MARCKS and Lung Disease

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Abstract

MARCKS (myristoylated alanine-rich C kinase substrate) is a prominent PKC substrate expressed in all eukaryotic cells. It is known to bind to and cross-link actin filaments, to serve as a bridge between Ca^{2+} /calmodulin and PKC signaling, and to sequester the signaling molecule phosphatidylinositol 4,5-bisphosphate in the plasma membrane. Since the mid-1980s, this evolutionarily conserved and ubiquitously expressed protein has been associated with regulating cellular events that require dynamic actin reorganization, including cellular adhesion, migration, and exocytosis. More recently, translational studies have implicated MARCKS in the pathophysiology of a number of airway diseases,

including chronic obstructive pulmonary disease, asthma, lung cancer, and acute lung injury/acute respiratory distress syndrome. This article summarizes the structure and cellular function of MARCKS (also including MARCKS family proteins and MARCKSL1 [MARCKS-like protein 1]). Evidence for MARCKS's role in several lung diseases is discussed, as are the technological innovations that took MARCKS-targeting strategies from theoretical to therapeutic. Descriptions and updates derived from ongoing clinical trials that are investigating inhalation of a MARCKS-targeting peptide as therapy for patients with chronic bronchitis, lung cancer, and ARDS are provided.

Keywords: MARCKS; lung disease; inflammation; cancer

Properties of MARCKS Protein

Structure

MARCKS binds and cross-links actin, bridges calmodulin (CaM) and PKC signaling, and sequesters phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane (1–6). Associated with cellular events that require actin, including adhesion, migration and exocytosis (7–21), it is involved in pulmonary disorders such as chronic obstructive pulmonary disease (COPD), asthma, acute respiratory distress syndrome (ARDS) and lung cancer (12, 17, 20–23). In 1982, Wu and colleagues described an “87 kD” PKC substrate protein that is known today as “MARCKS” (6, 24). MARCKS has an actual molecular weight of 32 kD, but it was first described as 87 kD owing to its

apparent molecular weight on SDS-PAGE gels (6). This anomalous migration behavior is due to its extended, nonglobular conformation and weak binding of SDS molecules to this highly acidic protein (2). MARCKS family proteins are comprised of MARCKS and MARCKSL1, also known as MacMARCKS, MRP (MARCKS-related protein), MLP (MARCKS-like protein), and F52 (brain protein F52) (25). MARCKS is ubiquitously expressed, whereas MARCKSL1 is expressed primarily in brain, reproductive tissue, and macrophages (1, 26).

MARCKS belongs to a class of “natively unfolded” proteins known for having very little secondary structure and no hydrophobic core (27). MARCKS and MARCKSL1 have three evolutionarily conserved amino acid domains. The first is a 24-amino acid sequence at the N-terminus, which includes an N-terminal

myristoylation in which myristic acid, a C14 saturated fatty acid, is linked via an amide bond to the N-terminal glycine. The myristic acid moiety inserts hydrophobically into lipid bilayers to stabilize membrane binding of MARCKS (28). The second conserved region is the MH2 (multiple homology 2) domain. The function of the MH2 domain is currently unknown, but it contains the only known intron in the genes for MARCKS and MARCKS family proteins (29, 30). The final conserved region is located at the center of the protein and is known as the effector domain (ED; amino acid residues 151–175). The ED is lysine rich with four serine residues and a highly basic charge. The positively charged lysines play a key role in electrostatic interactions between the ED and negatively charged phospholipids in membranes, and they likely contribute to

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the “unfolded” conformation of the protein due to electrostatic repulsion (2). The four serine residues provide a site for phosphorylation by conventional and novel isoforms of PKC (31). The ED is also the site of CaM and actin binding (29, 32–34).

Binding Interactions

Myristoyl-electrostatic switch. In most resting cells, MARCKS is anchored to the plasma membrane through membrane interactions with both the N-terminal myristoyl moiety and the positively charged lysine residues of the ED. The myristate associates hydrophilically with the inner leaflet of the plasma membrane (28), and the basic ED associates electrostatically with negatively charged phospholipids, namely PIP₂ (35). Both the myristoyl moiety and the lysine-rich ED are required for MARCKS to optimally associate with the plasma membrane (36–38). When cells receive signals that activate conventional or novel PKCs, MARCKS is phosphorylated at the ED, and the electrostatic interaction with PIP₂ weakens, leading to MARCKS translocation from the plasma membrane to the cytosol (16, 18, 39–42). After dephosphorylation, MARCKS can return to the cell membrane (10, 37, 43) or target other intracellular membranes (44, 45), depending on the cell type. This fundamental framework relating cellular activation signals to reversible MARCKS membrane binding was first described by Graff and colleagues (6) and Hartwig and colleagues (3) and was later termed the “myristoyl-electrostatic switch” by McLaughlin and Aderem (36).

PIP₂. PIP₂ is continuously present in the plasma membrane of most mammalian cells at an effective concentration of approximately 10 μ M, comprising about 1% of the total lipids (46). PIP₂ regulates numerous cellular functions by serving as a precursor for production of lipid second messengers, including diacylglycerol (DAG), inositol 1,4,5-trisphosphate (IP₃), and phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) (47); acting as a “lipid” anchor for actin-binding proteins (48); and activating membrane proteins, such as ion channels and transporters (49, 50).

In the resting state, MARCKS binds to PIP₂ in the plasma membrane, thereby sequestering it and keeping it unavailable for hydrolysis by phospholipase C (PLC). Upon cell stimulation and phosphorylation

of MARCKS by PKC, or a rise in intracellular Ca²⁺/CaM, MARCKS desorbs from the membrane, allowing PLC to hydrolyze PIP₂ and produce a measurable burst of IP₃ and DAG (51). Computational modeling, as well as research in large unilamellar vesicles and lipid monolayers, reveals that the MARCKS ED forms an electroneutral complex with approximately four PIP₂ molecules (52–54). The functional significance of MARCKS–PIP₂ binding is supported by several lines of evidence. First, MARCKS and PIP₂ are present at similar concentrations ($\sim$ 10 μ M) within cells (26, 55). Second, overexpression of MARCKS increases the amount of PIP₂ present within cell membranes (44, 56). Third, similar to reports by Glaser and colleagues on MARCKS–PIP₂ binding and PLC, recent studies in both lipid bilayer systems and living cells show that MARCKS ED blocks PIP₃ production by the lipid kinase PI3K α owing to sequestration of its substrate, PIP₂ (44, 56–58). In glioblastoma multiforme cells, the MARCKS ED is capable of regulating nuclear membrane PIP₂ with downstream effects on gene transcription (44). These findings, together with evidence from *in vitro* single-molecule and cell migration models, provide definitive evidence of MARCKS/PIP₂ binding and support PKC and Ca²⁺/CaM regulation of MARCKS as an important mechanism used by cells to control the localization, availability, and downstream cell signaling of PIP₂ (46, 52, 53, 57–63).

Actin. Interest in MARCKS as an actin-binding protein first emerged when Hartwig and colleagues determined that both MARCKS and a synthetic peptide corresponding to the MARCKS ED directly bind to and cross-link F-actin and that this cross-linking is diminished when PKC phosphorylates, or when Ca²⁺/CaM binds, MARCKS at the ED (3, 64, 65). MARCKS has two actin-binding sites in the ED (66), and both MARCKS and MARCKSL1 ED peptides induce rapid polymerization of G-actin and bundling of F-actin (64, 67). The current theory is that nonphosphorylated membrane-bound MARCKS stabilizes the cytoskeleton of quiescent cells by cross-linking F-actin, and cellular activation signals that cause PKC-mediated MARCKS phosphorylation or Ca²⁺/CaM binding displace MARCKS to the cytosol while also decreasing actin cross-linking, leading to areas permissive of cytoskeletal

reorganization (5, 56, 58, 68). Although significant evidence still points to MARCKS as an important actin-binding protein, recent theories regarding MARCKS regulation of actin-dependent processes such as cellular migration have shifted toward MARCKS regulation of PIP₂ availability as a mechanism for coordinating signals, including Ca²⁺/CaM, PKC, PI3K, and DAG, to generate dynamic cytoskeletal reorganization (58).

CaM. The same MARCKS ED that serves as the site for PKC phosphorylation also binds Ca²⁺/CaM. Nonphosphorylated MARCKS binds CaM (when complexed with Ca²⁺) with high affinity (6, 69–71). Although it was initially proposed that the MARCKS ED assumes an α -helix conformation consistent with many known CaM-binding domains (6, 72), later studies using circular dichroism, nuclear magnetic resonance, and X-ray crystallography confirmed that MARCKS binds Ca²⁺/CaM in a nonhelical, extended conformation, making MARCKS family proteins among a novel class of CaM-binding proteins (73–76). Ca²⁺/CaM binding to MARCKS is disrupted by PKC-dependent phosphorylation (2, 3, 5, 6, 14, 37, 69–71, 76–79).

Role in Cellular Function

Evidence for MARCKS’s role in fundamental biological processes is extensive and ranges from events guiding embryogenesis, such as gastrulation (80) and neural tube closure (81); to reproductive functions, such as oocyte maturation (82); to neuronal functions, including neurite and axon outgrowth (56, 83), dendrite branching (84), and maintenance of memory (85). In addition to roles in health and homeostasis, MARCKS family proteins are also essential for growth and survival, because knockout of either MARCKS or MARCKSL1 in mice leads to embryonic fatality owing to abnormal brain and central nervous system development (86–88). Although a complete description of the numerous biological functions of MARCKS is beyond the scope of this review, evidence for MARCKS’s role in cellular events relevant to the pathophysiology of inflammatory, allergic, and neoplastic airway diseases is presented.

Before reviewing the roles of MARCKS in respiratory/pulmonary disease, it is worthwhile to briefly describe technologies that have been developed to investigate MARCKS function in living cells and

Table 1. MARCKS Translational Research Summary: Model Types and Significant Findings

	Model/Cell Type	Investigational Strategy to Inhibit MARCKS Function	Significant Finding(s) for MARCKS Function	References
Acellular models	Computational models	ED peptide	MARCKS sequesters PIP ₂ through electrostatic interactions	53
	Lipid models	ED peptide	MARCKS sequesters PIP ₂ /PIP ₃ , regulates PIP ₂ availability as PI3K substrate	54, 60, 61
Cells <i>in vitro</i>	Neutrophils	N-terminal peptide (MANS), BIO-11006	MARCKS involved in directed migration, adhesion, respiratory burst, cytokine production, and degranulation	8, 21, 93, 94, 100
	Macrophages	MANS, siRNA	MARCKS involved in directed migration and cytokine production	13, 96
	Mast cells	ED peptide	MARCKS involved in degranulation	130
	Fibroblasts	MANS, siRNA	MARCKS involved in directed migration	15, 16
	Stem cells	MANS	MARCKS involved in directed migration	95
	Airway goblet cells	MANS, BIO-11006	MARCKS involved in mucin secretion	18, 92, 97, 100, 108–111, 113–115
	Vascular smooth muscle cells	siRNA, mutated protein expression	MARCKS involved in adhesion and $\alpha_5\beta_1$ integrin function	138
	Endothelial cells	siRNA	MARCKS involved in directed migration	63, 139
	Lung cancer cells	MANS, siRNA, BIO-11006, ED peptide, mutated protein expression	NSCLC cells: elevated p-MARCKS expression, MARCKS involved in cancer cell migration, MARCKS enhances radiation sensitivity	12, 104, 141–143, 163
<i>In vivo</i> , animal models	Mouse mucus hypersecretion/allergic inflammation models	MANS, BIO-11006	MARCKS involved in airway mucin secretion and inflammation	17, 20, 98, 107
	Mouse ALI/ARDS models	BIO-11006, MANS	MARCKS pulmonary inflammation and ALI	22, 23
	Mouse lung cancer models	MANS, BIO-11006, ED peptide	MARCKS involved in metastasis and tumor growth	12, 99, 164
Human clinical trials	Healthy volunteers	Inhaled BIO-11006	Safe pulmonary and systemic profile	Clinical Trials BIM-CL-001, BIM-CL-002
	Chronic bronchitis	Inhaled BIO-11006: 172 subjects with COPD, double-blind, placebo-controlled, multicenter dose escalation study for 21 d	75 mg twice daily increased proportion of clinically significant FEV ₁ response	136, Clinical Trial BIM-CL-003
	ARDS	Inhaled BIO-11006: 40 ventilated ICU patients, randomized, placebo-controlled trial	Expected 2019	Clinical Trial BIM-CL-005
	NSCLC	Inhaled BIO-11006: randomized, SOC controlled trial	Expected 2019	Clinical Trial BIM-NSCLC-001

Definition of abbreviations: ALI = acute lung injury; ARDS = acute respiratory distress syndrome; COPD = chronic obstructive pulmonary disease; ED = effector domain; ICU = intensive care unit; MANS = myristoylated N-terminal sequence; MARCKS = myristoylated alanine-rich C kinase substrate; NSCLC = non-small cell lung cancer; PIP₂ = phosphatidylinositol 4,5-bisphosphate; PIP₃ = phosphatidylinositol (3,4,5)-trisphosphate; p-MARCKS = phosphorylated MARCKS; SOC = standard of care.

organisms. As previously mentioned, knockout of MARCKS results in embryonic lethality in mice; therefore, adult knockout mice are unavailable for MARCKS investigations. Deletion studies have been limited to cells such as macrophages and megakaryocytes collected from bone marrow or liver of MARCKS-knockout mouse embryos (89, 90). Recently, investigators have used newer technologies such as CRISPR/Cas9 to conduct MARCKSL1 deletion studies in epithelial cells (91). In addition to these approaches, structural/functional analysis, targeted mutation, siRNA knockdown, cell fractionation, fluorescence microscopy, and other newer analytic techniques have been applied to studies of MARCKS.

However, before 2000, inhibition or alteration of MARCKS function relied on silencing MARCKS, mutating MARCKS, or altering MARCKS regulation by targeting phosphatases or PKC. In a breakthrough in 2001, Li and colleagues described a synthetic, myristoylated peptide identical to the first 24 amino acids of the N-terminus of MARCKS that inhibited mucin granule secretion in human airway epithelial cells (92). The sequence for this peptide, known as MANS (myristoylated N-terminal sequence) is MA-GAQFSKTAAGGEAAERPGEEAVA (MA denotes myristic acid). The control peptide (random N-terminal Sequence [RNS]) contains the same amino acid composition as MANS but arranged in random order (MA-GTAPAAEGAGAEVK RASAEAKQAF). Because the N-terminus of these peptides contains a hydrophobic myristate moiety, these peptides are readily taken up by cells (8, 92). Since its discovery in 2001, the MARCKS-specific MANS has been used in numerous investigations of MARCKS function *in vitro* and *in vivo* (8, 12, 13, 15, 17, 20–22, 92–96). This method of MARCKS inhibition marked a turning point for investigators looking for ways to target this omnipresent protein and opened the door to development of a new class of airway therapeutics. The MANS peptide is not suitable as a drug because of its limited solubility, large size, and rapid hydrolysis by lung-lining fluid and blood, so attention was turned toward creating a smaller and more soluble peptide that encompasses the active site on MANS. After screening numerous peptides with amino acid and myristic acid substitutions, a peptide with a sequence encompassing the first 10 amino acids of the MARCKS N-terminus was developed.

The solubility of this peptide was greatly enhanced by substituting an acetyl group for the myristic acid moiety. This peptide, called BIO-11006, is resistant to hydrolysis in airway lining fluid and has been shown to be equally as effective as MANS in attenuating mucus secretion, inflammation, and cell motility in several *in vitro* and *in vivo* systems (22, 23, 97–100). BIO-11006 was developed as an inhaled aerosol drug by BioMarck Pharmaceuticals and is the basis for several clinical trials in different lung diseases, discussed below.

MARCKS inhibition has also been achieved with an ED (amino acids 151–175) peptide, known as the ED peptide, which has been described in numerous biophysical studies of MARCKS interactions with lipids (51, 52, 54). Solubility of this peptide, however, can be problematic (96, 101–104), consistent with its extremely basic nature, although this peptide can be modified to enhance cell permeability, such as by C-terminal addition of a tyrosine aminotransferase (105).

Reports on mechanisms of MARCKS inhibition afforded by these two different peptide inhibitors have varied, likely owing to differences in cell type and function. The MANS peptide seems to displace MARCKS from normal membrane associations, alter cytoskeletal dynamics, and decrease MARCKS phosphorylation (8, 12, 92). The ED peptide has been reported to decrease MARCKS phosphorylation, to directly bind and sequester lipopolysaccharide (LPS), and to act as a nuclear localization signal and regulator of nuclear membrane PIP₂, with downstream effects on gene expression (44, 105, 106).

MARCKS and Lung Disease

Mucin Secretion

Excess mucus secretion is a common lesion in patients with inflammatory airway diseases, predisposing to obstruction, infection, and even destruction of airway walls and contiguous tissues. As a result, cellular regulators of airway mucus production and secretion are attractive therapeutic targets. To date, both *in vitro* and *in vivo* evidence strongly supports a role for MARCKS in regulation of airway mucin (the solid component of mucus) secretion. Using an *in vitro* model system of well-differentiated normal human bronchial epithelial (NHBE) cells, Li and

colleagues (92) showed that, consistent with other cell types, PKC activation led to MARCKS phosphorylation and translocation to the cytosol. They further showed that subsequent PKG (protein kinase G) activation resulted in protein phosphatase 2A-mediated MARCKS dephosphorylation and that downregulation of MARCKS protein, or inhibition of MARCKS phosphorylation/dephosphorylation, significantly inhibited mucin secretion. In addition, these investigators showed for the first time that treatment of NHBE cells with the MANS peptide significantly attenuated mucin hypersecretion in response to PKC activation. Using radiolabeled immunoprecipitation, these investigators determined that dephosphorylated cytoplasmic MARCKS associated with myosin and actin, and they hypothesized a role for MARCKS in actin-dependent mucin granule exocytosis (92). Further evidence for MARCKS's role in mucin granule exocytosis comes from an *in vivo* study in mice. Using ovalbumin-sensitized mice as a model for allergic inflammation and goblet cell metaplasia, Singer and colleagues showed that intratracheal MANS peptide pretreatment attenuated methacholine-induced mucin secretion into the airway lumen (20). Subsequent *in vivo* studies using intranasal administration in the same mouse model showed that MANS blocked mucin release and subsequently improved physiological measurements of airway obstruction, as determined by plethysmographic measures of airway conductance (17). In a model of mucin hypersecretion provoked by exposure of rats to the occupational pollutant and cigarette smoke component, acrolein, Chen and colleagues found that intratracheal administration of MANS blocked the secretory response (107). In each of the above-described studies, the RNS control peptide was without effect.

Although it is clear that MARCKS plays a significant role in airway mucin secretion, mechanisms of regulation are not fully understood. When NHBE cells were exposed to human neutrophil elastase (a possible model of mucin hypersecretion, as seen in chronic bronchitis), human neutrophil elastase-induced mucin release was shown to be dependent on a PKC-mediated mechanism, specifically the delta isoform of PKC (PKC δ) (108), which acted by phosphorylating MARCKS (18). Additional studies in NHBE cells showed

that two chaperone proteins—HSP70 (heat shock protein 70) and CSP (cysteine string protein)—were integral to MARCKS regulation of mucin secretion. Both of these chaperones appeared to form a complex with MARCKS and with mucin granule membranes. Inhibition of either chaperone attenuated mucin release by these cells (109). Additional morphological studies using fluorescent markers of MARCKS and HSP70 revealed tight physical associations between these two proteins that were enhanced when mucin secretion was stimulated with PMA (110). The aforementioned MARCKS–HSP70–CSP complex was shown to bind to the cytoskeleton, specifically an unconventional myosin isoform, myosin V (111), which is associated with moving intracellular vesicles and granules along actin filaments (112). When mucin granule membranes from NHBE cells were isolated via immune isolation techniques, the mucin granule membrane marker hCLCA1 (human calcium-activated chloride channel accessory 1), together with MARCKS, HSP70, CSP, myosin V, and protein phosphatases, was associated with mucin granule membranes, supporting the above-described paradigm (113). These data are consistent with a mechanism whereby MARCKS is released from its membrane attachment after it is phosphorylated by PKC and then goes into the cytoplasm, where, in concert with the chaperones HSP70 and CSP, it binds to both the cytoskeleton and mucin granule membranes and thereby regulates granule release (92, 109, 110).

Another explanation of MARCKS's role in mucin secretion points to actin as a barrier of mucin granule exocytosis and to MARCKS phosphorylation as a key step regulating the removal of that barrier upon cell stimulation. This hypothesis is based on observations reported by Ehre and colleagues (114) using SPOC1 cells, an immortalized rat tracheal epithelial cell line. Unstimulated SPOC1 cells have a subplasmalemmal sheet of β - and γ -actin between granules and the apical membrane, and the actin sheet disappeared when the cells were exposed to secretory agonists. In contrast, stabilization of the actin sheet inhibited mucin secretion. These investigators also showed, similarly to Li and colleagues, that MARCKS is phosphorylated and displaced from the membrane to the cytosol upon stimulation

of goblet cells. They concluded that MARCKS phosphorylation/translocation is a key step in dissolving the apical actin that otherwise presents a barrier to mucus secretion in these cells (114).

There are numerous other proteins that could be involved in mucin exocytosis, such as SNAREs (SNAP receptor proteins), SNAPs (soluble N-ethylmaleimide-sensitive factor attachment proteins), and VAMPs (vesicle-associated membrane proteins) (115, 116). Exactly how MARCKS and these related proteins interact to regulate exocytotic release of mucin in airways has not been fully established. However, it seems probable that the highly complex secretory mechanisms used by airway mucin-secreting cells, or similar mechanisms, would also be involved in other secretory pathways. To this point, MARCKS has been implicated in secretion of colonic mucin (117, 118), insulin (119), apolipoprotein E (120), cathepsin K (121), amylase (122), neurotensin (123), adrenocorticotropin (124), and acrosomal exocytosis (125). It is conceivable that other cell types have similar secretory mechanisms involving MARCKS in a central role.

Inflammation

Exocytosis/degranulation. With a demonstrated role for MARCKS in the release of secretory granules from respiratory epithelial cells (described above), the possibility that MARCKS could also be involved in regulating release of granules from other cell types was addressed. Attention was turned to release of inflammation-associated granules from inflammatory cells. Several cell types feature prominently in the pathophysiology of airway inflammation, including leukocytes such as neutrophils and mast cells, and a role for MARCKS in the secretion/exocytosis of inflammatory mediators by these cells was investigated.

Neutrophils are required for normal host defense, but when dysregulated in their response, they contribute to pathological airway inflammation in patients with chronic bronchitis, severe forms of neutrophilic asthma, cystic fibrosis, and acute lung injury (ALI)/ARDS (126). As a normal mechanism for host defense, neutrophils synthesize and store degradative enzymes in membrane-bound granules within their cytoplasm.

Inflammatory signaling during airway disease can stimulate exocytosis of these granules, the contents of which can damage host tissue. Inhibition of MARCKS with the MANS peptide attenuated PMA-stimulated release of myeloperoxidase from isolated human peripheral blood neutrophils in a concentration-dependent manner (21). MANS also attenuated secretion of 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-9222 (21).

Pretreatment of canine neutrophils with MANS significantly reduced both mRNA and protein expression of several proinflammatory cytokines, including IL-8 and TNF- α . The observed reduction in cytokine secretion was associated with reduced transcript synthesis (93). Treatment of isolated macrophages with MANS or a peptide identical to the MARCKS ED attenuated LPS-induced expression of TNF- α through suppression of p38 and JNK (c-Jun N-terminal kinase) mitogen-activated protein kinases, as well as NF- κ B (96). Treatment of mice *in vivo* with the MANS peptide reduced serum TNF- α and IL-6 concentrations and resulted in 40% survival of mice after the administration of a lethal dose of LPS (96).

Mast cells also contribute to the pathophysiology of inflammatory airway diseases, including asthma, COPD, and idiopathic pulmonary fibrosis, primarily through exocytosis of granules containing mediators such as histamine and tryptase capable of eliciting a programmed inflammatory response (127). Exocytosis of mast cell granules depends on elevated intracellular Ca^{2+} (128). In an *in vitro* model using antigen-stimulated rat basophilic leukemia (RBL-2H3) cells, Gadi and colleagues showed that MARCKS dissociates from the membrane at the same time that activated PKC- β 1 translocates to the membrane (129). Furthermore, by expressing a mutated, nonphosphorylatable MARCKS-ED, these investigators showed that MARCKS sequestration of membrane PIP_2 delayed intracellular Ca^{2+} mobilization and inhibited granule exocytosis. They concluded that MARCKS regulates mast cell granule exocytosis in a PKC-dependent manner by regulating availability of membrane phosphoinositides required for granule fusion with the plasma membrane (129).

Inflammatory cell migration.

MARCKS has been shown to bind to and to cross-link actin in different cell types, as well as to colocalize with actin in the leading edge of migrating cells (10). Interestingly, MARCKS constitutes 90% of all proteins synthesized in neutrophils in response to exposure to TNF- α or LPS (130). Given the known relationship between MARCKS, the actin cytoskeleton, and inflammatory signals, it was hypothesized that MARCKS could be involved in inflammatory cell motility and that MANS could have additional antiinflammatory effects related to inhibiting leukocyte and inflammatory cell migration into the lung. Treatment of isolated human neutrophils with MANS, but not RNS, dramatically inhibited migration toward the chemotactic agents formyl-methionyl-leucyl-phenylalanine peptide, IL-8, and LTB₄ *in vitro*. MANS treatment also inhibited β_2 -integrin-dependent adhesion of these cells (8), as well as platelet-activating factor- and LTB₄ (leukotriene B₄)-induced chemotaxis and chemokinesis of equine neutrophils (94). Treatment of primary human neutrophils with the PKC δ inhibitor rottlerin blocked MARCKS phosphorylation and attenuated fMLP-, LTB₄-, and IL-8-induced chemotaxis in a concentration-dependent manner (16).

Treatment with MANS, but not RNS, also inhibited migration of macrophages in response to the chemoattractant MCP-1 (monocyte chemoattractant protein 1), and visual and biochemical evidence of a transient interaction between MARCKS and actin during the process of migration was observed (13). MANS treatment also blocked *in vitro* migration of mesenchymal stem cells in response to injury-associated chemokines, such as complement component 5a, stromal cell-derived factor 1 α , and MCP-1 (95).

As described above, MARCKS binds to and sequesters PIP₂ in membranes and subsequently releases PIP₂ after phosphorylation by PKC. This putative mechanism is related to MARCKS regulation of leukocyte migration and trafficking. Indeed, directed migration of neutrophils involves PIP₂ and downstream signaling molecules, especially PI3K (131). The leading edge of migrating neutrophils and macrophages contains many PIP₂-related signaling molecules, including MARCKS, PIP₃, PI3K, and numerous actin-binding and regulatory proteins (58,

132, 133). A series of recent publications by Ziemba and colleagues offered significant insight into the MARCKS-PIP₂ role in cellular migration. These investigators first used single-molecule fluorescence and total internal reflection fluorescence microscopy of supported lipid bilayers to measure the surface densities and activities of proteins and phospholipids involved in a hypothesized Ca²⁺-PKC-MARCKS-PIP₂-PI3K-PIP₃ amplification module (57, 58, 62, 134, 135). These studies confirmed that MARCKS sequesters PIP₂ at the lipid membrane and prevents PI3K α -mediated generation of PIP₃, until Ca²⁺-activated PKC α phosphorylates MARCKS (57). A second study also using single-molecule fluorescence microscopy and supported lipid bilayers showed that Ca²⁺/CaM binding to MARCKS ED also causes MARCKS to release sequestered PIP₂, leading to increased production of PIP₃ by PI3K α (62). Interestingly, the kinetics of PIP₂ release from MARCKS is much more rapid with Ca²⁺/CaM binding than with PKC α phosphorylation, taking seconds rather than minutes, suggesting two different pathways for regulation of membrane PIP₂ availability by MARCKS. Finally, studies with live cell fluorescence of polarized macrophages confirmed that the pathway activators PDGF-BB (platelet-derived growth factor BB) and ATP promote PIP₃-enriched leading edge expansion that is regulated by a Ca²⁺-PKC-MARCKS-PIP₂ signaling feedback module (58). These authors proposed that this signaling network is relevant not only to cells with amoeboid-like movement (i.e., neutrophils and macrophages) but also for mesenchymal cell chemotaxis and, perhaps, cancer cell migration.

Chronic Bronchitis

Based on *in vitro* and *in vivo* results showing a relationship between MARCKS, inflammation, and mucin secretion, a potential therapy related to chronic bronchitis, which is characterized by both mucus hypersecretion and inflammation in the airways, was considered. As indicated above, the biotech company BioMarck Pharmaceuticals developed the MANS derivative BIO-11006 as an inhaled aerosolized anti-MARCKS drug. Preclinical studies in dogs and rats for as long as 90 days indicated an absence of toxicity of inhaled BIO-11006, including no adverse effects on body weight; food consumption;

respiratory function; neurological function; cardiovascular function; or laboratory, microscopic, or macroscopic parameters. BIO-11006 showed no evidence of mutagenicity or clastogenicity. The U.S. Food and Drug Administration (FDA) then was approved BIO-11006 as an investigational new drug for a clinical trial in patients with chronic bronchitis. Two phase I clinical studies were performed in healthy volunteers ($n = 67$). A single 75-mg dose of inhaled BIO-11006 was well tolerated by eight normal adult volunteers in the first study (BIM-CL-001). In the second study (BIM-CL-002), a single inhaled dose of up to 1,000 mg and multiple inhaled doses of up to 250 mg/d for 14 days were well tolerated by 59 healthy adult volunteers. Headache, pyrexia, and cough were the most common adverse events seen with multiple inhaled doses.

On the basis of the foregoing, the FDA approved the phase IIa study (BIM-CL-003), BREATH 1 (BIO-11006: Respiratory Effectiveness of a Targeted Therapeutic), consisting of a double-blind, placebo (half-normal saline)-controlled, multicenter dose escalation study in subjects with chronic bronchitis. The primary objective was to evaluate the safety and tolerability of BIO-11006 when administered once (75 and 150 mg) or twice (75 and 125 mg) daily for 21 days by inhaled aerosol to subjects with COPD experiencing chronic bronchitis by assessing adverse events, physical examinations and vital signs, pulmonary function (including FEV₁), 12-lead electrocardiogram, clinical blood chemistries, hematology, and urinalysis. The secondary objective of the study was to evaluate the efficacy of BIO-11006 by assessing the change from baseline in pulmonary function as measured by FEV₁. A total of 172 subjects with stable COPD (mostly Global Initiative for Chronic Obstructive Lung Disease stages 2 and 3) were randomized in the double-blind study. Trough FEV₁ (primary endpoint) was measured at baseline and on Days 3, 7, 14, 21, 28, and 49. Secondary endpoints of Breathlessness, Cough and Sputum Scale and St. George's Respiratory Questionnaire were measured regularly.

Although the primary and secondary efficacy endpoints did not meet statistical significance at Day 21, the conclusion of the study researchers was that the 75-mg twice-daily dose of BIO-11006 proved to be

efficacious by increasing the proportion of FEV₁ responders statistically significantly as compared with half-normal saline on Days 14 and 28 of the study. In addition, bronchitic symptoms associated with COPD decreased (136).

ARDS

Given the above-described *in vitro* effects of MANS and BIO-11006 on leukocyte trafficking and cytokine/chemokine secretion, and given the antiinflammatory effects in lungs of mice exposed to ozone (22), it was hypothesized that inhibition of MARCKS with inhaled peptides might be beneficial for patients with the lung disease ARDS. The United States has about 200,000 cases of ARDS annually. It is a disease associated with sepsis, pneumonia, smoke inhalation, near drowning, among others, and is characterized by a massive influx of neutrophils into the lung. Mortality is approximately 40%, and the current standard-of-care treatment is mechanical ventilation. There are no drugs proven effective in this disease.

The possibility of an inhaled anti-MARCKS peptide affecting disease progression was looked at, using intratracheal instillation of bacterial LPS as a murine model of ALI/ARDS. BIO-11006 was delivered via inhalation either just before intratracheal instillation of 5 µg of LPS into BALB/c mice or 4, 12, 24, or 36 hours after LPS instillation. Treatment with aerosolized BIO-11006 at 0, 4, 12, 24, and even 36 hours after LPS instillation reversed the disease process: Mouse behavior returned to normal after two treatments 12 hours apart with the inhaled peptide after LPS injury, whereas control LPS-instilled animals treated with PBS only remained moribund. Histological appearance of inflammation, BAL fluid protein concentrations, leukocyte and neutrophil numbers, KC (keratinocyte chemoattractant; CXCL1, mouse IL-8 equivalent) and TNF-α gene and protein expression, and NF-κB activation were all significantly attenuated by inhaled BIO-11006 at all time points (23). The fact that the treatment was effective as long as 36 hours after administration of LPS to the mice suggests that inhaled MARCKS inhibitors could be an effective treatment for patients who present with already-active ARDS. In another related study, treatment of mice with the MANS peptide administered intravenously reduced

serum TNF-α and IL-6 concentrations and resulted in 40% survival of mice after the administration of a lethal dose of LPS (96).

On the basis of the data reported above, the FDA approved a phase IIa clinical study in ARDS using inhaled BIO-11006, which already had been shown to have a good safety profile in human volunteers and patients with chronic bronchitis in a previous clinical trial (*see above*). This clinical study of ARDS (BIM-CL-005) commenced in the spring of 2018. The title of the study is “A Phase 2 Multicenter Study to Evaluate the Safety and Efficacy of BIO-11006 Inhalational Solution in Patients with Acute Respiratory Distress Syndrome” (NCT03202394). This study is placebo controlled and funded by BioMarck Pharmaceuticals. All patients are receiving ventilation in an intensive care unit, with half randomized to receive BIO-11006 and half to receive placebo. The primary endpoint is survival at Day 28. Other factors such as days on ventilator and percentage arterial oxygen are secondary endpoints.

Lung Cancer

Clearly, MARCKS plays an integral and probably critical role in the motility and migration of different cell types, including inflammatory cells, fibroblasts, endothelial cells, stem cells, and epithelial cells (8, 13, 15, 16, 95, 137–140). The potential role of MARCKS in migration of lung cancer cells, and possibly in lung cancer metastasis, was investigated. Results of studies looking at effects of MARCKS-inhibitory peptides on migration of cancer cells in chambers showed that both MANS (12) and BIO-11006 (99, 141–143) blocked *in vitro* migration of non-small cell lung cancer (NSCLC) cells, suggesting that MARCKS could potentially be an effective target for attenuating metastasis of lung cancer *in vivo*.

Certainly, MARCKS appears to be a valid therapeutic target in lung cancer. There are many published reports indicating that the presence of MARCKS, especially the phosphorylated, activated form, correlates with aggressiveness and poor prognosis in numerous neoplasias, including melanoma (9, 11), cholangiosarcoma (144), bladder (145), glioma (146, 147), breast (148, 149), colorectal (150, 151), intestinal (152), kidney (153), liver (154), pancreas (155), prostate (156, 157), multiple myeloma

(158), osteosarcoma (159), ovarian (160, 161), and, indeed, lung (105, 162). On the basis of these reports, Chen and colleagues looked retrospectively at lung cancer archived tissues (163). Samples from a cohort of over 100 patients with lung cancer were analyzed by immunohistochemical staining with a monoclonal antibody recognizing phosphorylated MARCKS (p-MARCKS). High concentrations of p-MARCKS were found in tumor tissues compared with normal lung tissues, and, importantly, the concentrations of MARCKS phosphorylation correlated directly and significantly with the stage of the cancer; samples from stages III and IV had the highest concentrations. This also correlated with lymph node metastatic status (163).

Studies were then performed to determine the potential role of MARCKS and phosphorylated MARCKS in metastasis *in vivo*. An orthotopic model of lung cancer in which PC-9 cells, an aggressive human lung cancer cell line, were injected into the left lobe of immunocompromised mice was used. Treatment with the MANS peptide, injected intraperitoneally into these mice every 3 days, dramatically inhibited the metastasis of these cells to other regions of the lung and to distal organs (12). Another study with the same orthotopic model used a 25-mer peptide targeting the MARCKS phosphorylation site domain (ED motif). This peptide suppressed tumor growth and metastasis *in vivo* and reduced concentrations of p-MARCKS, PIP₃, and Akt activity. The peptide also enhanced the sensitivity of lung cancer cells to erlotinib treatment, especially those cells with sustained activation of PI3K/Akt signaling (164). These inhibitory effects on metastasis in models of lung cancer also held true for treatment *in vivo* with inhaled BIO-11006 (99, 141).

A third phase II clinical trial is currently ongoing, again under the auspices of BioMarck Pharmaceuticals, for NSCLC (A Randomized Study of the Safety and Efficacy of BIO-11006 in Treatment of Advanced Non-Small Cell Lung Cancer in Patients Who Are Not Candidates for Curative Surgery and/or Radiation and Who Are Receiving Pemetrexed and Carboplatin; BIO-NSCLC-001 [NCT03472053]). This study is controlled by standard-of-care therapy (pemetrexed and carboplatin). All patients receive standard of care,

and half are randomized to receive nebulized BIO-11006 in addition for a 3-month period. The primary endpoint is progression-free survival at 3 months, but patients will be followed for a total of 12 months to assess overall survival and weight. Patients will be monitored for adverse events and serious adverse events during the 12-month study. The study commenced in early 2018.

Conclusions

A summary of relevant translational research involving MARCKS is provided in Table 1. From the time that MARCKS was identified as a molecule that plays a role in secretion of mucin in the airways, its role as a potential therapeutic target in the airways and lungs has expanded from COPD to include airway inflammation and ARDS, and even NSCLC. Means of

inhibiting MARCKS pharmacologically include the MANS peptide, which is identical to the 24-amino acid stretch encompassing the MARCKS amino terminus, and BIO-11006, which contains the active portion of MANS but is smaller (10 vs. 24 amino acids) and more soluble (acetate substituted for myristic acid on N-terminal amino acid). Peptides containing the ED of MARCKS, including the MRP peptide, also have been shown to be effective in inhibiting MARCKS and p-MARCKS actions in preclinical models. At this point, BIO-11006, under FDA investigational new drug status, has been tested in phase I studies in human volunteers, as well as in 172 patients with COPD, and it has shown a good safety profile. Currently, there are ongoing phase IIa clinical trials with BIO-11006 in patients with ARDS and NSCLC, with potentially

other trials to come. The fact that the same therapeutic target might occur in three different respiratory/pulmonary diseases goes back to the fact that MARCKS, found ubiquitously in all eukaryotic cells and conserved evolutionarily for such a long time, clearly has an important and basic function in cells, and it is therefore not surprising that its inhibition or attenuation could have diverse effects. Whatever the outcome(s) of these trials, it is clear that MARCKS and p-MARCKS represent “druggable” targets in several lung diseases. Pending results of current investigations, specific MARCKS-inhibitory peptides could be available as airway therapeutics in the near future. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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