

ABSTRACT

GREEN, TERESA DARLENE. The Role of MARCKS Protein in Macrophage Migration. (Under the direction of Dr. Kenneth B. Adler).

Uncontrolled inflammation is often a fatal pathological feature characterizing several airway diseases such as asthma, cystic fibrosis, and chronic bronchitis. During an inflammatory response leukocytes, including neutrophils and macrophages, are recruited to infected or injured areas in response to chemical substances through a process of chemotaxis. Macrophages located in the submucosal tissues are phagocytes that are involved in clearing of debris in both innate and cell mediated immunity in vertebrates. However, exacerbated inflammation can cause severe damage and can ultimately lead to death. The precise mechanisms involved in regulation of the accumulation of macrophages and potential targets for effective treatment have not yet been determined.

The purpose of this work was to examine mechanisms of macrophage migration. Although it is known that Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) is involved in cell motility, membrane trafficking, and phagocytosis in various cells, the distinct role that MARCKS plays in macrophages during such processes is unknown. A synthetic peptide corresponding to the first 24 amino acids of the amino (N) terminal region of MARCKS, referred to as Myristoylated N-terminal sequence or MANS peptide, was utilized to explore the regulation of MARCKS in migration of the murine macrophage cell line J774A.1 cells and primary murine macrophages.

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The Role of MARCKS Protein in Macrophage Migration

by
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DEDICATION

This dissertation is dedicated to my parents and in loving memory of Kamari Micah Green.

BIOGRAPHY

Teresa Darlene Green was born August 31, 1977 in Durham, North Carolina. She is the daughter of John and Patricia Green, sister of Katrice, and aunt of Tendai & Destiny. She attended Hillside High School in Durham and graduated in 1995 as class valedictorian. She received her undergraduate degree from North Carolina Agricultural and Technical State University in Greensboro, NC and graduated magna cum laude with a BS in Biology in 1999. After graduation she worked at Laboratory Corporation of America in Burlington, NC as a lab technologist in the Bone Marrow Department, where she was recognized as Laboratorian of the Year in 2002. She aspired to obtain a graduate degree and was accepted into a Post-Baccalaureate Research Education Program at Wake Forest University School of Medicine in Winston-Salem, NC where she successfully completed the program in 2004. In August 2004, she was accepted into the PhD program in Comparative Biomedical Sciences with a concentration in Cell Biology at North Carolina State University in Raleigh, NC under the direction of Dr. Kenneth B. Adler.

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CHAPTER 1

LITERATURE REVIEW: THE 3M's: MACROPHAGES, MARCKS, & MIGRATION

Introduction

The immune system is a complex organization of biological structures designed to protect organisms from invading pathogens and fight against disease. Inflammation is a key response of the immune system and serves as a defensive barrier to infection. Unfortunately, when inflammation is uncontrolled there is potential for development of chronic airway diseases categorized as chronic obstructive pulmonary disease (COPD). Macrophages are prominent cells involved in the inflammation process. The ability to understand the underlying mechanisms involved in regulating macrophage migration towards sites of inflammation and injury could be an important component for evaluating potential therapeutic targets to combat inflammatory airway diseases.

It is already known that Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) is involved in cell movement. Therefore, we investigated a relationship between MARCKS and macrophage recruitment. This research was designed to study the mechanisms of migration in murine macrophages *in vitro*. In the introductory chapter, I explore background information to link current knowledge, and in the second chapter I discuss the possible role of MARCKS specifically in regulation of mucin secretion and inflammation in asthma. In the third chapter, the role of MARCKS in migration of both a murine macrophage-like cell line and primary murine macrophages *in vitro* is explored, as is the distribution of MARCKS and the contractile protein, actin, in macrophages. Finally, I conclude the dissertation with a summary and delineate future directions.

Inflammation and chronic inflammatory disease

One hallmark feature of several chronic inflammatory diseases that fall under the category of COPD is chronic inflammation characterized by an influx of macrophages and neutrophils as a defensive mechanism [1]. COPD is the fourth leading cause of death in the US and worldwide [2]. In particular, macrophages function as phagocytes that are essential for clearance of unwanted inhaled particles. Pathological inflammation is often attributed to macrophages that release cytokines, such as tumor necrosis factor alpha (TNF- α), and chemokines, such as macrophage monocyte chemotactic protein 1 (MCP-1), which trigger an unrestricted sequence of proinflammatory responses [3]. This leads to damaging accumulation of various cell types with the role of macrophages as conductors and destroyers of infiltration still under investigation.

Together the detrimental effects of COPD reveal symptoms related to a combination of inflammation, mucus hypersecretion, and goblet cell hyperplasia [4-5]. The physical evidence of structural changes, referred to as remodeling, is a manifestation of airway obstruction, airway wall thickening, and eventually fibrosis [6-7]. Although the mechanisms behind inflammation are complex, one potential resolution is to focus on regulating the cells that are involved. Furthermore, a major aspect of resolution would be elimination of inflammatory cells that are involved in exacerbation of diseased airways [8]. Here we concentrate on migration of macrophages in an *in vitro* setting.

Development and function of macrophages

Since the early discovery of macrophages by Metchnikoff as he observed phagocytosis in starfish, they have been studied vigorously in processes related to cellular immunity. The functional significance of macrophages is complex, as they are mainly responsible for pathogen clearance and resolution of inflammation by way of phagocytosis and cytokine production [9]. Macrophages are classified as leukocytes or white blood cells and they are generated in the bone marrow derived from a myeloid lineage which differentiate into monocytes [10]. Monocytes are immune effector cells that circulate in the blood. Upon infiltration into tissues, monocytes differentiate into macrophages [11]. Unlike neutrophils which have a short tissue life span of 1 to 2 days, macrophages have a long tissue life span, up to many months under normal circumstances. Nonetheless, macrophages are elevated in the lungs of smokers and those patients with COPD, more specifically they accumulate in the alveoli, bronchioli, and small airways [12].

Macrophages are critical in the process of phagocytosis, which is the uptake of large particles ($>0.5\mu\text{m}$) into cells an actin-dependent mechanism. Moreover, macrophages are often referred to as “professional phagocytes” which are efficient in internalizing particles which are important aspects of tissue remodeling, immune response, and inflammation. The complete study of phagocytosis requires a collaboration of the mechanisms of signal transduction, actin-based motility-membrane trafficking, and infectious disease. The process of phagocytosis is complex due to a variety of receptors capable of stimulating phagocytosis and the variety of microbes that are involved in internalization of particles. However, most of the studies involving the signaling pathway leading to phagocytosis in macrophages

traditionally involves the opsonic receptors, including Fc receptors and complement receptors [13]. Professional phagocytes secrete a variety of important molecules, including cytotoxic radicals of oxygen and nitrogen, enzymes that degrade the extracellular matrix, lipid mediators of inflammation, and cytokines that influence phagocytosis and other processes [14]. The general shared features of complex phagocytic mechanisms are as follows: 1) Particle internalization is initiated by the interaction of specific receptors on the surface of the macrophage plasma membrane; 2) localized actin polymerization and extension of membrane pseudopodia around particles via actin-based mechanisms; 3) after internalization actin is shed from the phagosome and the phagosome matures by a series of fusion and fission events; 4) subsequent endosome-lysosome trafficking in association with microtubules together with phagosome maturation, which requires the coordinated interaction of the actin- and tubulin-based cytoskeleton [13, 15-16]. There is overlap in the function of macrophages as migrating cells and their prominent role as phagocytic cells, including the role of MARCKS protein and cytoskeletal proteins throughout the processes.

Myristoylated alanine-rich C kinase substrate

Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) which has long been identified as a well known substrate of protein kinase C (PKC) is thought to be involved in regulation of mucin secretion and inflammation in asthma. Since its discovery, MARCKS and MARCKS family proteins have been found to be widely expressed and largely associated with membranes in various cell types, which supports the ability of MARCKS to participate in a variety of regulatory roles. Structurally the MARCKS protein is rod-shaped

consisting of three highly conserved domains: the N-terminal domain, the multiple homology II (MH2) domain, and the phosphorylation site domain (PSD).

The identification of MARCKS protein dates back to 1982 when it was found that an '87 kDa' substrate in rat brain nerve endings could be regulated by calcium and calmodulin ($\text{Ca}^{2+}/\text{CaM}$) through the activation of PKC [17]. A similar substrate for PKC was purified from bovine brain and it was found to have widespread species, tissue, and subcellular distribution [18-19]. This '87 kDa' protein captured the interest of several groups and subsequently the protein was officially named Myristoylated Alanine Rich C Kinase Substrate (MARCKS) [20]. The classical members of the MARCKS family are: MARCKS, an 87 kDa protein ubiquitously expressed in bovine, chicken, mice, rat, cow, and human, and MARCKS related protein (MRP, also known as MacMARCKS, F52, or MLP), a 20 kDa protein highly expressed in brain, reproductive tissues, and macrophages [21-22]. This diverse family of abundant proteins with unique structures has several functions that play important roles in a variety of cellular processes.

MARCKS is an 87 kDa ubiquitously expressed PKC substrate, that contains three highly conserved regions. This rod-shaped protein contains three distinct domains: the N-terminal Myristoylated domain, the multiple homology domain 2 or the MH2 domain, and the phosphorylation site or effector domain. The N-terminus displays a consensus sequence for myristoylation, which is a co-translational lipid modification attaching a myristic acid. Myristic acid is a C_{14} saturated fatty acid that is attached via an amide bond to the amino group of the N-terminal glycine residue, which aids in the anchorage of MARCKS to the plasma membrane. The MH2 domain, of unknown function, resembles the cytoplasmic tail

of the cation-independent mannose-6-phosphate receptor. The PSD or effector domain is highly basic and is essential to the function of MARCKS, since it serves as the phosphorylation site as well as the site that binds and crosslinks actin filaments and also binds calcium and calmodulin. Overall, MARCKS is an acidic protein rich in alanine, glycine, proline, and glutamic acids which contribute to pI's ranging from 4.12 to 4.42 in various species [23].

Membrane interactions of MARCKS and actin

MARCKS has been known to bind plasma membranes of various cell types including macrophages [24] neurons [25] and fibroblasts [26]. It has been determined that this interaction is dependent upon two major features that are important characteristics of MARCKS. The MARCKS protein has the ability to bind membranes due to the myristate insertion hydrophobically into the phospholipid bilayer and electrostatic interaction of the basic effector domain with acidic phospholipids [27-29]. Both interactions are required, neither myristate nor electrostatic interactions alone are sufficient to anchor the protein to the plasma membrane [30-31].

The association with MARCKS protein to the plasma membrane is critical for the underlying mechanisms which ultimately lead to regulation and function. MARCKS cycles from the plasma membrane in various cell types [32-33]. Phosphorylation of MARCKS by PKC leads to the attachment of negatively charged phosphate groups to the serine residues within the effector region, which weakens the electrostatic interactions. Since myristoylation on its own is not sufficient enough to anchor the protein, MARCKS is released and moves

into the cytosol [34-35]. Subsequently dephosphorylation leading to the removal of phosphate groups by protein phosphatase I, protein phosphatase 2A, or calcineurin, allows MARCKS to return to the plasma membrane [31, 36-37]. This mechanism describes the reversible translocation of MARCKS yet there are other factors that may be involved and influence this model, such as calcium and calmodulin.

In addition to PKC, the MARCKS protein PSD domain is also a target for Ca^{2+} and calmodulin (CaM) binding. Upon activation due to increased Ca^{2+} concentrations, calcium-bound CaM is able to bind MARCKS, which aids in the release of MARCKS from the membrane both *in vitro* and *in vivo*. This process is reversible so once intracellular Ca^{2+} concentrations decrease CaM will not bind to MARCKS promoting the ability of MARCKS to bind to the membrane again [38-40]. There has been evidence to suggest that there is cross-talk between PKC and CaM via MARCKS protein. It has been found that CaM binds with high affinity to the effector region of MARCKS and MARCKS related protein [41-42], yet the phosphorylation of MARCKS by PKC significantly decreases this affinity [43-44]. Therefore, Ca^{2+} /CaM regulation in conjunction with PKC activation has an influence on MARCKS binding as well.

Another influence on the binding and subsequent translocation of MARCKS is the cytoskeletal protein actin. In fact, the discovery that MARCKS binds and cross-links actin was a profound contribution to the understanding of MARCKS. Additionally, both the phosphorylation of MARCKS by PKC and Ca^{2+} /CaM were found to inhibit cross-linking, connecting two possible signal pathways that control the downstream target, actin [45]. Although the general concept of actin binding and cross-linking was agreed upon, there were

several explanations of the particular mechanism by which they take place. The following explanations were given: 1) MARCKS may contain a single actin-binding site in the effector domain which allows for actin cross-linking via dimerization 2) MARCKS may contain two actin binding sites within the effector domain 3) The effector domain of MARCKS might bundle actin filaments by lowering the electrostatic repulsion between the filaments [46-49]. However, unlike the evidence found in MARCKS for cross-linking, in the case of MRP there is no such evidence for cross-linking [50]. Although it appears that even today there is still controversy over the mechanisms involved in the regulation of actin via MARCKS interactions, it is clear that actin is essential to the overall functioning of MARCKS.

MARCKS protein is a potential candidate as a major regulator of cell migration, as the pathway is linked and leads to regulation of the actin cytoskeleton which is prominent in movement of all cells. It has been demonstrated that inhibitors of PKC significantly inhibit chemotaxis of RAW 264.7 macrophage-like cells via regulation of MRP expression [51]. Recently, it has been shown that MARCKS has a role in regulation of human neutrophil migration. In fact, the MANS peptide inhibited the migration of isolated human neutrophils in response to chemoattractant stimuli [52]. Also, MANS peptide inhibited neutrophil elastase induced bronchoalveolar lavage (BAL) inflammation in a mouse bronchitis mouse model *in vivo* [53].

Inflammatory Mediators

Chronic inflammatory diseases of the airways, including asthma, involve the activation of many inflammatory and structural cells, which release inflammatory mediators that result in pathophysiological changes that ultimately can lead to damage or death. More than 50 different inflammatory mediators, cell products that are secreted and exert functional effects, have been identified in asthma. Inflammatory mediators have been classified into subcategories such as amine mediators, lipid-derived mediators, peptide mediators, cytokines, chemokines, proteases, and other small molecules. Regardless of the subcategory that the inflammatory mediator falls under, they ultimately are responsible for producing adverse effects in the airways, including bronchoconstriction, enhanced mucus secretion, increased thickness of the airway smooth muscle layer (hyperplasia) along with increase in their size (hypertrophy), and fibrosis [54]. Although several inflammatory mediators are essential during inflammatory responses, we focus specifically on chemokines and complement, which are peptide mediators.

Initially chemokines were identified as key regulators of leukocyte trafficking [55-56]. Chemokines are a family of small molecules with a molecular weight of 8-14 kDa. These low molecular weight secreted proteins play several roles, however they mainly control the migration of neutrophils, monocytes, macrophages, lymphocytes, antigen-presenting cells, and dendritic cells [57]. Chemokines can be divided into four subgroups, the CC, CXC, C, and CX₃C families, based on their genetic organization and the position of two highly conserved cysteine residues at the N-terminus [58]. Specifically, members of the CC family are known to primarily target monocytes and T cells, whereas CXC chemokines target

neutrophils. Within the subfamily of CC chemokines are monocyte/macrophage chemoattractant proteins (MCP). To date, five members of the family of monocyte/macrophage chemoattractant proteins have been identified MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), and MCP-5 (CCL12) [59].

MCP-1 (CCL2) has been described as the most potent monocyte/macrophage attractant, and was the first discovered human CC chemokine (although it was originally isolated from mouse 3T3 fibroblasts [60]). A variety of cell types produce MCP-1 either constitutively or after induction by oxidative stress, cytokines, or growth factors. Although many cell types such as epithelial, endothelial, smooth muscle, and fibroblasts all produce MCP-1, the major source is monocytes and macrophages. Once released from cells, MCP-1 exerts its effects by binding G-protein-coupled receptors on the surface of leukocytes. MCP-1 binds mainly to classic MCP-1 receptors known as CCR2 and CCR11; however, it has been shown to bind to alternative receptors such as D6, the Duffy antigen receptor for chemokines (DARC) and US28 [61]. In summary, MCP-1 and its receptors play vital roles in monocyte/macrophage recruitment during inflammatory responses.

As mentioned previously, another effective inflammatory mediator is complement, a well known peptide mediator. The complement system is comprised of a series of 30 distinct circulating proteins, including proteolytic proenzymes, nonenzymatic components that form functional enzymes when activated and corresponding receptors [62]. These enzymes induce inflammation in a cascade manner when activated by bacterial antigens and immune complexes. Subsequently, complement activation occurs via the classical, alternative, or lectin-mediated pathways and activated complement such as C3a and C5a help eliminate

foreign substances. Elimination occurs by inducing inflammatory reactions, phagocytic chemotaxis, and opsonisation [63-65]. Specifically, C5a is a 74-amino acid peptide with a molecular weight of 11.5 kDa with multiple functions in various cells [66]. Similarly to MCP-1, C5a binds to G-protein coupled receptors on the plasma membrane of various cells. The corresponding 7 transmembrane spanning receptors for C5a are C5aR (CD88) and C5L2 [67]. C5a has a functional role in mast cell degranulation, monocyte/macrophage migration, smooth muscle contraction, recruitment of immune cells, and increased vascular permeability at inflammatory sites [68]. Inflammatory mediators such as MCP-1 and C5a are essential promoters of migration that can aid in the further study of the underlying mechanisms of movement.

Mechanisms of cell migration

The fundamental processes underlying cell migration are complex, beginning with a cell's response to an external signal that leads to the polarization and the extension of a protrusion in the direction of movement [69-70]. In the case of macrophages, in which their primary function requires them to migrate in order to perform proficient phagocytosis, understanding this is important during an inflammatory response. Yet, there still remains much to be discovered regarding the molecular mechanisms that regulate such migration. Macrophages are responsive to a variety of signals including bacterial components, leukotrienes, complement factors, and most importantly chemokines which are a significant family of attractants controlling directed migration of macrophages toward chemotactic gradients, a process known as chemotaxis [71]. Macrophage Monocyte Chemotactic Protein (MCP-1)

which binds to the receptor CCR2 is one such potent chemokine that plays a crucial role in the recruitment of macrophages and various cell types under *in vitro* and *in vivo* conditions [72].

Transmembrane signal transduction has been extensively studied leading to discovery of several intracellular signaling pathways. It has been suggested that chemoattractants that bind receptors on the surface of cells ultimately activate protein kinase C (PKC) [56], which can phosphorylate MARCKS. In addition, there have been indications that phosphorylation of MARCKS protein can be the connection for some PKC effects on the cytoskeleton [73]. Along with the collaborative ability of MARKCS to serve as an important factor for the anchoring of the actin cytoskelton to the plasma membrane, there is the relationship of the phosphorylation of MARKCS leading to regulation of movement. Regulation of the cytoskeleton has been shown in myoblasts [74], and it is possible that macrophages employ a similar mechanism resulting in migration.

Conclusions

Macrophages are essential to the complex process of an immune response. The diverse functional roles of macrophages during a defensive response are critical for resolution of inflammation to prevent development of chronic airway diseases. In events in which inflammation potentially gets out of control, it is necessary to prevent the accumulation of macrophages so that irreversible damage may be avoided. It is important to understand the key players and mechanisms that influence and control the regulation of macrophage migration. In this work, I investigate on a molecular level the downstream effects of

chemoattractant stimulation on macrophage migration. In Chapter III, I explore the role of MARCKS protein in macrophage migration and look at the interactions and distribution of MARCKS and actin within macrophages.

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CHAPTER II. REGULATION OF MUCIN SECRETION AND INFLAMMATION IN
ASTHMA; A ROLE FOR MARCKS PROTEIN?

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Abstract

A major characteristic of asthmatic airways is an increase in mucin (the glycoprotein component of mucus) producing and secreting cells, which leads to increased mucin release that further clogs constricted airways and contributes markedly to airway obstruction and, in the most severe cases, to status asthmaticus. Asthmatic airways show both a hyperplasia and metaplasia of goblet cells, mucin-producing cells in the epithelium; hyperplasia refers to enhanced numbers of goblet cells in larger airways, while metaplasia refers to the appearance of these cells in smaller airways where they normally are not seen. With the number of mucin-producing and secreting cells increased, there is a coincident hypersecretion of mucin which characterizes asthma. On a cellular level, a major regulator of airway mucin secretion in both *in vitro* and *in vivo* studies has been shown to be MARCKS (Myristoylated Alanine-Rich C Kinase substrate) protein, an ubiquitous substrate of protein kinase C (PKC). In this review, properties of MARCKS and how the protein may regulate mucin secretion at a cellular level will be discussed. In addition, the roles of MARCKS in airway inflammation related to both influx of inflammatory cells into the lung and release of granules containing inflammatory mediators by these cells will be explored.

Introduction

In asthma and many other chronic respiratory diseases, excessive mucus production and airway inflammation can ultimately contribute to morbidity and mortality in many patients. Subsequently, the development of drugs that inhibit overproduction of mucus and chronic inflammation are necessary. Although some conventional therapies, such as anticholinergics, β -2-adrenoceptor agonists, and corticosteroids are available, they have variable effectiveness. In recent years, MARCKS (Myristoylated Alanine Rich C-Kinase Substrate) protein has emerged as a new target for inhibition of mucus hypersecretion and inflammation. This review will focus on MARCKS and its apparent roles in both secretion and inflammation, making it an attractive potential therapeutic target for asthma and other respiratory diseases characterized by mucus hypersecretion and inflammation.

The identification of MARCKS protein dates back to 1982 when it was found that an '87 kDa' substrate in rat brain nerve endings could be regulated by calcium and calmodulin ($\text{Ca}^{2+}/\text{CaM}$) through the activation of PKC [1]. A similar substrate for PKC was purified from bovine brain and it was found to have widespread species, tissue, and subcellular distribution [2-3]. This protein captured the interest of several groups and subsequently the protein was officially named Myristoylated Alanine Rich C Kinase Substrate (MARCKS) [4]. The classical members of the MARCKS family are: MARCKS, an 87 kDa protein ubiquitously expressed in bovine, chicken, mouse, rat, cow, and human, and MARCKS related protein (MRP, also known as MacMARCKS, F52, or MLP), a 20 kDa protein highly expressed in brain, reproductive tissues, and macrophages [5-6]. This diverse family of abundant proteins with unique structures has several functions that play important roles in a variety of cellular processes.

STRUCTURE & MEMBRANE BINDING

MARCKS has been identified as an 87 kDa substrate for Protein Kinase C (PKC) that is expressed ubiquitously in eukaryotic cells. This rod-shaped protein contains three distinct evolutionarily-conserved regions: the N-terminal myristoylated domain, the multiple homology2 (MH2) domain, and the phosphorylation site domain (PSD) or effector domain. The N-terminus displays a consensus sequence for myristoylation, which is a co-translational lipid modification attaching a myristic acid. Myristic acid is a C₁₄ saturated fatty acid that is attached via an amide bond to the amino group of the N-terminal glycine residue, which aids in the anchorage of MARCKS to the plasma membrane. The amino terminus makes up the first 24 amino acids of MARCKS. The highly basic PSD domain consists of 25 amino acids containing several serine residues that can be phosphorylated by PKC. It also serves as the site for MARCKS binding and cross-linking actin filaments and binding to calcium/calmodulin. The MH2 domain, of unknown function, resembles the cytoplasmic tail of the cation-independent mannose-6-phosphate receptor. Overall, MARCKS is an acidic protein rich in alanine, glycine, proline, and glutamic acids which contribute to pI's ranging from 4.12 to 4.42 in various species [7].

MARCKS binds to plasma membranes in various cell types including macrophages [8] neurons [9] and fibroblasts [10]. It has been determined that this interaction is dependent upon two major features that are important characteristics of MARCKS; the protein has the ability to bind to membranes due to the myristate insertion hydrophobically into the phospholipid bilayer, as well as electrostatic interactions between the basic PSD and acidic head groups of membrane phospholipids [11-13]. Both interactions are required, since neither myristic acid insertion alone nor electrostatic interactions are sufficient to anchor the protein to the plasma membrane [14-15].

The association with MARCKS protein with the plasma membrane is critical for the underlying mechanisms which ultimately lead to regulation and function. MARCKS cycles from the plasma membrane to the cytoplasm in various cell types [16-17]. Phosphorylation of MARCKS by PKC leads to the attachment of negatively charged phosphate groups to serine residues within the effector region, which weakens the electrostatic interactions. Since myristoylation on its own is not sufficient to anchor the protein, MARCKS is released and moves into the cytosol [18-19]. Subsequently dephosphorylation leading to the removal of phosphate groups by protein phosphatase I, protein phosphatase 2A, or calcineurin allows MARCKS to return to the plasma membrane [15, 20-21]. Other factors, such as binding of MARCKS to calcium and calmodulin, may further influence these reactions and MARCKS translocation.

As alluded to earlier, in addition to PKC, the MARCKS PSD is also a target for Ca^{2+} and calmodulin (CaM) binding. Upon activation due to increased Ca^{2+} concentrations, calcium-bound CaM is able to bind MARCKS, which aids in the release of MARCKS from the plasma membrane. This process is reversible, so once intracellular Ca^{2+} concentrations decrease, CaM will not bind to MARCKS, facilitating binding of MARCKS to the plasma membrane again [22-24]. There has been evidence to suggest that there is cross-talk between PKC and CaM via MARCKS protein. It has been found that CaM binds with high affinity to the effector region of MARCKS and MARCKS related protein [25-26], yet the phosphorylation of MARCKS by PKC significantly decreases this affinity [27-28]. Therefore, Ca^{2+} /CaM regulation in conjunction with PKC activation can influence MARCKS binding characteristics.

Another influence on binding and subsequent translocation of MARCKS is the cytoskeletal protein actin. In fact, the discovery that MARCKS binds and cross-links actin was a profound contribution to the understanding of MARCKS function in cells. Additionally, both the

phosphorylation of MARCKS by PKC and Ca^{2+} /CaM were found to inhibit cross-linking, connecting two possible signaling pathways that control the downstream target, actin [29].

Although the general concept of actin binding and cross-linking is agreed upon, there are several explanations of the particular mechanism by which this takes place. Firstly, MARCKS may contain a single actin-binding site in the effector domain which allows for actin cross-linking via dimerization. Secondly, MARCKS may contain two actin binding sites within the effector domain, and thirdly the effector domain of MARCKS might bundle actin filaments by lowering electrostatic repulsion between the filaments [30-33]. These mechanisms are suggested for MARCKS, but in the case of Marcks-related proteins (MRP), there is no such evidence for cross-linking [34]. While there is still some controversy over the mechanisms involved in the regulation of actin via MARCKS interactions, it is clear that actin is essential to the overall functioning of MARCKS.

MUCUS, INFLAMMATION, AND ASTHMA: A ROLE FOR MARCKS

MARCKS protein has a prominent role in regulation of secretion in various cell types. Previous experiments revealed that the phosphorylation of MARCKS mediated by PKC was important in neurotransmitter release [35]. Similarly, MARCKS was pivotal in glucose-induced secretion in isolated rat pancreatic islets [36]. It was also found that, upon stimulation, the phosphorylation of MARCKS caused rapid and early release of adrenocorticotropin (ATCH) in ovine anterior pituitary cells [37], and stimulation of platelets by thrombin induced MARCKS phosphorylation and serotonin release [38].

In studies from this laboratory, MARCKS protein has been found to be a key molecule regulating mucin secretion in airway epithelial cells. Pivotal to these studies was utilization of a

synthetic peptide corresponding to the first 24 amino acids of the N-terminal region of MARCKS, named the MANS (Myristoylated N-terminal sequence) peptide. This peptide was developed at a time when there were no known reagents to inhibit MARCKS function, and the MANS peptide had profound effects on a number of cell functions, while a control missense peptide consisting of the same amino acids but arranged randomly (the RNS peptide) was without effect. In the first published studies using well-differentiated normal human bronchial epithelial (NHBE) cells in vitro in an air/liquid interface system, pretreatment of the cells with MANS, but not RNS, resulted in attenuation of mucin hypersecretion in these cells in response to PKC activation [39], implicating MARCKS protein in the secretory response. In addition, the cycles of MARCKS phosphorylation/dephosphorylation and binding to actin and myosin were shown to be critical to the secretory response.

We then turned our attention to mucin hyperproduction and secretion in asthma. Goblet cell hyperplasia and metaplasia are well-characterized features of the asthmatic airway, and we looked at the possibility of the MANS peptide affecting mucin secretion in a well-defined model of allergic inflammation, the ovalbumin (OVA) sensitized mouse. OVA sensitized and challenged mice develop a goblet cell metaplasia and, in response to methacholine challenge, secrete large amounts of mucin. However, pretreatment of these mice with the MANS, but not RNS peptide, for 15 min via intratracheal instillation attenuated, in a concentration-dependent manner, mucin secretion into the airway lumen in response to methacholine aerosol. This study implicated MARCKS in the process of mucin secretion *in vivo* in allergically inflamed mouse airways [40].

Additional studies with this model were performed in which inhibition of mucin secretion *in vivo* in OVA sensitized and challenged mice in response to intratracheal administration of the

MANS peptide was again demonstrated. Of great interest, these studies also showed that inhibition of mucin secretion via treatment with the MANS peptide correlated with a substantial and significant lowering of airway obstruction and resistance when the mice were subjected to pulmonary function testing [41]. Similar effects of MANS on mucin secretion and pulmonary function were demonstrated in work using the elastase instillation model of airway inflammation and goblet cell hyperplasia/metaplasia in mice [42]. Finally, in a translational study in which human airway epithelial cells were derived from asthmatic patients, the MANS peptide was shown to inhibit expression of the mucin gene, MUC5AC, in epithelium from these patients (but not in epithelial cells from control non-asthmatic patients) after infection with *Mycoplasma pneumonia* [43].

The precise intracellular mechanisms by which MARCKS regulates secretion have not been fully elucidated, but additional studies from this laboratory have implicated the PKC• isoform [44], chaperone proteins such as cysteine string protein (CSP) and heat shock protein 70 (HSP70) [45] and a novel non-muscle myosin isoform, myosin V [46] in the MARCKS-regulated mechanism.

MARCKS AND INFLAMMATION

Excessive inflammation is a key component of asthma and other airway diseases, including chronic bronchitis, bronchiectasis, and cystic fibrosis. Influx into the lung and airways of neutrophils, eosinophils, and other leukocytes can result in severe tissue damage. Since we had shown that mucin secretion was regulated by MARCKS and could be alleviated by treatment with the MANS peptide, we speculated that perhaps degranulation of inflammatory cells, a process similar to mucin secretion in that membrane-bound granules are released by cells, also could be linked to a MARCKS-dependent mechanism. In studies with isolated human neutrophils

and other human leukocyte cell lines, release of myeloperoxidase from neutrophils, eosinophil peroxidase from the eosinophil-like cell line HL-60 clone 15, lysozyme from the monocytic leukemia cell line U937, and granzyme from the lymphocyte natural killer cell line NK-92 were each attenuated by pre-incubation of the cells with MANS but not with the missense control peptide. The results indicate that MARCKS protein also may play an important role in degranulation of leukocytes, and thus inhibition of MARCKS could be anti-inflammatory [47].

Another potential therapeutic target in asthma and other inflammatory airway diseases is actual migration of leukocytes, especially neutrophils and eosinophils, into the lung in response to chemoattractants released in the airway, such as IL-8. Since MARCKS is known to be an actin-binding protein, it was examined as a potential regulator of neutrophil migration towards a chemoattractant. The MANS peptide, but not the RNS peptide, was shown to attenuate migration of isolated human neutrophils *in vitro* towards the chemoattractants fmlf, IL-8 or LTD4 [48]. Furthermore, pretreatment of mice with the MANS, but not RNS peptides, greatly attenuated the increase in levels of pro-inflammatory cytokines (IL-6, KC) and neutrophil migration in a mouse model of airway inflammation induced by exposure to ozone [49]. The same results were seen in the elastase model of airway inflammation in mice, where levels of inflammatory cytokines (KC, IL-6, IL-1 α , TNF α , MCP-1) as well as influx of neutrophils, eosinophils and lymphocytes, were significantly decreased in BAL fluid of animals pretreated with the MANS peptide [42]. Thus, MARCKS protein appears to play an important role in both migration of inflammatory cells into the lung and release of granule-stored inflammatory mediators by these cells.

CONCLUSIONS

Asthma and other inflammatory airway diseases are characterized by excess mucin production and secretion as well as influx of inflammatory cells into the lungs and airways and increased levels of pro-inflammatory cytokines and mediators. In both of these processes, mucin production and secretion as well as inflammatory cell influx and release of inflammatory mediators, MARCKS protein appears to play a major role, as inhibition of MARCKS function with a peptide identical to the MARCKS N-terminus attenuates significantly, and in some cases dramatically, all of these processes *in vitro* and *in vivo*, and also enhances lung function in murine models of asthma and allergic inflammation. Thus, MARCKS represents a potentially important therapeutic target for treatment of airway diseases characterized by mucus hypersecretion and inflammation. The mechanisms of MARCKS protein regulation of both of these processes are still under investigation, and certainly several other proteins that may associate or interact with MARCKS no doubt are integrally involved in these responses; their exact contributions and interactive dynamics remain to be elucidated.

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CHAPTER III. MARCKS PROTEIN IS INVOLVED IN REGULATION OF MIGRATION
OF MURINE MACROPHAGES *IN VITRO*

(Manuscript in preparation for *Respiratory Research*)

ABSTRACT

Uncontrolled inflammation often can be a fatal pathological feature in several airway diseases, with neutrophils and macrophages serving as key contributing inflammatory cells. We have previously shown that a peptide identical to the N-terminal sequence of the myristoylated alanine-rich C kinase substrate (MARCKS protein) blocks directed migration of human neutrophils *in vitro*. Here, we investigated whether or not this MARCKS-related peptide (the MANS peptide) could affect migration of macrophages as well, using the macrophage-like J774A.1 cell line and primary murine macrophages. We used a transwell migration assay to determine if these cells migrate in response to the chemoattractant macrophage/monocyte chemotactic protein (MCP-1; 25-100ng/ml). Cells were pre-incubated (15 min) with MANS or a missense control peptide (RNS), both at 50 μ M, and effects on migration determined at 3 hours. The results showed that the MANS, but not RNS, attenuated macrophage migration in response to MCP-1. Additionally, MARCKS was rapidly phosphorylated within these cells after exposure to MCP-1. Using confocal microscopy, we followed the movement and interactions of MARCKS and actin, visualizing them with a fluorescently-labeled antibody to MARCKS and Alexa Fluor 488 Phalloidin for identifying actin. Exposure of cells to MCP-1 resulted in translocation of MARCKS from plasma membrane to cytosol within a few minutes, while actin spread out through the cell and cell protrusions. The results suggest that MARCKS protein is involved in migration of macrophages, and is phosphorylated by exposure of these cells to the chemoattractant MCP-

1. It would appear that this phosphorylation of MARCKS is involved integrally in directed migration of macrophages.

INTRODUCTION

In several airway diseases such as chronic bronchitis, asthma, and cystic fibrosis the inflammatory component is a major contributor that leads to airway obstruction and lung function complications. During an inflammatory response macrophages migrate to infected or injured areas in response to pro-inflammatory signaling molecules including cytokines and chemokines. However, uncontrolled recruitment produces inappropriate inflammation although mechanisms and potential therapeutic targets have not been determined. Macrophages are the predominant cells in the bronchoalveolar spaces in individuals with and without asthma. However, the absolute number of airway macrophages is significantly increased in asthma. The control of macrophage accumulation may be important in regulating the severity of airway inflammation [75].

MARCKS (Myristoylated Alanine-Rich C Kinase Substrate), an important substrate for PKC, is a 87kDa protein that is highly conserved and known to be involved in several important cellular processes such as membrane trafficking, phagocytosis, and cell movement in general. MARCKS protein contains three highly conserved domains the N-terminal domain, the MH2 domain, and the phosphorylation site domain (PSD), with the N-terminus and PSD having important roles in MARCKS [76]. Previous work from our lab has shown that MARCKS is a key molecule regulating mucin secretion in airway epithelial cells. A

synthetic peptide corresponding to the first 24 amino acids of the amino (N) terminal region of MARCKS, referred to as Myristoylated N-terminal sequence, or MANS peptide, blocked secretion in these cells [77-79]. Additionally, we demonstrated that MARCKS plays a role in secretion of membrane bound granules in various leukocyte cell lines [80]. More recently it has been demonstrated that MARCKS protein also plays a prominent role in the regulation of neutrophil migration in vitro [81].

The concept of cell migration is rather complex, yet there are some key aspects such as cell morphology, adhesion dynamics, and actin polymerization with which MARCKS has been associated [69-70]. During embryogenesis, regulation of actin cytoskeletal dynamics ultimately controls movement and thus is critical to the process. It has been shown that MARCKS function is essential in cortical actin formation during gastrulation movements associated with *Xenopus* embryogenesis [82]. MARCKS also participates in the regulation of another important aspect of cell motility, is cell adhesion, in human embryonic kidney cells [83], while MacMARCKS regulates the cytoskeletal-integrin link in J774A.1 mouse macrophage cells [84-85]. It has also been reported that MARCKS and PKC regulate actin-dependent cell spreading and cell migration in various cell types [86-87].

There are several studies that link MARCKS protein functionally to cell motility, yet MARCKS does not act alone. The actin cytoskeletal network is also required to regulate intracellular and extracellular movement. An aim of this study was to determine if MARCKS has a specific role in regulating migration of macrophages. Here we stimulated macrophage migration with chemoattractant proteins MCP-1 and C5a, then utilized the cell-permeant MANS peptide to investigate macrophage migration. We hypothesized that

phosphorylation of MARCKS in response to chemotactic stimuli causes translocation of MARCKS from plasma membrane to cytoplasm, where it is linked to association, spreading, and crosslinking of actin. We also looked at the distribution and interactions of MARCKS and actin in response to chemoattractant stimulation. Our results using the mouse J774A.1 macrophage cell line and primary mouse macrophages indicate that MANS peptide, but not the missense control RNS peptide, inhibits chemoattractant-induced macrophage migration. The results suggest that MARCKS protein is involved in migration of macrophages, and is phosphorylated by exposure of these cells to the chemoattractant MCP-1. It would appear that this phosphorylation and resultant cytosolic translocation of MARCKS is involved integrally in directed migration of macrophages.

MATERIALS AND METHODS

Materials

The mouse Macrophage Cell line J774A.1 was obtained from American Type Culture Collection, ATTC (Manassas, VA). Dulbecco's Modified Eagle's Medium, DMEM and RPMI 1640 were from Mediatech, Inc. (Herndon, VA). Fetal bovine serum (FBS) and penicillin-streptomycin were from Fisher (Pittsburgh, PA), and amphotericin B from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was from Gemini Bio-Products (West Sacramento, CA). Transwell plates with inserts were from Corning, Inc. (Corning, NY) and rat tail collagen type I was from BD Biosciences (San Jose, CA). Recombinant mouse MCP-1 and C5a were from R&D Systems (Minneapolis, MN). The MANS and RNS peptides were synthesized by Genemed Synthesis Inc. (San Francisco, CA). The Diff-Quick® Stain Set was purchased from Dade Behring Inc. (Newark, DE). The Bradford Assay Reagent and trans-blot nitrocellulose membranes were from Bio-Rad Laboratories (Hercules, CA). For Western blot analysis of MARCKS expression in J774A.1 cells, the MARCKS antibody was purchased from Upstate (Lake Placid, NY). Antibodies for phosphorylated MARKCS, horseradish peroxidase-conjugated •-rabbit IgG, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA). The enhanced chemiluminescence development kit was from GE Life Science Products (Piscataway, NJ). Alexa Fluoro 488 and TO-PRO-3 iodine were from Molecular Probes (Eugene, OR). Dako mounting medium was from Dako North America Inc. (Carpenteria, CA). All other chemical reagents were purchased from Sigma (St. Louis, MO).

Peptides

The MANS and RNS peptides were synthesized by Genemed Synthesis Inc. (San Francisco, CA). The MANS peptide is identical to the first 24 amino acids of MARCKS: MA-GAQFSKTAACKGEAAAERPGEAAVA (MA=N terminal myristate acid). The RNS control peptide contains the same amino acids as MANS but in random sequence: MA-GTAPAAEGAGAEVKRASAEAKQAF. The peptides have been previously described [78].

Cell Culture

Mouse macrophage J774A.1 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin 100µg/ml streptomycin, 0.25µg amphotericin B. Cells were seeded in T75 tissue culture flasks and cultured at 37°C in an atmosphere containing 5% CO₂ until cells reached ~80% confluency. Cell viability was >90%, as assessed using the Cellometer™ by trypan blue dye exclusion.

Mice

This study was approved by the Institutional Animal Care and Use Committee of North Carolina State University (Raleigh, NC). CD-1 mice were purchased from Charles River (Wilmington, Massachusetts). The mice were 6-10 wks old and were maintained under proper animal housing conditions and provided with food and water *ad libitum*.

Isolation of Lung Murine Macrophages

The animals were heavily sedated with ketamine (100 mg/kg body weight; Vedeco Inc., St. Joseph, MO) and exsanguinated by cutting the left renal artery and abdominal aorta. The abdomen was carefully exposed by cutting through the ribcage without damaging the lungs. Briefly, red blood cells were flushed using a 10ml syringe and a 21 g needle used to inject 5-10ml of 0.9% sterile NaCl through the right ventricle of the heart. Surgical exposure of the trachea was performed and a 20 g catheter was inserted into the trachea. Next, the lungs were filled with 3ml of dispase through the catheter, followed immediately by instillation of 0.45 ml agarose into the lung through the catheter, and then the thorax was covered with crushed ice for 2 min to solidify the agarose. Carefully, the trachea was sealed with surgical silk and the heart and lungs were removed from the body, then rinsed with 5 ml sterile PBS. The heart was removed and lungs transferred to 2ml of dispase in a sterile culture tube and then placed in the 37° C water bath for 30 min. The lung was placed in 7ml of freshly prepared DMEM with 100 ul of 0.7% DNase I type II, then put into a 60 mm Petri dish, and then carefully teased apart and gently swirled for 5-10 min to release cells. Next, the mixture was filtered through a 100 micron sterile cell filter into a 50 ml Falcon tube, then centrifuged at 1000 rpm for 12 min at 4°C. The supernatant was aspirated and the cell pellet was re-suspended in 10 ml of DMEM with 10% FBS then added to the dish and incubated for 30 min at 37°C with 5% CO₂. Macrophages stuck to plastic, while unattached cells were washed away 2X with sterile PBS, then fresh media was added to the cells. To ensure that the suspension cell preparation was enriched with lung macrophages, differential staining on Cytospin cells was performed. Cells were stained with Diff Quick for differential cell

counting, and counted under a light microscope. Viability of isolated lung macrophages was assessed using the Cellometer™ by trypan blue dye exclusion and was >90%.

Transwell Migration Assay

Migration assays were performed in transwell plates 6.5mm in diameter with 8- μ m pore filters. The upper side of the transwell insert was thinly coated for 1 hr with rat tail type I collagen. Murine macrophages (1×10^6 /ml) resuspended in chemotaxis buffer (RMPI 1640 plus 0.02% BSA) were incubated with MANS or RNS peptide (50 μ M) for 15 min at 37°C. Untreated or pre-treated J774A.1 cells (100 μ l) were added to the upper chamber and 600 μ l of migration medium with or without chemotactic factor MCP-1(25ng/ml, 50ng/ml, 100ng/ml), PMA (10nM, 50nM, 100nM), or C5a (5ng/ml, 10ng/ml, 20ng/ml). Cells were allowed to migrate through the insert membrane for 3 hours at 37°C under a 5% CO₂ atmosphere. The inserts were then washed with PBS, and non-migrating cells remaining on the upper surface of the insert were removed with a cotton swab. The migrated cells on the insert were fixed, stained with Diff-Quick, and mounted on glass slides. Migration was measured visually by counting using a light microscope at 40X magnification. The mean number of cells of ten randomly chosen fields was calculated for each treatment. A migration index was calculated by dividing the number of migrated cells in response to the chemokine by the number of cells that migrated randomly (RPMI/0.02%BSA) with a reference index exceeding 1 indicating chemotaxis.

Western Blotting

Protein expression of MARCKS and phosphorylated MARCKS was measured by Western blot. Unstimulated and MCP-1 (100ng/ml), PMA (100nM), or C5a (10ng/ml) stimulated J774A.1 cells were washed with PBS, scraped, lysed in lysis buffer, sonicated, then centrifuged at 14,000 X g for 15 minutes @ 4°C. The protein concentrations of cell lysates were quantified by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Proteins were denatured by boiling in 2X SDS sample buffer for 5 minutes. The sample lysates (30µg) were loaded on 4-15% SDS polyacrylamide gels and then transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked with 5% nonfat milk, then incubated in primary antibodies to MARCKS and phospho - MARCKS. Specific bands were visualized after incubation with secondary antibodies, either mouse or rabbit anti-mouse IgG conjugated to horseradish peroxidase, by enhanced chemiluminescence followed by exposure to HyBlot film (Denville Scientific, Metuchen, NJ). The optical density of protein bands from Western blotting was analyzed by LabWorks software (Upland, CA) data not shown.

Immunofluorescence

J774A.1 cells were seeded on glass coverslips that were placed in 6 well tissue culture plates. The cells were treated with vehicle or MCP-1 (100ng/ml) for 1.5, 3, 5, or 10 minutes, then washed with PBS, and fixed in 4% paraformaldehyde in PBS for 20 minutes. Cells were blocked in 10% normal donkey blocking serum with 1.0% Triton X in PBS for 1 hr at room temperature. For MARCKS staining, the cells were incubated in primary MARCKS antibody overnight at 4°C, washed with PBS, then incubated in secondary donkey anti-

mouse fluorescent (red) antibody for 1 hour at room temperature. For actin staining, cells were incubated in phalloidin with Alexa 488 (green) for 30 minutes, and for nuclear staining with TO-PRO-3 iodine (blue) for 15 minutes, then washed with PBS. Stained cells were mounted on glass slides using Dako mounting medium. Confocal imaging was conducted using a C1 Nikon confocal microscope at the Laboratory for Advanced Electron and Light Optical Methods at North Carolina State University College of Veterinary Medicine.

Statistical Analysis

The experiments shown are a summary of the data from at least three experiments and presented as mean \pm SEM. Statistical significance of the data was determined using one-way ANOVA; $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity

All reagents at the concentrations used were tested for cytotoxicity using a Promega Cytotox 96 non radioactive cytotoxicity assay kit according to the manufacturer's instructions. The data were expressed as the ratio of released lactate dehydrogenase to total lactate dehydrogenase. There was little to no cytotoxic effect observed for any of the reagents at the concentrations used (data not shown).

MCP-1, PMA, and C5a are chemoattractant agents for J774A.1 cells.

We tested the effects of various concentrations of MCP-1 (25ng/ml, 50ng/ml, 100ng/ml), PMA (10nM, 50nM, 100nM), and C5a (5ng/ml, 10ng/ml, 20ng/ml) on J774A.1 cell migration after 3 hours in an *in vitro* transwell migration assay. A concentration - dependent stimulation of J774A.1 cell migration in response to MCP-1 was shown (Figure 1A). MCP-1 at 50ng/ml or 100 ng/ml significantly increased migration of J774A.1 cells toward the lower surface of the polycarbonate membrane through 8- μ m pores ($P < 0.01$) compared with medium alone. Figure 1B shows that PMA also stimulated concentration dependent migration by J774A.1 cells at 50nM and 100nM. In response to C5a (5ng/ml-20ng/ml), Figure 1C shows that J774A.1 cells responded significantly with a peak response at 10ng/ml. MCP-1, PMA, and C5a all enhanced directed migration of J774A.1 cells above control (random migration).

Effect of MANS peptide on MCP-1, & C5a induced J774A.1 cell migration.

Next we examined the effect of MANS peptide (50 μ M) on MCP-1 induced cell migration at 3 hours. Figure 2 shows micrographs of representative fields of cells that migrated in response to MCP-1 corresponding to data in Figure 3. Pre-treatment (15 min) with MANS peptide but not the RNS (control) peptide resulted in inhibition of MCP-1 induced migration (Figure 3A&3B). The MANS peptide also inhibited migration in response to C5a (10ng/ml) as illustrated in Figure 3C. In an additional study, we also found that MANS peptide inhibits the migration of 3T3 fibroblasts in response to C5a (data not shown).

Effects of MCP-1, PMA, & C5a on MARCKS phosphorylation in J774A.1 cells.

MCP-1 was used to determine if addition of a chemoattractant agent would affect MARCKS phosphorylation, since MCP-1 and other chemokines have been shown to activate PKC [84]. Stimulation of J774A.1 cells with 100ng/ml of MCP-1 for 10 to 180 sec resulted in rapid phosphorylation followed by dephosphorylation of MARCKS (Figure 4). PMA (100nM) which is known to provoke phosphorylation of MARCKS in various cell types, also stimulated a rapid phosphorylation followed by dephosphorylation of MARCKS, as did C5a (10ng/ml).

MANS peptide inhibits migration of primary macrophages in response to MCP-1.

The optimal concentration of MCP-1 to effectively induce migration of isolated primary mouse macrophages was determined to be between 25 and 100 ng/ml (Data not shown). We then evaluated the ability of MANS peptide to attenuate macrophage migration in response to MCP-1 100ng/ml. Isolated macrophages were pretreated with 25 μ M, 50 μ M, or 100 μ M MANS for 15 minutes and effects on directed migration measured. As shown in Figure 5, MANS significantly ($p < 0.05$) inhibited the ability of the cells to migrate into the lower wells toward MCP-1, whereas the RNS control peptide at both 25 μ M and 50 μ M did not. (However, the RNS peptide at 100 μ M did have an inhibitory effect). These results suggest that MANS peptide prevents migration of isolated primary lung macrophages.

Effect of MCP-1 and C5a on MARCKS phosphorylation in primary macrophages.

The protein expression of phosphorylated MARCKS after stimulation by MCP-1 or C5a was investigated by Western blotting. Unstimulated CD-1 macrophages did not express detectable amounts of phosphorylated MARCKS protein. Cells stimulated with MCP-1 (100ng/ml) expressed enhanced amounts of P-MARCKS at 60 seconds. When cells were stimulated with C5a (10ng/ml) rapid phosphorylation of MARCKS was also detected at 30 and 60 sec time points (Figure 6).

Effect of MCP-1 on MARCKS movement in J774A.1 cells.

It has been previously shown that phosphorylation of MARCKS can result in its translocation from the plasma membrane to the cytosol. To determine whether this occurs in macrophages under the influence of MCP-1, we visually analyzed the response within J774A.1 cells. Cells treated with 100ng/ml of MCP-1 appeared to translocate within time points from 1.5 to 10 minutes (Figure 7). Although MARCKS appears to be abundant throughout the cell, the results suggest that MARCKS translocates in response to MCP-1. In addition, we found that actin rapidly responds to MCP-1 stimulation by spreading and extending to the cell periphery (Figure 8). Therefore, MARCKS translocates while actin appears to spread within the cells in response to MCP-1 and, presumably, in response to phosphorylation of MARCKS.

DISCUSSION

In this study, we demonstrate that, *in vitro*, the MANS peptide inhibits migration of J774A.1 cells and primary murine macrophages in response to MCP-1 supporting the hypothesis that MARCKS protein is involved in regulation of macrophage movement. The effect is paralleled by the rapid phosphorylation of MARCKS, which is responsible for the translocation of MARCKS from plasma membrane into the cytosol, possibly influencing actin association and movement. Furthermore, we demonstrate that MCP-1 directly influences MARCKS and actin, which may serve as part of a mechanism for regulating cell movement. The ability of the MANS peptide to inhibit the migration of murine macrophages provides strong evidence for a role for MARCKS in macrophage directed migration.

It is well known that chemoattraction of macrophages and neutrophils into tissues is an essential step in the host response to infection, however the adverse effects of exacerbated inflammation can cause severe damage. The forward movement of macrophages in response to chemoattractant stimulation involves: protrusion of filopodia and lamellipodia at the leading front, adhesion of the protruding edge to the substratum via focal complexes, contraction of the cytoplasmic actomyosin, and finally release from contact sites at the tail of the cell [71, 88]. It is important to fully understand the molecular events necessary to allow movement, with an eye towards developing potential molecular targets to regulate migration. Previous studies have demonstrated that MANS peptide attenuates neutrophil migration in response to fMLP, IL-8, or LTB₄ [81]. The chemokine MCP-1 is typically expressed in tissues during inflammation and is induced in a variety of cell types by proinflammatory

mediators such as TNF- α , IL-1 and endotoxins [59, 89]. Another prominent chemokine is C5a which exerts its chemotactic effect on various immune cells, including monocytes and endothelial cells [90-91]. In this study we utilized MCP-1 and C5a to induce the migration of J774A.1 cells and primary macrophages *in vitro* (Figure 1). Both MCP-1 and C5a exert effects through G-protein coupled receptors, specifically MCP-1/CCR2 and C5a/C5aR. These receptors, once activated, trigger a set of cellular reactions that result in inositol triphosphate formation, intracellular calcium release, and PKC activation [92]. Once directed migration toward chemoattractants was established, the next set of experiments determined the effects on migration of pretreatment of cells with the MANS peptide. This study provides evidence that MANS peptide also inhibits macrophage migration in response to MCP-1, PMA, and C5a (Figures 1-3).

In terms of transmembrane signal transduction it has been previously shown that when chemokines such as MCP-1 bind receptors on the surface of cells, this activates protein kinase C [56], leading to MARCKS phosphorylation, which promotes changes in the cytoskeleton and facilitates or drives cell spreading and migration [73]. Elevating levels of PKC promotes migration of endothelial cells [93] and colon carcinoma cells [94], although the phosphorylation target of PKC in these cells was not examined. As expected in our studies, stimulation of J774A.1 cells or primary murine macrophages with MCP-1, PMA, or C5a caused the rapid phosphorylation of MARCKS (Figures 4, 6) presumably via activation of PKC and correlating with enhanced migration.

It has previously been demonstrated in muscle cells that integrin-mediated cell spreading is regulated by the ability of MARCKS to translocate sequentially between the membrane

and cytosol, thereby controlling the dynamics of actin cytoskeletal organization [74]. Our studies suggest that J774A.1 cells respond to MCP-1 in a similar manner. Immunofluorescent experiments showing the distribution of MARCKS and actin in response to MCP-1 (Figures 7, 8) suggest a correlation between the phosphorylation of MARCKS and downstream effects on the cytoskeleton.

In conclusion, we demonstrate that the MANS peptide inhibits directed migration of J774A.1 cells and primary murine macrophages in response to MCP-1, PMA, and C5a. Our study provides support for a signaling pathway involving the phosphorylation of MARCKS by PKC followed by translocation leading to regulation of cell spreading and migration. We speculate that MANS peptide competes with the ability of the N-terminal region of endogenous MARCKS to bind to the inner face of the plasma membrane in these cells and to function normally in regulating cell movement and migration. Clearly, the exact mechanism by which the peptide inhibits macrophage migration is not fully understood, and additional studies directed at elucidating fully this mechanism(s) are needed. Eventually, it would appear that inhibition of MARCKS function in macrophages (and perhaps neutrophils) via MARCKS-directed reagents could be the basis for novel anti-inflammatory therapeutic approaches.

FIGURES

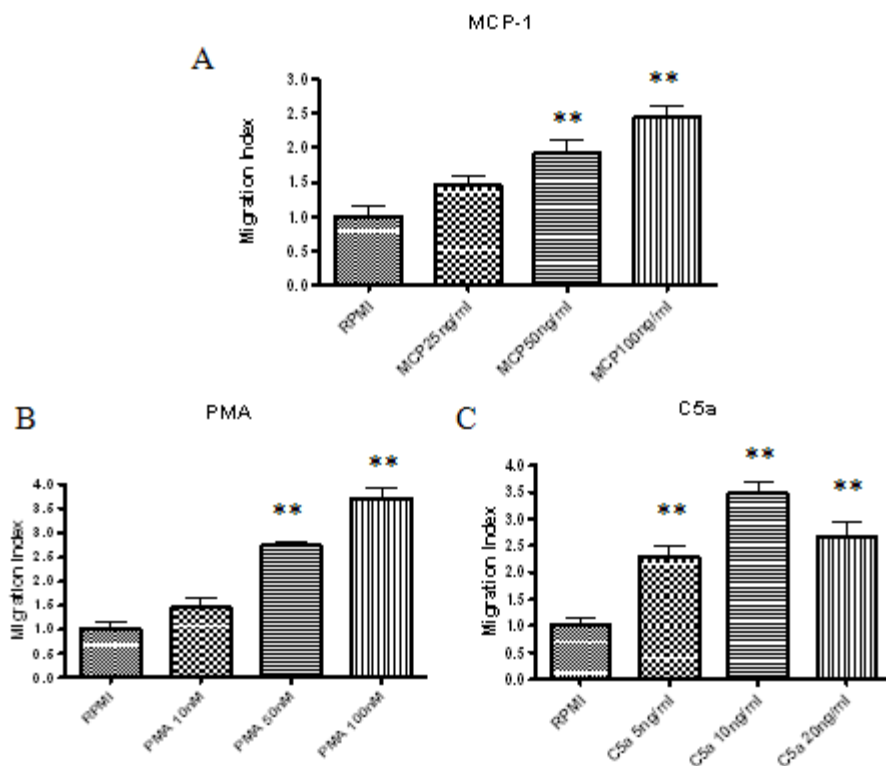


Figure 1 J774A.1 cells migrate in response to (A) MCP-1 (25, 50, or 100ng/ml) (B) PMA (10, 50, 100nM) (C) C5a (5, 10, 20ng/ml) in transwell plates. Cells were allowed to migrate for 3 hours, at which time migrating cells were counted. Data represent mean \pm SEM, n=3, **, p<0.01.

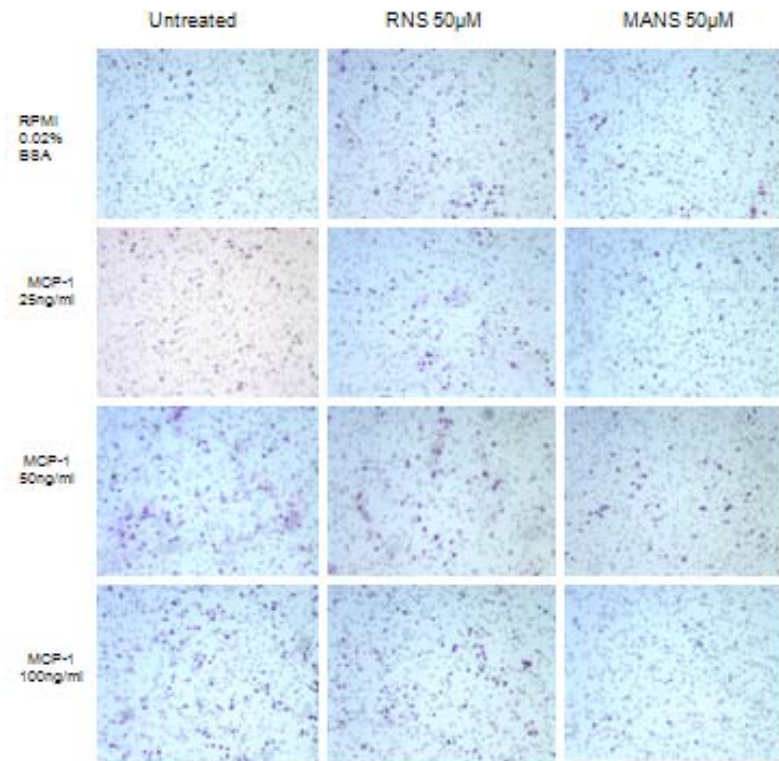


Figure 2 MCP-1 acts as a chemoattractant for J774A.1 cells. The MANS, but not RNS peptide, inhibits migration. Cells were untreated or treated with 50 μ M MANS or RNS prior to adding MCP-1 in a transwell migration assay. Cells that had migrated to the lower side of the insert were fixed with Diff Quick stain. Images represent stained inserts of fields that were counted at 40X in a light microscope.

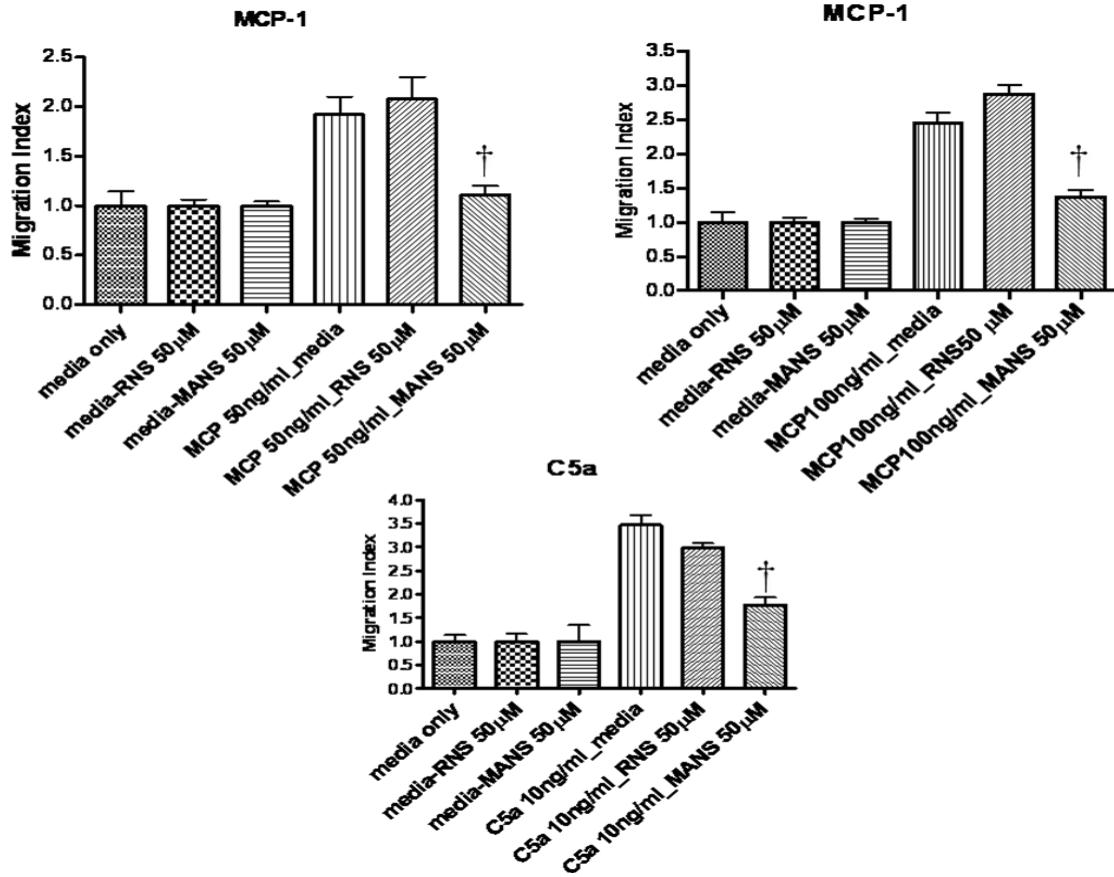


Figure 3 Inhibitory effect of MANS peptide on MCP-1 induced migration. J774A.1 cells were pretreated with 50 μ M MANS or RNS peptide. Migration in transwells was measured in response to (A) MCP-1 50ng/ml (B) MCP-1 100ng/ml (C) C5a 10ng/ml. Cells were treated for 3 hrs, at which time migrating cells were counted. Data represent mean \pm SEM, n=3, †, p<0.05.

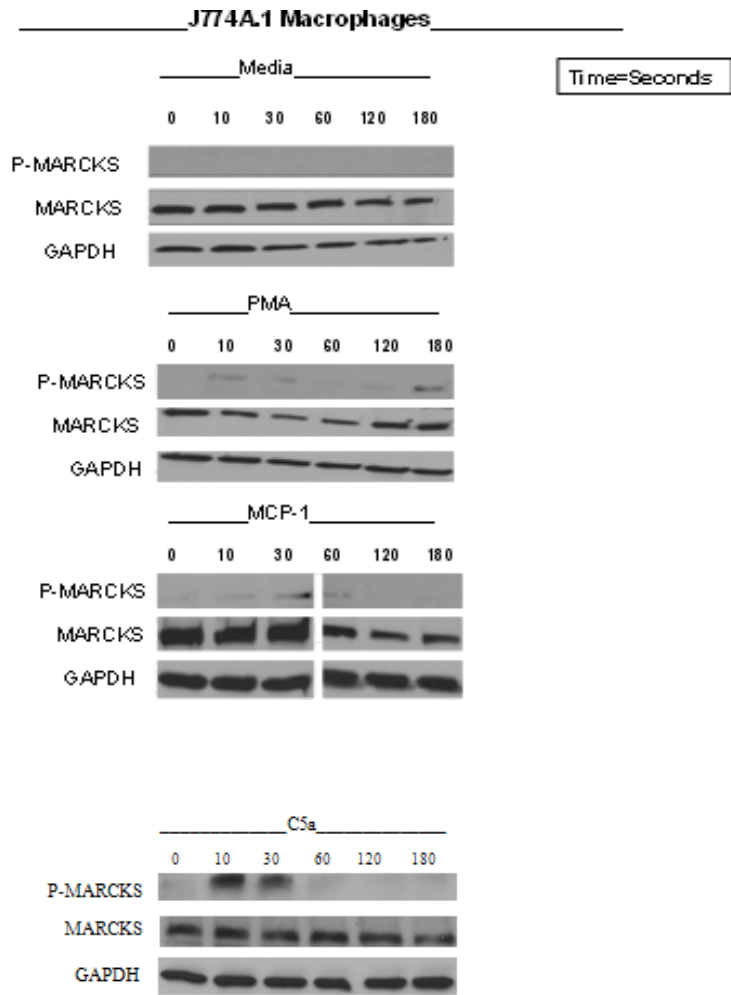


Figure 4 Western blot Analysis of expression of phosphorylated MARCKS and total MARCKS protein. J774A.1 cells were exposed to media only, PMA (100nM), MCP-1 (100ng/ml), or C5a (10ng/ml). Upon stimulation with PMA, MCP-1, or C5a the cells were rapidly phosphorylated. Blots are representative of three replicate experiments.

CD-1 Primary Murine Macrophages

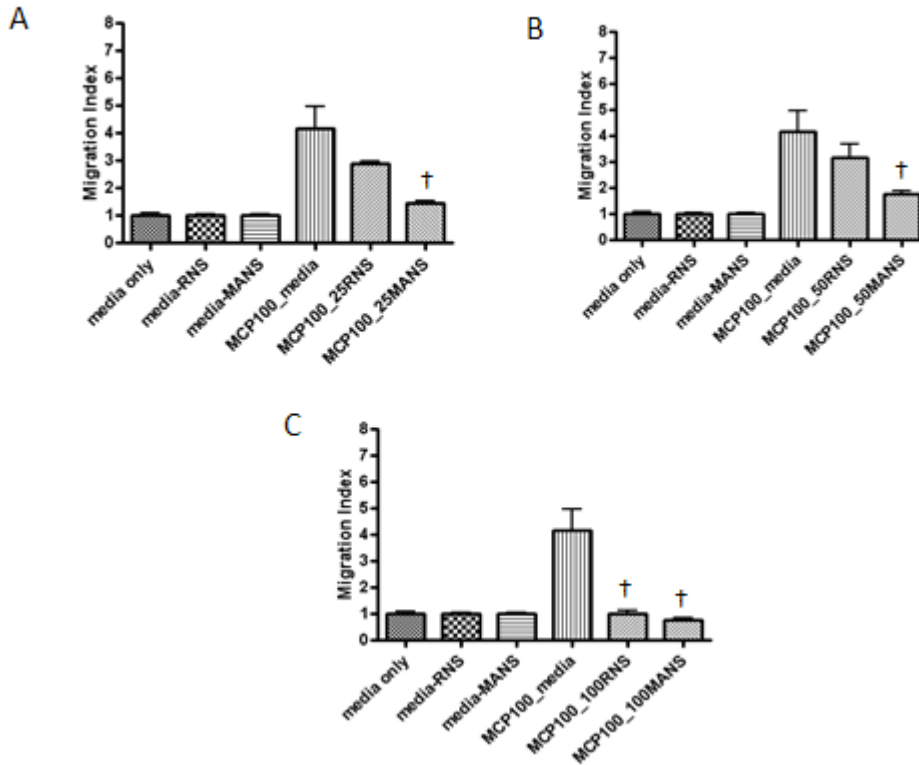


Figure 5 Inhibitory effect of MANS peptide on MCP-1 induced migration of **primary mouse macrophages**. Isolated cells were pretreated with (A) 25µM (B) 50µM (C) 100µM MANS or RNS peptide for 15 min. Migration was measured in response to MCP-1 100ng/ml in a transwell plate. Cells were allowed to migrate for 3 hrs, at which time migrating cells were counted. Data represent mean ± SEM, n=3, †, p<0.05.

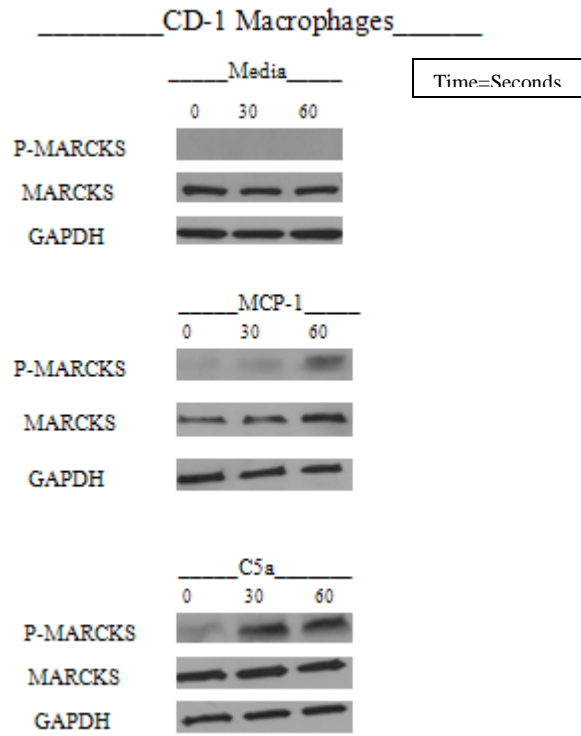


Figure 6 Western blots of phosphoylated MARCKS protein expression. CD-1 primary macrophages were exposed to with media only, MCP-1 (100ng/ml), or C5a (10ng/ml). The cells were rapidly phosphorylated upon stimulation (within 30 seconds).

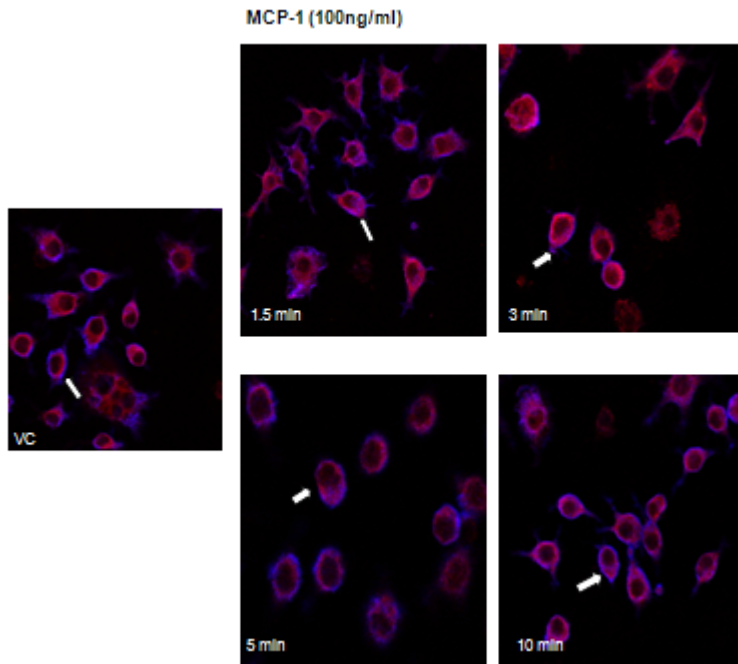


Figure 7 MARCKS and actin movement in response to MCP-1 in J774A.1 cells. Cells were stained for MARCKS with anti-MARCKS antibody conjugated to donkey-anti-mouse fluorescent (red) antibody and phalloidin (blue) for actin staining. MARCKS translocates within these cells in correlation with actin movement in response to MCP-1. Representative fields analyzed by confocal microscopy are shown.

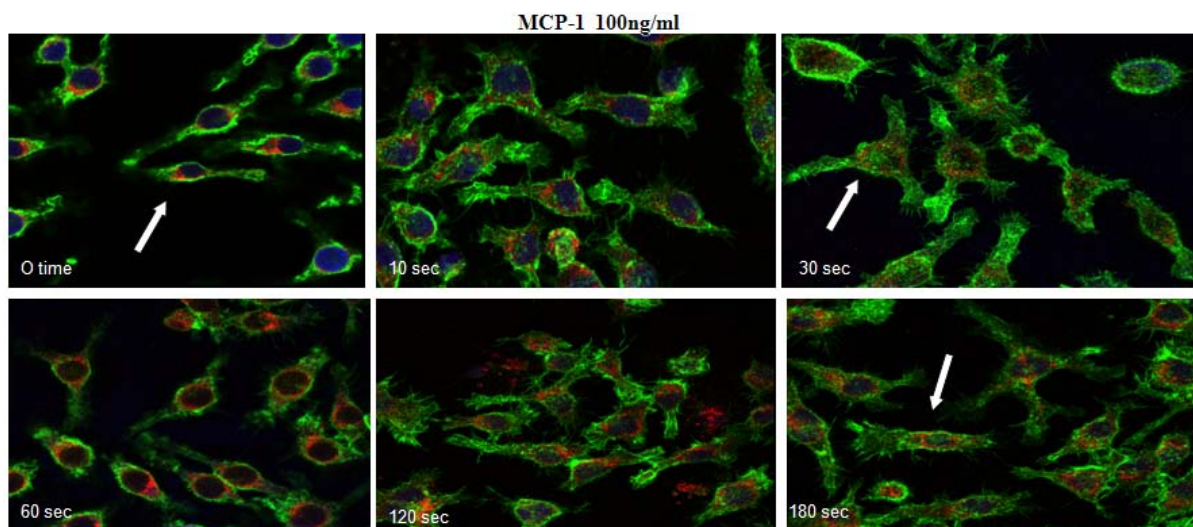


Figure 8 MARCKS and actin movement in response to MCP-1 in J774A.1 cells. The cells were stained for MARCKS with anti-MARCKS antibody conjugated to donkey-anti-mouse fluorescent (red) antibody and Alexa Fluor 488 phalloidin (green) for actin staining, and TO-PRO-3 iodide (blue) for nuclear staining. MARCKS translocates within these cells while actin extends to periphery in response to 100ng/ml MCP-1. Representative fields analyzed by confocal microscopy are shown.

IV. SUMMARY AND FUTURE DIRECTIONS

The studies presented in this work investigated the mechanisms of cell migration using J774A.1 cells and primary murine macrophages *in vitro*. Here we investigated the migratory capacity and utilized various stimuli such as MCP-1, PMA, and C5a, to determine if the MANS peptide could inhibit migration. Additionally, phosphorylation of MARCKS protein in response to chemoattractant stimuli appeared to be involved in the translocation of MARCKS and in actin spreading, and presumably in cell migration.

Chapter I reviewed the fundamental concepts, key players, and functional mechanisms of macrophages relating to cell migration. Chapter II provided a manuscript that explored the role of MARCKS protein in the regulation of mucin secretion and inflammation. Chapter III provided a manuscript in preparation that investigated the role of MARCKS protein in murine macrophage migration. We utilized the MANS peptide, which is identical to the first 24 amino acids of MARCKS, to assess a role for MARCKS in the cells' response to chemoattractant stimuli. Previous work has shown that MANS peptide interferes with both airway mucin hypersecretion and neutrophil migration [77, 81], implicating MARCKS in these responses. In our study, pretreatment with MANS peptide attenuated migration of J774A.1 cells and CD-1 mouse primary macrophages. The MANS peptide presumably competes with endogenous MARCKS for discrete membrane binding sites and therefore interferes with the function of MARCKS. Stimulation with chemotactic factors such as MCP-1 caused the rapid phosphorylation of MARCKS protein, which influenced the translocation of MARCKS and actin spreading.

Future directions include determining the effects of MANS peptide on the translocation of MARCKS within these cells, and examining in more detail polarization and adhesion mechanisms. Finally, the perfect complement to these *in vitro* studies would be to see if the MANS peptide can inhibit macrophage migration in an *in vivo* setting also. It is interesting that MARCKS, a ubiquitous protein found in all eukaryotic cells, has such an apparently wide range of functions and actions in various cell types, and could represent an important target for anti-inflammatory therapy since it plays a major role in directed migration of both neutrophils and, as reported here, macrophages as well.

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APPENDICES

Appendix 1. Neuroprobe Chemotaxis System Assay Protocol

Prior to settling on the transwell migration assay used herein, an alternative method to assess chemotaxis was tried. However, the results using this method did not appear to be consistent. Therefore, we chose to use the transwell migration method instead.

Chemotaxis Assay: This assay was modified from Jones et.al.¹ Briefly, murine macrophages were incubated with 1 mg/ml calcein in Hanks Buffer Saline Solution (HBSS) for 30 minutes at room temperature prior to assay. The cells were re-suspended in chemotaxis buffer (HBSS⁺⁺ with 2% Fetal Calf Serum). After pre-treatment chemotaxis was assayed using a 96-well chemotaxis system (Neuroprobe, Gaithersburg, MD). The bottom wells were filled with media only (HBSS) or MCP-1 at varying concentrations (0.0001-1 μ g/ml). A 3- μ m pore diameter membrane filter was placed over the wells, and 1X10⁴ cells/ml suspended in chemotaxis buffer were added to the top side of the filter and allowed to migrate for varying time points (30, 60, 90, and 120 minutes) at 37°C with 5% CO₂. After incubation the top side of the filter was washed with HBSS and scraped with cell scraper, then washed with EDTA (0.5mM) and incubated at 4°C for 30 minutes . The EDTA was removed, the plate was centrifuged at 500 rpm for 10 minutes, the filter was removed and fluorescence was measured at 485 nm excitation, 530 nm emission wavelength.

Mouse Models of Allergic Airway Disease

ABSTRACT

In vivo animal models of allergic airway disease have been frequently used to gather information regarding our current understanding of the features of human disease. In particular, the mouse model is used to mimic the complex features of asthma such as inflammation, airway hyperresponsiveness (AHR), and airway tissue remodeling. This is accomplished by sensitizing and challenging animals with a variety of foreign antigens to provoke the desired response. As a small rodent animal model the mouse is an ideal model because of its widespread availability, relative cost effectiveness, ease of use, ease in genetic alterations, and the large variety of reagents available. There are several similarities and differences between common murine models that are often used including ovalbumin, house dust mite, lipopolysaccharide (LPS), and ozone which have all contributed to our increasing knowledge. This review will assess and compare these different mouse models that continue to serve as useful models of allergic airway disease.

INTRODUCTION

The key characteristic features in allergic airway disease have been identified and subsequent narrowing of the airways as a result of or leading to the following: chronic inflammation, airway hyperresponsiveness (AHR), airway tissue remodeling, and accumulation of airway smooth muscle [95]. The similarities and differences between

experimental mouse allergic airway diseases and human asthma have been determined. More specifically, it has been found that some common features of an allergen induced response are T helper 2 (Th2) cytokine production of interleukins (IL-4, IL-13, and IL-5), goblet cell hyperplasia, neutrophil response followed by eosinophil response, and mast cell degranulation. Additionally, there is IgE production, AHR, rapid and delayed development of increased airway resistance, smooth muscle hyperplasia, and subepithelial fibrosis [96-99]. Although it is understood that under proper experimental conditions several factors must be taken under consideration including, age, sex, and strain for developing such models, we focus mainly on the effects of exposures to ovalbumin, house dust mite (HDM), LPS and ozone respectively.

Studies in laboratory animals provide practical tools for the study of human respiratory disease by providing relative understanding of the cellular and molecular mechanisms, effects of structural changes on function, and the ability to test possible therapeutic interventions [100]. The mouse model has been well characterized in terms of the inflammatory and remodeling process as it relates to asthma. Despite often debated advantages and disadvantages of the mouse model it is known to replicate the pulmonary features observed in human allergic lung disease. In this review, we compare commonly used sensitization and challenge allergens and their contribution to the inflammatory response, structural remodeling, and ultimately airway obstruction.

OVA

There are many different protocols that have been established for sensitization and challenge via specific allergens with AHR often being the cardinal feature [101]. The most commonly used allergen is ovalbumin (OVA) which is derived from chicken egg, and the most commonly used mouse strain for antigen challenge models is the BALB/c, known for developing a profound Th2 biased response, although other strains have been used successfully as well [102-104]. Two other important variables to consider are the dose of antigen for sensitization and the route of administration. The dose of ovalbumin that is often used in mouse models ranges from 1 µg to 8000 µg per animal [105-106] for intraperitoneal sensitization of mice. Also, the route of administration varies and sensitization is most often accomplished by multiple intraperitoneal injections [107] subcutaneous [108] intranasal [109], intratracheal [110] delivery or inhalational challenge alone [111]. All of these variables have an influence on the presence or absence and intensity of specific key features mentioned previously.

Another important variable is duration of the challenge. This variable is important as it determines whether the model will be acute or chronic, which affects the display of characteristic features. In order to foster a response there is usually multiple administration of ovalbumin which is sometimes accompanied by the presence of an adjuvant, such as aluminum hydroxide or potassium aluminum sulfate, to enhance the development of Th2 cytokines and boost the immune response [112-113]. When adjuvant-free protocols are used they require a greater number of exposures to achieve the desired sensitization [114-115]. While the acute model is normally generated when the animal is challenged by inhalation of

aerosolized ovalbumin, by intratracheal administration or by intranasal instillation, repeatedly for several days, the chronic model is generated within weeks or months [116]. Several examples of commonly used “classical” models are described and summarized by Kumar *et al.* [18].

In terms of response to challenge there are some key differences between the acute and chronic models and there is still some controversy on the relevance to human asthma in terms of the acute model. Acute models are thought to be useful for examining immune-mediated events, while chronic models examine more prominent asthma related features such as airway remodeling [117-119]. Some key features of the acute model are elevated levels of IgE, airway inflammation, goblet cell hyperplasia, epithelial hypertrophy, and AHR. Moreover, it has been found that the influx of inflammatory cells is dominated by eosinophils in bronchoalveolar lavage and lung tissue, which are known to contribute to AHR leading to the decline in lung function [120]. However, many of these key features of the acute challenge appear to be short-lived in some models, and the airway remodeling changes are absent [19,121]. Key features of chronic allergen exposure are more profound and are believed to be more relevant to human asthma, such as Th2 dependent allergic inflammation characterized by eosinophilic influx into the airway mucosa, AHR airway remodeling with goblet cell hyperplasia, epithelial hypertrophy, and subepithelial fibrosis. Unlike acute models in which the features are short-lived, in chronic models the features tend to persist after the challenge [122-124]. Although both acute and chronic models have certain limitations, such as tolerance [125], both are valid tools for investigating allergic disease in the mouse.

HOUSE DUST MITE

Although the OVA model is known as the “classical” model, other models such as house dust mite (HDM) are commonly used. HDM is considered as a more clinically relevant allergen [126-128]. *Dermatophagoides farinae* (*Der f*) and *D pteronyssinus* are 2 major house dust mites that are used in experimental animal models to trigger allergic asthma [129-132]. While sensitization of the airways can be induced using both OVA and HDM [19], the inhaled delivery of HDM is successful in inducing the desired response either alone [133] or along with an adjuvant. In contrast to mice exposed to OVA, when exposed to HDM the response is persistent Th-2 driven airway inflammation [134-136] with severe and sustained eosinophilia [137] via mechanisms that have a direct effect on lung macrophages [138] and mast cells [139]. One difference compared to the OVA model is that when animals are exposed to HDM they do not develop inhalation tolerance to the allergen when exposed for a prolonged period of time [140]. In terms of similarities, chronic HDM exposure results in airway remodeling structural changes that are similar to the chronic OVA model [39,[141]. Specifically, there is evidence of goblet cell hyperplasia, collagen deposition, and peribronchial accumulation of contractile tissue following HDM exposure in mice [43]. The HDM as an allergen appears to be an effective and relevant tool for studying allergic asthma.

LIPOPLYSACCHARIDE

Another factor that has been found to influence asthma is lipopolysaccharide (LPS) which is classified as an endotoxin. LPS is a cell wall component of gram-negative bacteria known to enhance immune-stimulating and pro-inflammatory activities [142-143]. However, there is much controversy over whether endotoxin exposure leads to a protective or an exacerbating effect on disease progression in humans and animals [144-146]. Studies have shown that these opposing effects can be explained easily and are dependent upon dose and timing of exposure [147]. The response to endotoxin is significantly different with low-dose compared to high-dose and early versus late administration, which ultimately determines the contradictory results. For example, it was shown that early LPS administration (day 1 to day 4) prevented lung inflammation and eosinophilia, while later administration (day 6 to day 10) significantly increased airway inflammation and edema in a rat model [148]. Similar contradictions have been found in the mouse model of asthma following exposure to LPS.

Studies involving OVA-sensitized mice exposed to LPS showed that LPS appeared to down-regulate Th2 response to the allergen and suppressed the development of airway inflammation unlike what has been shown in the HDM model and other OVA models. Subsequently, it prevents IgE production, Th2 responses and development of airway eosinophilia [149]. These effects were dependent upon administration of high doses of LPS by stimulating dendritic cells [150]. Alternatively, when LPS is administered during what is known as the effector phase of allergic reactions it has been found that LPS increased airway eosinophilia [49,[151] creating the opposite effect of exacerbation of asthma via mechanisms involving mast cells. The specific mechanism by which this occurs is via toll-

like receptor 4 mediated mast cell activation and modulation leading to increased production of Th2 cytokines which control the severity of eosinophil airway inflammation [152-153]. It is apparent that different factors determine either the inhibition or exacerbation of inflammation when LPS is involved, so unlike other mouse models it influences two alternative outcomes.

OZONE

There are also many oxidants that are known to contribute to the pathogenesis and exacerbation of respiratory diseases, including ozone. Ozone or trioxygen is an air pollutant that can cause harmful effects on the respiratory system of animals and humans at concentrations as low as 0.06 ppm [154]. In humans, exposure to ozone is known to increase airway inflammation [155], however the effect has been found to be a result of either increased neutrophilic inflammation [156] or eosinophilic inflammation [157-158]. The battle over whether eosinophils or neutrophils regulate inflammatory effects in response to ozone is also found in the mouse model. It has been suggested that the features of AHR and/or airway remodeling is due to the role of eosinophils [159-160]. Alternatively, investigators have also found that neutrophils may regulate AHR in mouse models of allergic airway disease [161]. Additionally, Pichavant *et al.* have recently found that in order for ozone exposure to induce AHR it requires the presence of natural killer T (NKT) cells and IL-17 production [162]. It has also been shown that asthmatic subjects may be particularly sensitive to the effects of ozone. Both the effect of ozone on lung function and bronchoconstrictive effects of asthmatic subjects versus healthy subjects have been extensively studied [157, 163]. Several investigators have demonstrated via epidemiological

studies that ozone pollution increases the risk of asthma exacerbations in asthmatic subjects [164-165]. Nonetheless, there is agreement that ozone acts as an environmental factor that influences AHR and pulmonary inflammation in asthma.

MOUSE MODEL LIMITATIONS

Although all of the above-mentioned models have been helpful many would argue that mice are just not a good model for human airway disease. The most basic evidence is that the mouse lung is considerably different in structure from the human lung. There are several specific features related to size and organization that are different: 1) The total lung capacity of the lung (TLC) of the lung of the mouse is about 1ml compared to 6,000 ml of a human lung. 2) The alveoli of the mouse lung are smaller: an 80 μ m mean linear intercept (MLI) compared to a human MLI of 210 μ m. 3) Mouse lungs have fewer respiratory bronchioles and airway generations (13-17 generations) than human lungs (17-21 generations). Other significant differences that have been noted are that mouse lungs have thin respiratory epithelium, have a relatively large airway lumen, lack submucosal glands, and exhibit high numbers of Clara cells unlike the human lung [166]. These features are obviously believed to have an important bearing on the function of the mouse lung.

Due to anatomical differences it has been suggested that the inflammatory processes that compromise lung function in humans and is not relative to mice. Moreover, many believe that mice simply do not have nor can they develop asthma, and therefore asthma can not be effectively replicated in a mouse model. Even the most so called “hyperresponsive” strains do not exhibit spontaneous symptoms consistent with asthma that are found in

humans [167]. Additionally, the mouse is not considered to be a good model since there is little plasma exudation in the mouse, which is a cardinal feature of human bronchial asthma, and there is an alternative distribution of eosinophils that do not degranulate unlike in human asthma [168]. Nonetheless, with all of the mouse model imperfections it still serves as a valuable animal research model based on past contributions and future prospects [169].

MOUSE MODEL CONTRIBUTIONS

There is no questioning the fact that the mouse model has allowed for the identification of several therapeutic targets, and the mouse system has outperformed various other species in this category [170]. Several targets have been identified and evaluated in mouse models such as anti-cytokine approaches, specific mediator agonists, monoclonal antibodies, and specific enzyme inhibitors. For example, IL-5 knockout mice appeared to be protected from acute allergic inflammation, AHR, and chronic airway remodeling [113, 171-172]. Unfortunately, some targets that are evaluated and shown to be effective in the acute model do not appear to have clinical benefit, as with IL-5 antagonists [173]. Genetically manipulated mouse models have been utilized to determine the role of several other cytokines such as IL-4 and IL-13 in various aspects of asthma pathophysiology [174]. Chronic models have been proven to be more clinically relevant when considering the effects of anti-inflammatory agents. Specifically, corticosteroids, leukotriene receptor antagonists, and phosphodiesterase 4 inhibitors are some examples of potential therapeutic agents that have been profiled in chronic mouse models [175-177].

CONCLUSIONS

The pathophysiologic events underlying the inflammation and remodeling response in allergic airway disease are complex. Over the years, researchers have investigated and optimized various mouse models that have been useful to increase our understanding of the mechanisms involved throughout development and progression of disease. By taking into consideration the specific trigger, choosing the adequate strain, manipulating dose, route of administration, duration and timing of exposure, scientists have developed several mouse models to mimic asthma in humans. Beyond limitations and despite similarities and differences described above, the mouse models all have contributed to our general knowledge of airway disease.