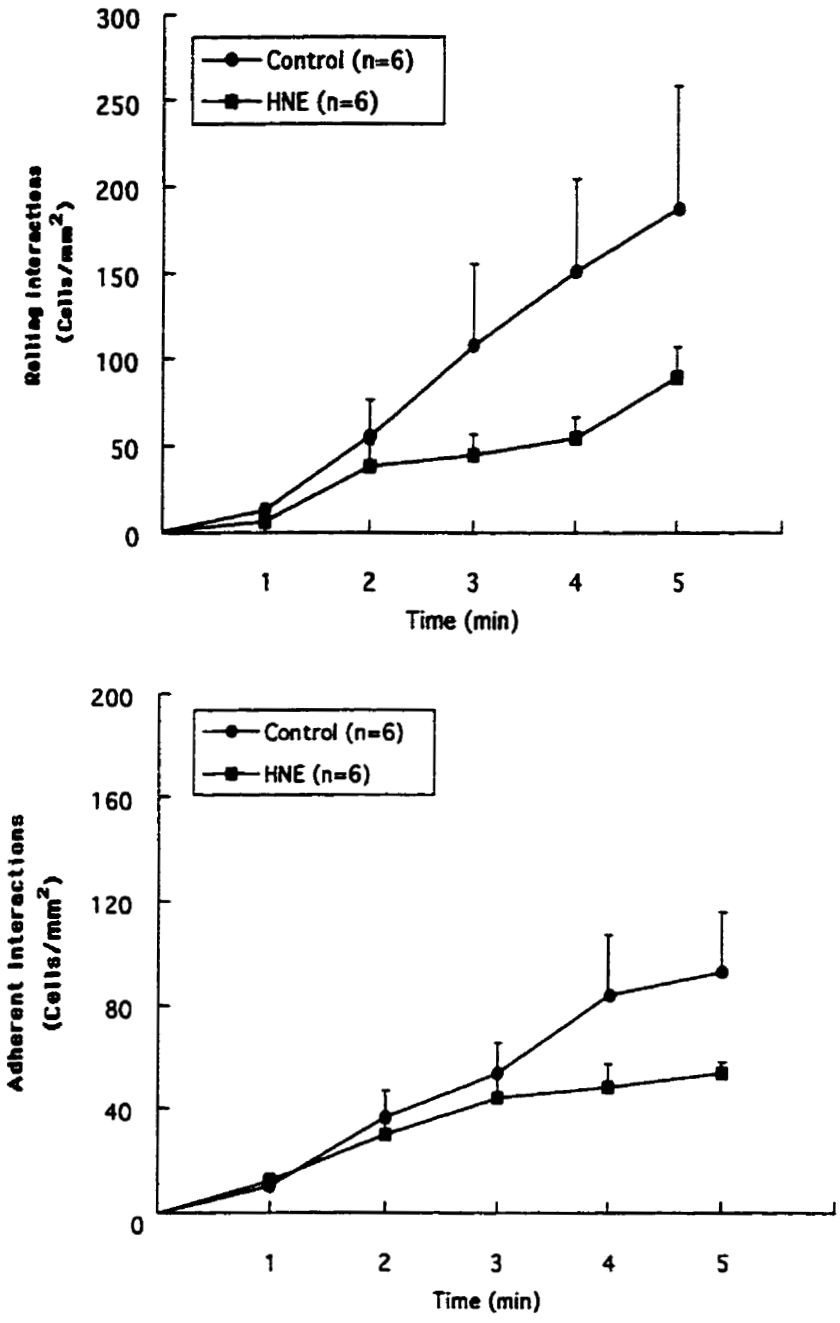


Figure 6.4 Neutrophils (control and HNE-treated) interacting on 25ng/ml TNF-α activated HUVEC. There is a trend for CD43-shed neutrophils to interact less than control neutrophils on activated HUVEC.

Table 6.1. Summary of rolling and adhesive interactions (after 5 min perfusion) taking place on HUVEC after stimulation with various concentrations of TNF-α.

TNF-α (ng/ml)	Rolling	Adhesion
2.5 (n=9)	255 ± 54	206 ± 37
25 (n=9)	254 ± 43	351 ± 40
100 (n=8)	207 ± 43	307 ± 64

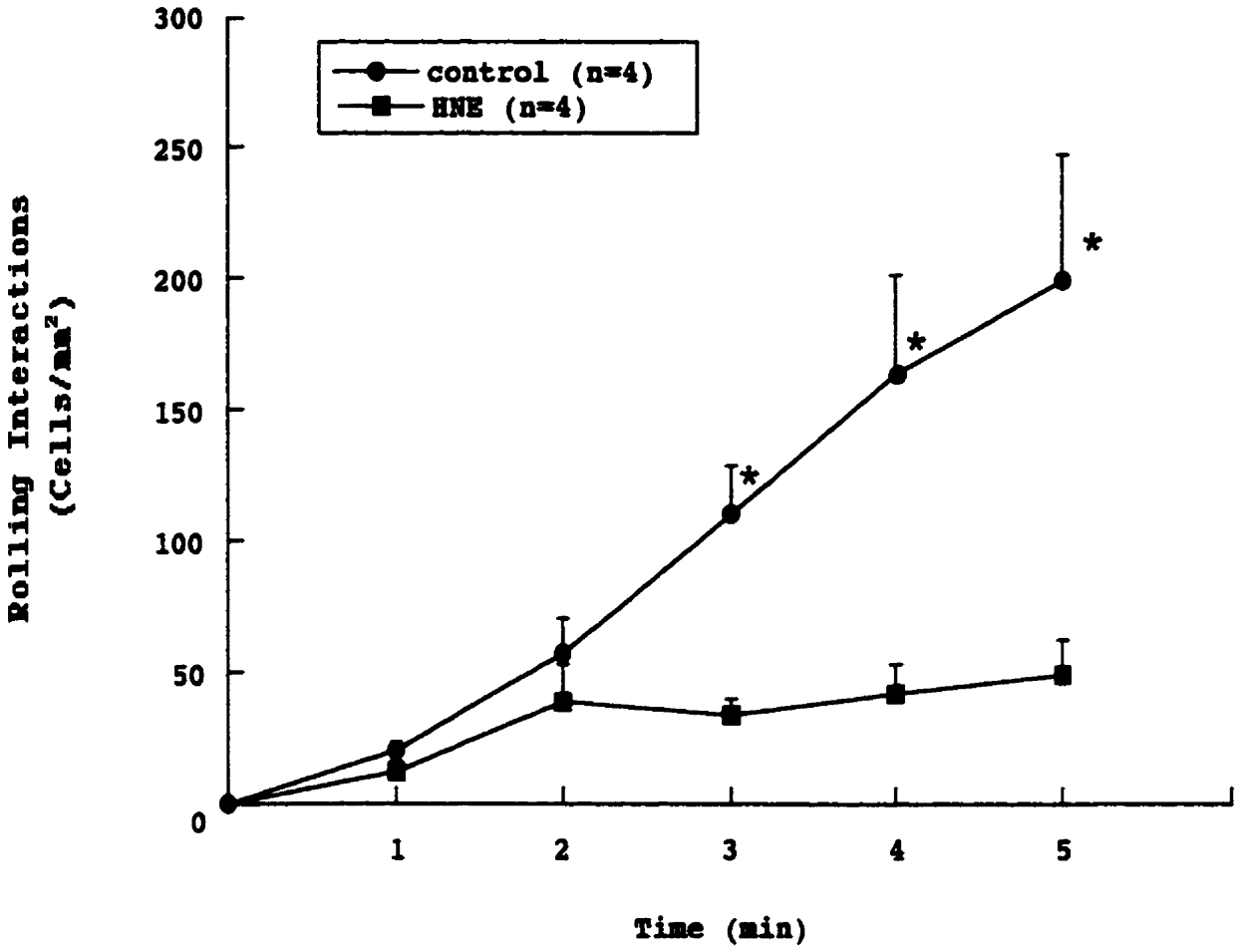


Figure 6.5 Neutrophil rolling interactions (control and HNE-treated) on P-selectin transfectants. CD43 shedding resulted in significantly less rolling compared to control (untreated) neutrophils. *comparing control and HNE-treated neutrophils, $P < 0.05$

Chapter Seven

Conclusion and Future Directions

7.1 Conclusion

In the previous chapters, we characterized PAF's ability to mediate CD43 shedding *in vitro*. This downregulation was preceded by cellular activation of neutrophils and required granule translocation to the plasma membrane. We further established that the adhesion event mediates CD43 shedding from neutrophils. We were not able to distinguish if endothelial-bound PAF mediated CD43 shedding from neutrophils. By examining the effects of a non-specific (DIDS) and specific (WEB 2086) inhibitor of PAF on neutrophils, we demonstrated that both blocked PAF-induced CD43 shedding, although DIDS was more effective at inhibiting PAF-mediated CD43 shedding.

I also examined the functional effect of CD43 shedding from neutrophils *in vitro*. Using E-selectin transfectants, CD43 shedding from neutrophils enhanced rolling interactions compared to control neutrophils. However, the same results were not observed with neutrophils interacting with activated HUVEC. It is possible that activated endothelium is upregulating factors that are capable of neutrophil degranulation, thus affecting CD43 expression. We concluded that interactions of neutrophils on E-selectin transfectants and activated HUVEC were mediated by E-selectin ligand(s) that is insensitive to HNE, unlike the P-selectin ligand, PSGL-1.

Based on the results of our studies, we would like to propose the following working model for CD43 shedding from neutrophils by endothelial-bound PAF.

The model:

CD43 provides a non-specific barrier to neutrophil-endothelial cell-cell interactions. In the unactivated state, neutrophil CD43, through its physical properties, minimizes homotypic and heterotypic interactions. Neutrophils marginate and roll on the endothelium of postcapillary venules. However, when neutrophils encounter activated endothelium expressing membrane-bound PAF, they become activated. The endothelial-derived PAF promotes translocation of neutrophil granules (that contain proteases capable of mediating CD43 shedding) to the cell surface. Local proteolytic shedding of CD43 reveals cryptic antigens and further augments adhesive interactions between the endothelium and the neutrophil. This leads to enhanced emigration of neutrophils out of the intravascular compartment into the tissues. The crucial step where CD43 shedding occurs is following the rolling event, and probably during the adhesion event.

7.2 Future directions

There is compelling evidence now accumulating that CD43 is important in the emigration of neutrophils into tissues. Proteolytic shedding of CD43 on neutrophils is never complete, as there is always a degree of persistent CD43 remaining on the neutrophil surface. Several reports have demonstrated that murine CD43-deficient leukocytes have impaired emigration suggesting an important role for the residual CD43 [73, 121]. Localization of CD43 to the uropod of the migrating cell as well as its ability to complex with cytoskeletal proteins further supports the hypothesis that

unshed CD43 promotes emigration of neutrophils into tissues [74–76, 101]. Precisely how and which ligand(s) interact with CD43 remains to be defined. The task at hand is to identify the substrates interacting with CD43 and perhaps then, we may determine the exact function(s) of CD43 in neutrophil recruitment.

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