[Frontiers in Bioscience 17, 1775-1794, January 1, 2012]

RHAMM and CD44 peptides-analytic tools and potential drugs

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1. ABSTRACT

CD44 and RHAMM are two extracellar matrix receptors whose principle ligand is the polysaccharide hyaluronan (HA). Both proteins are involved in wound repair and their aberrant regulation contributes to a variety of diseases including arthritis and cancer. Over the past decade, a number of peptide-based therapeutics that block the binding of CD44 or RHAMM-specific ligands have been developed and tested in experimental models of disease. Here, we review the structure of each of these proteins, the functions they control and the mechanisms, including their interactions with each other, responsible for these functions. We also review the development of peptide mimics that block the key functions of CD44 and RHAMM and their use in experimental models of disease.

2. INTRODUCTION

CD44 and RHAMM (1) play major roles in disease processes with an underlying inflammatory cell dysfunction. These include diabetes, arthritis and many types of cancer (1-3). There is some evidence that these two proteins interact either directly through protein-protein associations and/or by functionally influencing each other's signaling properties (3-6). For example, extracellular RHAMM binds to CD44 in wound mesenchymal cells and this association is necessary for the sustained activation and nuclear translocation of activated ERK1,2, as well as cell migration, robust fibroplasia and mesenchymal differentiation within wound sites (4). CD44 and RHAMM also influence each other's functions in a collagen-induced model of arthritis. In this

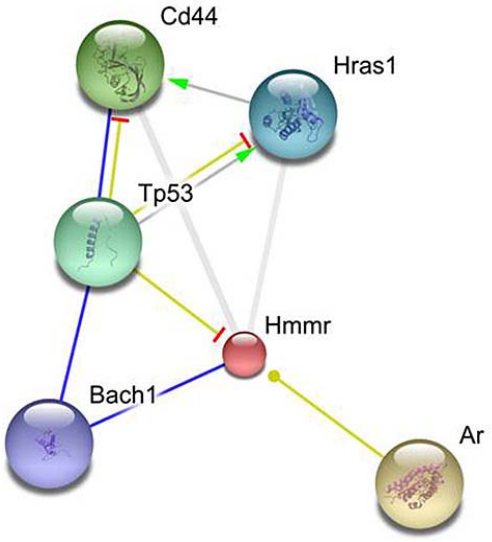

Figure 1. A CD44-RHAMM signaling network. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING (http://string-db.org)) that detects known and predicted protein-protein interactions, identifies a CD44-RHAMM interactome at >90% confidence levels, excluding annotated and curated pathways in the analysis. At this level of confidence, minimal signaling interactions between Ras and P53 are identified. CD44 =principle HA receptor; Hmmr= (RHAMM) Human receptor for HA mediated motility; H-Ras1=Harvey rat sarcoma viral oncogene homolog; Tp53=p53 tumor suppressor; Bach1=transcription regulator; Ar: androgen receptor.

model, blocking CD44 function attenuates the disease, clearly showing that CD44 is involved in arthritis initiation (5). Intriguingly, total loss of CD44 by genetic deletion in this model increases rather than decreases disease severity, which is an effect that is countered by blocking RHAMM function suggesting that RHAMM functionally compensates for CD44 genetic loss (i.e., in CD44 knock out mice). This study further illuminates dual functions of CD44 in arthritis, one that is required for disease manifestation but another that limits disease severity, at least in part by curbing pathology promoting effects of RHAMM. A similar duality in CD44 function appears to control progression of some cancers and has been related to tissue specific CD44 variant expression, and specific cell subpopulations such as tumor initiating cells (7, 8). RHAMM may similarly perform dual functions during cancer progression (3). For example, although RHAMM hyper-expression is most commonly linked to neoplastic aggression (e.g. breast cancer, colorectal cancer, multiple myeloma) (2, 9 ,10) its loss is associated with progression of malignant peripheral nerve sheath tumors (11). Further, increased risk of breast cancer associated with RHAMM polymorphisms can be linked to either increased or decreased germ line expression (12). This Janus-like nature of RHAMM and CD44 is similar to other oncogenic proteins including, NEDD1 (13), NF-kappa B (14), ErbA (15) and others, which can either promote or impair tumor progression depending both upon cellular context, their expression levels and the presence of other mutant signaling pathways in diseased cells. In the case of RHAMM, either high expression or loss can result in aberrant Ran-dependent mitotic spindle formation and chromosome segregation (16, 17). Thus, gain or loss of RHAMM has the potential of generating genomic instability that can fuel tumor progression. Net gain of RHAMM is more common in tumor cell populations as a whole, and this gain must confer advantages to the proliferation and survival of tumor cells that net loss does not. The underlying mechanisms for the cooperativity between RHAMM and CD44 is not yet well understood and the contribution of aberrant CD44/RHAMM partnering in tumor promoting vs. tumor suppressing functions of CD44 is also not understood. However CD44 and RHAMM most obviously share an ability to bind to the polysaccharide hyaluronic acid or hyaluronan (HA), which is an important factor in functionally linking these two otherwise very different proteins. Furthermore, co-expression of these two HA receptors has been linked to very poor prognosis in at least one cancer type, diffuse large B-cell lymphomas (18) suggesting that interactions of these two proteins likely has relevance in the clinical setting.

A survey of published literature documenting associations of these two proteins in cultured cells identifies pathways that RHAMM and CD44 co-regulate. For example, both receptors control signaling through RAS-ERK1,2 and PI3K/AKT pathways and the expression of both proteins is inhibited by P53 (19-22) (summarized in Figure 1). Published data sets can be probed with more rigor using Ingenuity pathways Analysis (www.ingenuity.com) (Figures 2,3). In this analysis, networks are algorithmically generated by identification of "Eligible Molecules" whose expression is significantly differentially regulated with disease or a physiological/morphogenetic function and overlaying

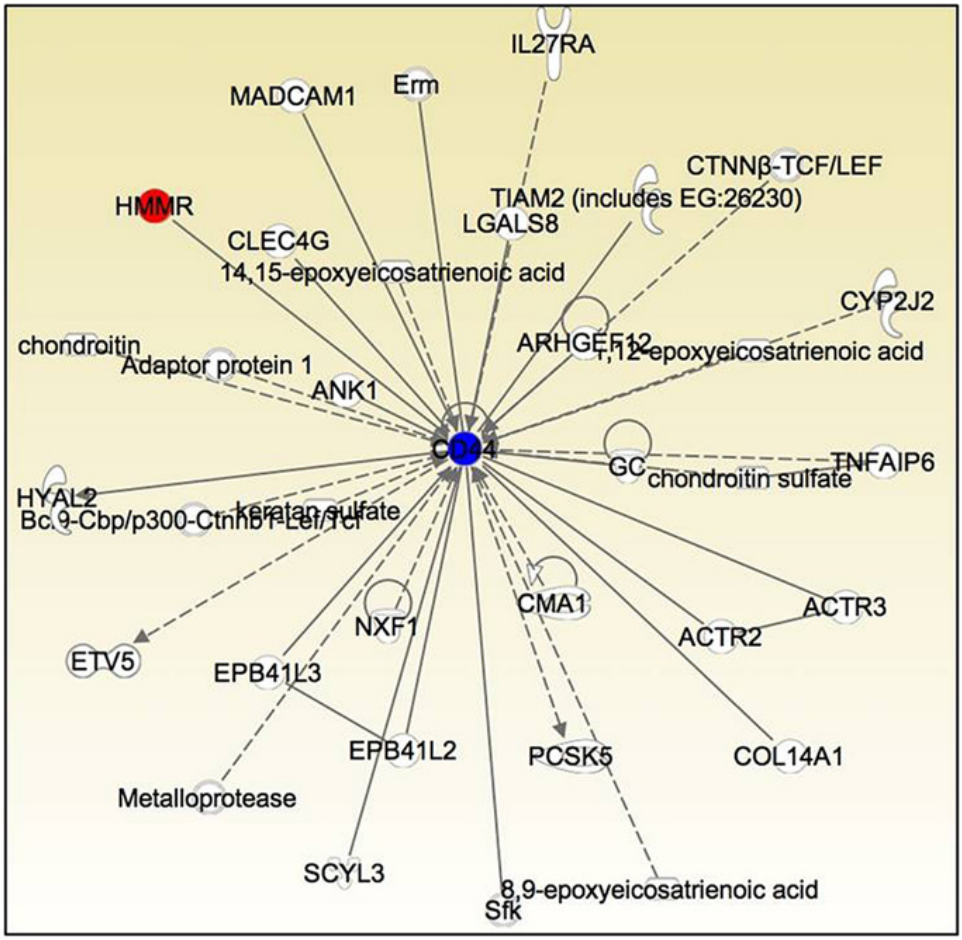

Figure 2. CD44 network of molecular interactions. Pathways associated with CD44 control cellular movement, growth and proliferation that contribute to cell-mediated immune responses and immune cell trafficking and that impact upon cancer and inflammatory disorders. The network was generated through the use of Ingenuity Pathways Analysis (Ingenuity®, www.ingenuity.com). The continuous lines represent protein-protein interactions while the dotted lines represent indirect interactions, which can include protein phosphorylation, effects on protein expression and translocation. The circular loops above genes represent homo-effects. CD44 and HMMR are highlighted with semi-transparent boxes. Full names and functions of genes are given in unigene on NCBI website (www.ncbi.nlm.nih.gov).

these onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. A network is a graphical representation of the molecular relationships between the identified molecules. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (23). All edges are supported by at least 1 reference from the literature, from a textbook or from canonical information stored in the Ingenuity Pathways Knowledge Base. These analyses highlight that RHAMM is one of many CD44 signaling partners (Figure 2). Conversely, CD44 connects with some but not all RHAMM-regulated networks (Figure 3). These include VEGF, HGF, HA and downstream effectors, Src, ERK1,2 and Fos, all of which have been linked to cell migration. Ingenuity analyses suggests that the mitotic spindle functions of RHAMM, e.g. that are mediated through MAFG, DYNLL1, MAFK and FAM83D (Figure 3), may be regulated by CD44-independent mechanisms. The identified CD44/RHAMM networks link significantly to cellular growth and proliferation as well as cellular movement ($p=0.00006$, and 0.00001 respectively) and to tissue injury, cancer and inflammatory responses ($p<0.0001$).

The dual nature, functionally interconnected properties and functional redundancy of important therapeutic targets such as CD44 and RHAMM is not a mere laboratory curiosity but a property that must ultimately be considered in the design of therapies that block the functions of these HA receptors for example by antibodies or peptides. Here, we review the development of peptide reagents that target CD44 or RHAMM.

3. CD44: STRUCTURE AND FUNCTION

The CD44 isoform repertoire and its wide spectrum of ligands have attracted much attention owing to their involvement in many physiological and pathological activities (24-30). The differential expression of distinct CD44 isoforms or their ligands on cells engaged in pathological or physiological activities has provided new opportunities for selective therapeutic

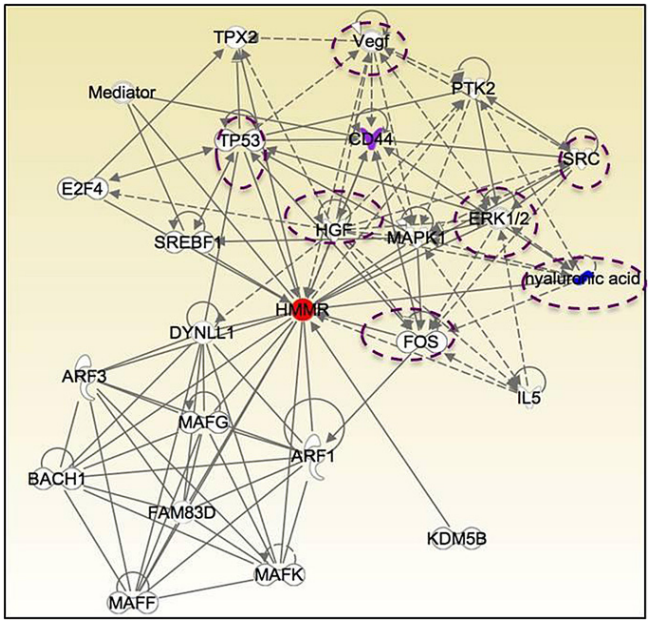

Figure 3. RHAMM network function. Pathways associated with RHAMM function that control cellular movement, growth and proliferation that contribute to connective tissue development and function and that play a role in cancer and tissue injury. The network was generated through the use of Ingenuity Pathways Analysis (Ingenuity®, www.ingenuity.com). The continuous lines represent protein-protein interactions while the dotted lines represent indirect interactions, which can include protein phosphorylation, effectson protein expression and translocation. The circular loops above genes represent homo-effects. The broken circles mark signaling elements that are also connected to CD44. Gene names are abbreviations provided by unigene (NCBI): TPX2: microtububule-associated protein homolog; Vegf: vascular endothelial growth factor; PTK2: protein tryosine kinase 2; SRC: Moloney murine sarcoma virus; E2FA: Transcription factor E2FA; TP53: tumor suppressor protein p53; CD44: CD44; SREBF: SREBF chaperone protein; HGF: hepatocyte growth factor; MAPK1: mitogen activated protein kinase 1; ERK1,2: Extracelllar regulated kinase 1,2; DYNLL1: dynein light chain 1; HMMR: (RHAMM) Human receptor for HA mediated motility; FOS: FOS Transcription factor; hyaluronan acid: hyaluronan (HA); ARF3: ADP ribosylation factor; MAFG: MAFG transcription factor; IL5: interleukin 5; BACH1: transcription regulator BACH1; FAM83D: mitotic spindle protein; KDM5B: MAFF: transcription factor; MAFK: transcription factor.

targeting – a long standing and as yet unattained goal of modern medicine. The multi-structured nature of CD44 is generated by alternative splicing of 9 (human) or 10 (mouse) variant exons inserted in different combinations between two constant regions, each one containing 5 constant exons (for review, see (25-27, 29, 30)). Theoretically, close to 800 CD44 variants (CD44v) can be generated by differential utilization of the variant exons (31), of which many already been detected (25-27, 29, 30). For example, splicing and insertion of variant exons v4, v5, v6 and v-7 (CD44v4-v7) between the two constant regions generate a CD44 isoform with a metastatic potency ((32) and review in (25)). Splicing and insertion of variant exons v3 to v10 in tandem generates the CD44v3-v10, which is preferentially expressed on normal keratinocytes (33) and synovial fluid cells from rheumatoid arthritis (RA) patients (34). Insertion of exons v8, v9 and v10 between the two constant exons, generates CD44v8-v10, preferentially expressed on epithelial cells and, therefore designated epithelial CD44 (25, 29). Direct splicing from constant exon 5 to constant exon 16, skipping all the variant exons, generates the shortest and most ubiquitous standard form, CD44s, which is preferentially expressed on hematopoietic cells (25, 29), and known also as hematopoietic CD44. All the CD44 variants share the 5' and the 3' constant regions; the 5' constant region includes the ligand (e.g., HA) binding site. The variable region, which includes the variant exon products of the differential alternative splicing, is inserted between the two constant regions.

N- and O-glycosylation, as well as glycosaminoglycan (e.g., heparan sulfate, chondroitin sulfate) attachments further increase the variability of CD44 (25-27, 29, 30). The multi-structured nature of CD44 is presumably associated with its multi-functionality, including cell-cell and cell matrix interactions, support of cell migration within blood vessels and inside the tissues, presentation of growth factors, cytokines, chemokines and enzymes to other cells or the surrounding tissues, as well as signal transmission from the cell surface to the interior of the cell, leading to programmed cell death or to cell survival and proliferation (for review see (26, 27)). The list of CD44 ligands is continuously growing, and it includes its principle ligand HA, as well as collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate, mucosal vascular addressin, serglycin/gp600, osteopontin (OPN), the major histocompatibility complex class II invariant chain (li), L-selectin and E-selectin (26). Again, the rich isoform-

repertoire of CD44 may explain its ability to bind a considerable number of different ligands, many of which may remain to be discovered. The interaction between CD44 and a specific ligand likely dictates the outcome, i. e., signaling leading to a defined physiological or pathological activity. If this is the case, it is important not only to illuminate the signal transduction of specific ligand-receptor interactions, but also to identify novel CD44 ligands that may be engaged in pathological functions and, consequently, could be used as therapeutic targets. It was recently found in Naor's laboratory that galectin-8, an apoptosis-inducing animal lectin, is an additional CD44 ligand that binds to the CD44 receptor with high affinity (Kd = 5.8x10-9) (35).

HA is the principle ligand of CD44 but it also interacts with a family of proteins collectively called hyaladherins, which include the link protein superfamily, the lymphatic vessel endothelial HA receptor-1 (LYVE-1) and Receptor for HA Mediated Motility (RHAMM) as well as Toll Like Receptors (TLRs),e.g., TLR-2 and TLR-4,involved in innate immunity and Stabilin1,2 involved in HA endocytosis in the liver and lymphatics (26, 28). HA consists of repeating disaccharide units of D-glucuronic acid (1-β-3) and N-acetyl D-glucosamine (1-β-4) and interacts with key basic and hydrophobic residues in its partner proteins. For example, the HA binding domain of RHAMM is a region containing a specific spacing of basic and hydrophobic amino acids as determined by computer modeling and site mutagenesis (36, 37). In contrast, HA binds to CD44 via a much more complicated "link module" that presents a shallow cleft, which HA fits into (38). Although CD44 sequence contains several RHAMM-like motifs and these bind to HA when synthesized as short peptides (39), their relevance to the HA binding capacity of CD44 as a protein has not been established.

4. CD44 PEPTIDES DERIVED FROM THE CD44 RECEPTOR

CD44 peptides compete with CD44 for shared ligands and are classified as CD44 peptide mimetics. They interfere with CD44-ligand interactions and ablate support for tumor cell invasion as well cell motility of autoimmune or allergic inflammatory cells (40).

To verify whether specific HA binding peptide motifs are relevant for tumor progression, a given sequence, e.g. a 42 amino acid peptide, assigned from GenBank human HABP2 sequence (accession number AY007241) was synthesized. This peptide (designated BH-P) contains three, RHAMM-like clusters of basic amino acids required for HA binding. BH-P was tested for its anti- tumor activity *in vitro* and *in vivo* assays (41). Injection of BH-P inhibited cell growth of B16 and MDA-435 melanoma cells in chick embryo chorioallantoic membrane assays. Similarly, transfection of human MDA-435 melanoma cells with a secreted version of the BH-P expression vector suppressed the xenograft tumor growth in nude mice. The anti-tumor activity of BH-P appears to be exerted through induction of apoptosis, as revealed by its ability to activate critical molecules of the pro-apoptotic pathway, such as caspase-8, caspase -3 or poly (ADR-ribose) polymerase (PARP). The authors suggest that BH-P binds to cell surface HA thereby initiating a signaling pathway that induces apoptosis in the tumor cells (41). However, these data can be interpreted differently: an interaction between CD44 expressed on tumor cells and HA in the surrounding extracellular matrix (ECM) likely delivers a survival signal to malignant cells (42). This interaction is competed with and abolished by the BH-P peptide, resulting in activation of programmed cell death.

The CD44 v6-containing variants are impressively expressed on many human and rodent metastatic cancer cells (26). CD44 v6-containing variants are CD44 isoforms inserted with a variable region, which includes the v6 variant exon either alone or in combination with additional variant exons (e.g., CD44v4-v7). For simplicity, all the CD44-containing variants are designated CD44v6, unless otherwise is indicated. The profound presentation of CD44v6 on metastatic tumor cells suggests that this variant is associated with their malignancy (26). Indeed, non-metastatic rat pancreatic tumor cell line, which was transfected with CD44v4-v7 (including variant exon v6) cDNA acquired metastatic activity (32, 43). Injection of anti-CD44v6 monoclonal antibodies (mAbs) into rats bearing CD44v6-expressing pancreatic adenocarcinoma substantially reduced their metastases in lymph nodes and lung (44). Cell surface CD44v6 of tumor cells expressing this variant (e.g., pancreatic adenocarcinoma cell lines as well as other v6-bearing tumors) cooperates with c-Met receptor tyrosine kinase (RTK), after its stimulation with hepatocyte growth factor (HGF). The collaboration between cMet, the cognate receptor of HGF, and CD44v6 generates an intracellular signal transmission leading to spread and invasiveness of carcinoma cells detected both *in vitro* (45, 46) and *in vivo* (40). This intracellular signaling is dependent on multi-component complex formation, which involves cMet, HGF, CD44v6 and ezrin (40). Ezrin is included in ezrin, radixin and moesin (ERM) family of proteins, which are associated with the cytoskeleton cellular function via interaction with the linker protein F-actin (47), hence indicating cytoskeleton integration in the HGF-induced signaling process. The complex formation, indicated above, activates Akt, c-Jun NH2-terminal kinase (JNK), Ras , Raf, MEK and Erk. Disruption of Ezrin or F-actin engagement by insertion CD44v6 deleted of cytoplasmic tail or interference with F-actin polarization blocks Erk activation (i.e., cMet to Erk signaling), but does not interfere with cMet phosphorylation. This finding suggests two collaboration steps between CD44v6 and HGF-stimulated cMet: extracellular step, where CD44v6 ectodomain involves in cMet phosphorylation and intracellular step where the above mentioned collaboration delivers signaling from the CD44v6 cytoplasmic tail to the cytoskeleton (48). However, the entire process cannot be completed without the initial partnership between CD44v6 and cMet, which finally potentiates tumor invasion and metastasis (40).

Additional collaborative activities between CD44v6 and other RTKs have been documented, revealing signaling pathways almost identical to the one generated by CD44v6-cMet cooperation. Ron, like cMet, is over-expressed in several cancer cell lines and it is activated by macrophage stimulating protein (MSP) (in analogy to HSF, which stimulates cMet), leading to Ron to Erk signal transduction and *in vitro* cell invasion into Matrigel by the tumor cells (46). The interaction between HGF and c-Met or between vascular endothelial growth factor-A (VGEF-A) and its cognate receptor (VEGFR-2) generated CD44v6-depended signaling process in endothelial cell lines as well. These interactions lead to Erk activation, which is also conditioned to association of the cytoplasmic CD44v6 carboxyl-terminus with ezrin, coupling the signaling to the cytoskeleton. These intracellular events subsequently lead to endothelial cell migration and tubule formation, detected in culture assays as well as to blood vessel development and tumor angiogenesis as shown *in vivo* (49). Listeria monocytogenes exploits cell surface protein such as c-Met in order to invade eukaryotic cells. The bacterial protein internalin B (InlB) phosphorylates and activates c-Met ,in CD44v6-dependent manner to allow signal delivery from cMet to Erk with cytoskeleton involvement , thus promoting invasion of the target cells by *L. monocytogenes* (50).

Collaboration between CD44v6 and RTKs in the delivery of cell signal transmissions leading to potential pathological activities has been demonstrated by targeting the CD44v6 molecule of the relevant cells with anti-CD44v6 mAb (46, 50), v6-specific RNA interference (RNAi) (46) or soluble CD44v6 ectodomain (49). The Substantial reduction in signaling delivery by these reagents implies that in the absence of an efficient collaboration with CD44v6, the RTKs-stimulating ligands, cannot deliver their pathological messages.

Five, 10 or 14 mer peptides derived from the v6 variant product of CD44v6 molecule (Table 1) can also disrupt the signaling process and the consequent pathological activities of malignant cells (46), endothelial cells (49) and infected eukaryotic cells (50), all expressing stimulated RTKs, which require CD44v6 cooperation (note that not all RTKs, e.g., PDGF, are CD44v6-dependent , see ref (46)). The peptides include a core trimer (EWQ in rat) a sequence, which is essential for the function of CD44v6 as a co-receptor (46). How does the v6 peptide work? The simplest explanation is that the CD44v6 ectodomain interacts with a specific site at the RTK ectodomain, which is also recognized by the v6 peptide, either by sequence or configuration, thus interfering with the interaction between the co-receptor and the receptor. Such a species-specific site that is shared by all the CD44v6-dependent RTKs has not yet been identified. Obviously, the CD44v6 peptides should be considered as promising candidates for clinical intervention. However, in this undertaking, maximal caution is advised, because CD44v6 is expressed on many cell types involved in physiological activities, such as normal keratinocytes. Indeed, patients with head and neck squamous cell carcinoma (CD44v6-expressing tumor) that were treated with toxin-conjugated anti-CD44v6 mAb developed a severe, even life threatening skin reaction, which led to the termination of this clinical program (51). The expression of CD44v6 on squamous cells in the upper layer of the human skin has been documented, using anti-CD44v6 mAb raised against a synthetic peptide derived from v6 variant exon (see Table 1). This information was published 14 years before this clinical study was accomplished (52). Therefore, it is taken for granted that this information was known at the time when the cancer patients were being treated with the CD44v6-specific antibodies. Note that this peptide shares a sequence of 7 amino acids (52) with the human v6 14 mer peptide ((46) underlined in Table 1). This sequence does not include the RWH core trimer of the human peptide (see below). Support that cell surface CD44v6 promotes cell migration is provided by liver peri-sinusoidal stellate cells derived from *in vivo* injured rat liver (designated activated stellate cells). The deleterious effect of chronic fibrosis initiated by the migrating cells may be assisted by v6-containing CD44 variant. Indeed, anti-CD44v6 antibody (but not anti-CD44v4 antibody) reduced by 50% the *in vitro* cell migration of activated stellate cells on HA substrate, but not on collagen substrate. Twenty mer synthetic peptides representing the N-terminal half of the v6 domain (Table 1), but not 23 mer peptides representing the C-terminal, block the anti-migratory effect of the anti-CD44v6 antibody (53). These results suggest that v6 epitopes of the stellate cell CD44v6 receptor influence the orientation of the HA binding sites on the constant region of the same molecule, allowing their interaction with the HA substrate and the subsequent support of cell migration. This remote pro-migratory (and pro-fibrosis) effect of v6 epitopes is disrupted after their interaction with anti-CD44v6 antibodies. Yet, the v6 N-terminal (designated v6N) peptide can block the anti-CD44v6 antibody, hence reversing the anti-migratory/ fibrosis effect of the antibody. Although the 14 Mer rat v6 peptide (46) and the 20 Mer rat v6N peptide (53) share the KEKWFE sequence (bolded in Table 1), this sequence does not contain the core trimer EWQ included in both 5 and 14 Mer v6 rat peptides ((46); mark italic in Table 1). Core trimers EWQ (in rat) and RWH (in man) are responsible for the cooperative activity of CD44v6 ectodomain and therefore for the inhibitory activity of v6 peptides (46). This fact suggests that the v6 peptides and the v6N peptide recognize different sequences on their acceptor molecules (RTK and anti-CD44v6 antibody, respectively).

5. THE TROJAN HORSE CD44-DERIVED PEPTIDE

Serine phosphorylation of human CD44 cytoplasmic tail at positions 323 and 325 (Ser323 and Ser325, respectively) enhances the cell migration potency, as indicated by a reduced motility in cells expressing CD44 mutants, in which serine was substituted for threonine at these sites. Mutation at position 325 only was sufficient to almost maximally reduce cell migration on HA substrate, implying the dominant status of phosphorylated Ser325 (pSer325). It was suggested that the role of serine at position 323 is to bind a kinase that

Table 1. Sequence of CD44-related peptides and their activity in experimental models

Ref.	Source	Mer	**SEQUENCE**	Effect of peptide and comments related to the peptide
41 (BH-P synthetic peptide)	Human CD44s	42	CNGRCGGRRAVLGSPRVKWTFLSRGRGGRGVRVKVNEAYRFR	Inhibits melanoma cell growth. Contains 3 HA binding sites (BX_7B). The N-terminal is acetylated and C-terminal is amidated to stabilize the peptide
46 (v6 14 mer synthetic peptide)	Rat CD44v6 Mouse CD44v6	14	**KEKWFE**N*EWQ*GKNP QETWFQNGWQGKNP	Inhibits c-Met signaling
46,49,50 (v6 14 mer synthetic peptide)	Human CD44v6	14	KEQWFGN*RWH*EGYR	Inhibits c-Met signaling Inhibits *in vitro* tumor cell migration and *in vivo* tumor cell growth Inhibits tumor angiogenesis , and infection of eukaryotic cells
46 (V6 5 mer Ref (synthetic peptide)	Rat CD44v6	5	N*EWQ*G	Inhibits c-Met signaling
46 (V6 5 mer synthetic peptide	Human CD44v6	5	N*RWH*E	Inhibits c-Met signaling Inhibits *in vitro* tumor cell migration
52 (v6 16 mer synthetic peptide)	Human CD44v6	16	STTEETATQKEQWFGN	Used to generate anti-CD44v6 monoclonal antibodies (mAb). An additional COOH-terminal cysteine was included in peptide for coupling purposes
53 (v6 N synthetic peptide)	Rat CD44v6	20	WADPNSTTEEAATQ**KEKWFE**	Revers the anti-fibrosis effect of anti-CD44v6 mAb
54 (pSer325-containg peptide)	Human CD44 cytoplasmic tail	13	**RQIKIWFQNRRMKWKK**LNGEASKpSQEMVH	Attenuating *in vitro* cell migration of melanoma cells. Conjugated to 16 mer Penetratin (bold and underlined); peptide C-terminal amidated and N- terminal biotinated
56 (pSer325-containg peptide)	Human CD44 cytoplasmic tail	13	**YGRKKRRQRR**LNGEASKpSQEMVH	Reduces *in vitro* cell migration of prostate cancer cells. Conjugated to 10 mer TAT (bold and underlined)
58-61, 65-67, 72 (Pep-1)	HA Phage display technology	12	GAHWQFNALTVR	Inhibits *in vitro* HA-dependent cell adhesion and *in vivo* cell migration of normal and tumor cells; suppresses antigen presentation to T cells; inhibits acute and chronic skin inflammation; detects pathological HA in inflammatory and malignant tissues; reduces HA-dependent lung metastasis of melanoma cells and attenuates IL-2-induced vascular leak syndrome
73 (P3,P6, P7)	Human osteopontin	10	p3=PDIQYPDATD; p6=HMESEELNGA; p7=ELNGAYKAIP	Reduce *in vitro* cell invasion of hepato cellular carcinoma by interaction with cell surface CD44. Derived from OPN-5kDa
76 (PI peptide)	COOH-terminal heparin binding domain of Human fibronectin	15	KNNQKSEPLIGRKKT	Attaches to glycosaminoglycan (GAG)-containing CD44
77 (Peptide V)	Hunan heparin-binding fibronectin fragment (HBFN-f)	8	WQPPRARI	Binds to cell surface GAG-containing CD44 ,thereby inhibits metalloproteinases (collagenases) induction by HBFN-f and the subsequent collagen cleavage in cartilage culture HBFN-f is a fragment of fibronectin.
78 (Peptide A5G27)	Laminin α5 globular domain	13	RLVSYNGIIFFLK	Inhibits melanoma local growth and lung metastasis as well as angiogenesis

subsequently phosphorylates Ser325. Although the mutation at position 325 reduced cell migration, it did not influence the ability of the cells to express CD44 or bind HA (54). This finding was further characterized (54), using CD44-derived 13 mer peptide, spanning phosphorylated Ser325 (pSer325), and linked to 16 mer Penetratin (derived from Antennapedia homeodomain, a Drosophila transcription factor, and nicknamed Trojan peptide). Inverted micelles, which form when the highly basic Penetratin (coupled to its cargo) interacts with the negatively charged phospholipid head groups, allowing effective plasma membrane translocation of the peptide (55). As expected, a Penetratin-conjugated peptide containing phosphoserine at residue 325 reduced *in vitro* migration of cells (derived from melanoma cell line) in wound closure assay. It was suggested that 325 phosphoserine of the CD44 cytoplasmic tail binds, directly or indirectly, unknown acceptor site of cytoskeletal or signaling molecule, involved in cell migration, while the corresponding peptide competes on this binding, thus attenuating the cell trafficking (54).

The same phosphoserine-containing CD44-derived peptide, but coupled to TAT (transactivator peptide) rather than to Penetratin, was exploited to elucidate the pathological cross-talk between cell surface CD44 and matrix metalloproteinase (MMP) 9 (56). These two molecules are co-localized on the prostate cancer cell line PC3. Transduction (as described in 57) of phosphorylated Ser325 (pSer325) or phosphorylated Ser323 and Ser325 (pSer323 and pSer325) peptides into PC3 cells not only disrupted the CD44/MMP9 complex, but also inhibited the activation of the MMP9 enzyme, which is essential for enhancement of the cancer cell migration. Indeed, the peptide insertion significantly reduced the cell motility as well, as indicated by transwell migration assay. Furthermore, down-regulation of MMP9 with siRNA reduced cell surface expression of CD44 (56). These findings point to a reciprocal relationship in the CD44/MMP9 cell surface complex. CD44 functions as a docking molecule for MMP9, using the pSer325 as an anchor that may interact, directly or indirectly, with hemopexin like domain (PEX) of MMP9, resulting in enzyme activation, which leads to cell migration. On the other hand, the presence of MMP9 influences CD44 expression. Insertion of pSer325 peptide deteriorates these vicious relationships by competing (directly or indirectly) with cell surface CD44 on binding to e.g., the PEX domain of MMP9, stressing the anti-metastatic therapeutic potential of the peptide.

6. HA BINDING PEPTIDE DERIVED FROM PHAGE-DISPLAY LIBRARY (PEP-1)

Pep-1 is a specific 12 mer HA binding peptide that was isolated from a phage-display library. The M13 phage library expressing 10^9 12-mer peptides, fused to pIII minor coat protein, was incubated on polystyrene plates coated with HA and counter coated with BSA. After removal of unbound phage, the enzyme hyaluronidase was added into the panning plates to elute only phage clones that had interacted with HA. After such four panning cycles, 13 isolated independent phage clones (of total of 10^9) showed an identical peptide sequence: GAHWQFNALTVR. This sequence, designated Pep-1, was synthesized (with amidated GGGS linker at the c-terminus) and its ability to bind HA (Kd=1.65 μM) and block HA function was demonstrated. Surprisingly, Pep-1 does not display any HA binding motif (including RHAMM-like motifs), which characterized known HA-binding proteins, such as CD44, RHAMM or link protein. Combining the knowledge of the secondary and tertiary structure of HA with mutation analysis of Pep-1, in which its binding potency to HA was verified following substitution of different amino acids with alanine, allowed a theoretical formulation, which described the mechanism of interaction between Pep-1 and its target HA (58). Such a prediction suggests that hydrophobic and/or polar aliphatic residues of Pep-1 interact with polar, hydrophobic, or formally charged groups of HA (hydrophobic–hydrophobic interaction). The oligomeric version of Pep-1 modified by tetravalent polyethylene glycol (PEG-Pep-1) displayed stronger inhibition of HA functions than the original peptide (59).

Pep-1 inhibits the binding of cells expressing HA receptors (e.g., CD44) to immobilized HA substrate as well as the binding of soluble HA to cells expressing such receptors, implying that Pep-1 and HA receptors compete for binding to the same ligand (58). The competition between Pep-1 (or oligomerized PEG-Pep-1) and cell surface HA receptors on HA binding can be exploited for attenuating various *in vivo* biological activities, which are dependent on interaction between cells expressing HA receptors (mostly CD44) and HA of the extracellular matrix (ECM), including the skin. To this end it should be stressed that both human and mouse skin are strongly stained with biotinynlated-Pep-1, indicating massive expression of HA in this tissue (60). Indeed, It has been shown that injection or topical administration of Pep-1 before sensitization with a reactive hapten inhibits the maturation of Langerhans cells and their migration from the epidermis (58, 61). Furthermore, administration of Pep-1 (either before the sensitization or prior to the elicitation phase) attenuated acute skin inflammation of contact hypersensitivity to dinitro-fluorobenzene as well as chronic skin inflammation following repeated applications of to the same hapten (58). Pep-1 inhibited also the secretion of the pro-inflammatory chemokine macrophage inflammatory protein-2 (MIP-2) from HA-stimulated macrophages (62).

The proliferation of T cells from mice transgenic to ovalbumin (OVA) T cell receptor (TCR) in response to stimulation with dendritic cells presenting OVA is suppressed when oligomerized Pep-1 is included in the antigen-presenting assay (59). This finding implies that HA is involved in the process of T cell activation by antigen presenting cells (APC). Yet, it is not clear whether expression of HA on APC, T cell or both is responsible for the activation of cell proliferation. Separated pre-incubation of either the APC or the T cell with Pep-1 before their co-culturing in the antigen-presenting assay may resolve this dilemma. One can be imagine that HA-

CD44 (or other HA receptors) interaction is used for co-stimulation, in analogy to B7 (CD80/CD86) - CD28 interaction, which assists the formation of cell surface synapses. These synapses coordinate the antigen presentation to the TCR and the subsequent T cell activation (63).

Although not always true, high HA expression correlates with an aggressive metastatic behavior in many cancer cells (64). Therefore, HA detection on cancer cells, using Pep-1 binding assay, may be exploited to determine the prognosis of cancer patients (65). Moreover, injection of Pep-1 can reduce lung metastasis and prolong survival of mice inoculated with cell line-derived melanoma cells, but this peptide neither influences the *in vitro* proliferation of these tumor cells nor their *in vivo* local growth (66). These findings imply that metastasis of the melanoma cells is HA-dependent, but the mechanism underling this malignant activity has not yet been elucidated. A preferential possibility is that binding of Pep-1 to HA of endothelial cells or ECM disrupts its ability to interact with cell surface CD44 of the melanoma cells, thus interfering with their migratory function and potency to establish distal metastatic colonies. However, other possibilities do exist (66) and further investigation is required.

HA in the tumor microenvironment is associated with the aggressiveness of human esophageal squamous cell carcinoma (ESCC). In *in vitro* assays, imitating the HA stromal effect, binding of HA to RHAMM on ESCC cells delivers intracellular signals leading to focal adhesion kinase (FAK) activation, filopodia formation and subsequent cell proliferation and migration, which characterizes the malignant phenotype of this tumor. FAK cleavage is detected after HA targeting by Pep-1, resulting in a blockade of the vicious sequence of events, which dictate the carcinoma invasive activity (67). Hence, Pep-1 may be considered as a potential therapeutic drug for the control of ESCC. These results also show that although Pep-1 sequence does not resemble RHAMM HA binding sequence it can nonetheless block HA-RHAMM interactions that are relevant to tumorigenesis, possibly by sequestering available signaling HA forms.

Injection of IL-2 into melanoma and renal cancer patients induces generation and/or expansion of lymphokine-activated killer (LAK) cells as well as activated T lymphocytes, resulting in complete (~10%) or partial (~10%) tumor regression (68). However, the anti-cancer effect is accompanied by IL-2-induced vascular leak syndrome (VLS). VLS is characterized by an increase in vascular permeability followed by extravasation of fluids and proteins resulting in interstitial edema and organ failure. Manifestations of VLS include fluid retention, increase in body weight, peripheral edema, pleural and pericardial effusions, ascites, anasarca and, in severe form, signs of pulmonary and cardiovascular failure (69). Therefore, VLS counteracts the anti-tumor effect of IL-2 treatment. Notable, IL-2 enhances the expression of HA on endothelial cells. In addition, CD44 targeting either by CD44-specific antibody (70) or as a result of genetic deletion (71) substantially reduced the VLS pathology. These findings suggest that IL-2-induced LAK cells that express CD44 attack vascular cells over-expressing HA after IL-2 treatment. This cell-cell CD44-HA-mediated interaction provokes severe damage to the endothelium. Indeed, injection of Pep-1, that inhibits the HA function, attenuated the VLS (72), exactly as did treatment with anti-CD44 antibody. As HA is not expressed on melanoma cells, injection of Pep-1 into melanoma-bearing mice, which were also treated with IL-2, can protect the endothelial cells from LAK cell attack, while the melanoma cells are still destroyed by the cellular killing machinery (72).

7. BLOCKING PEPTIDES DERIVED FROM CD44 LIGANDS

Peptides derived from CD44 ligands can block the cell surface CD44 receptor, thus interfering its interaction with the homologous natural ligand and inhibiting the subsequent pathological effect.

Hepatocellular carcinoma (HCC) is another aggressive metastatic human malignant disease that displays elevated expression of osteopontin (OPN) and matrix metalloproteinase-9 (MMP-9). The *in vitro* migration/invasiveness of cells derived from HCC- cell line (termed HCC cells) can be attenuated following incubation with OPN-related peptides. It was found that MMP-9 of HCC cells cleaves OPN alternatively spliced variant (OPN-c) of the tumor into three fragments, one of them, a fragment of 5 kDa (called OPN-5kDa), interacts with cell surface CD44 of the same tumor, activating *in vitro* cell invasion through Matrigel (73). This cell invasion is inhibited by 10 mer peptides assigned from OPN-5kDa, showing the therapeutic potential of these reagents. It should be stressed that the binding site recognizing CD44 (inserted in the OPN-5kDa) and that recognizing integrins via arginine-glycine-aspartic acid (RGD) sequence are distinct epitopes in the osteopontin molecule and they display divergent functions. After cleavage by thrombin, the C-terminal fraction (which includes OPN-5kDa) is involved in chemotactic attraction of cancer cells via activation of CD44, whereas the other fraction (which includes RGD) supports haptotaxis (directed cell crawling) of cancer cells via interaction with integrins (73). To this end, RGD-containing peptides can negate integrin-associated cell activities, which are dependent on interactions with OPN as well as other RGD-containing proteins (74, 75).

It has been reported that cell surface CD44 or soluble CD44 interacts with fibronectin, an ECM component, via glycosaminoglycans (GAGs; e.g., heparin, heparan sulfate, chondroitin sulfate) inserted in this receptor. Indeed, it was found that fibronectin-derived peptides containing GAG-binding sites can attach to GAG-containing cell surface CD44 and this attachment is blocked by excess of heparin or chondroitin sulfate as well as by chondroitinase (76). A different fibronectin-derived peptide, containing a heparin binding domain, was used to block the pathological interaction between CD44 and

MMPs. Elevated levels of fragments of fibronectin, which may include an heparin binding fibronectin fragment (HBFN-f), can be detected in osteoarthritis (OA) cartilage and in the synovial fluid of OA and rheumatoid arthritis (RA) patients. Such fragments may perpetuate and augment the pathological state of the joint inflammation. Indeed, it was found that that HBFN-f stimulates production of MMPs 1 and 13 (collagenases) as well as MMPs 2 and 9 (gelatinases) in human articular cartilage culture. It appears that HBFN-f binds to heparan sulfate of chondrocyte CD44 and subsequently this interaction stimulates the cellular MMPs, resulting in further cleavage of the cartilage type II collagen, including generation of new HBFN fragments. These HBFN fragments may perpetuate an unending cycle that gradually destroys the cartilage. Addition of peptide V, that was derived from HBFN-f blocks this loop, possibly by competing with HBFN-f on binding to CD44 (77). However, heparin or heparan sulfate, the binding site of HBFN-f, is expressed only on CD44 variants, which contain V3 exon (26).

Laminin is another source for CD44-recognizing peptides, that blocks cell surface CD44 functions by interfering with its interaction with the natural ligand. The globular domain of α5 chain of Laminin 10 (α5β1γ1) and 11 (α5β2γ1) was used as a template for generation of 113 overlapping peptides. Of these 113 peptides, 11 peptides bound to B16-F10 melanoma cells of which 4 peptides inhibited the *in vivo* local tumor growth and pulmonary metastasis. The same 4 peptides also reduced fibroblast growth factor-induced angiogenesis in chick chorioallantoic membranes as well as tumor cell invasion and cell migration *in vitro*. Additionally, these 4 peptides increased apoptosis in tumor cells. The molecular target of only one (A5G27) of the four peptides was identified. Using affinity chromatograpy, A5G27 peptide-coated column bound B16-F10 cell membrane fraction that after elution and de-glycosylation revealed bands of 60 and 90 kDa. These bands were identified as CD44 isoforms by Western blot and immunoprecipitation (78). Heparan sulfate (HS) and chondroitin sulfate (CS) decreased the adhesion of B16-F10 cells to A5G27 peptide, implying that the CD44 is a proteoglycan containing HS and CS chains (78). As the molecular mass of de-glycosylated standard CD44 (core CD44s) is 37 kDa, the 60 and 90 kDa CD44 de-glycosylated proteins (which bind the A5G27 peptide) must be CD44 alternatively spliced variants. These variants may include the v3 exon, because this exon is the only one that contains heparin or heparan sulfate (26). Therefore, v3 exon can bind heparin-binding growth factors (e.g., fibroblast growth factor 2 or vascular endothelial growth factor), concentrating and orienting them to cells expressing the cognate receptors, such as endothelial cells, thus enhancing the malignant process ((79, 80) and (34), which includes additional references). In conclusion, A5G27 peptide may not only compete with the tissue laminin on binding to cell surface CD44, hence attenuating tumor cell migration and invasion, but also block the binding of tumor-supporting growth factors to membrane CD44, preventing their involvement in the malignant cascade.

8. RHAMM: STRUCTURE AND FUNCTION

RHAMM appears to be an evolutionarily more ancient protein than CD44 since orthologues are expressed in chordates and arthropods (Figure 4). Both tribolium (arthropod, flour beetle) and ciona intestinalis (chordate, tunicate) RHAMM share approximately 30% identity (protein) with human RHAMM although only chordate and higher RHAMM forms contain a highly conserved HA and mitotic spindle binding region (Figures 4, 5). Arthropod RHAMM encodes an ERK docking site (16) but lacks HA binding sequence (39, 81) and a leucine zipper that is required for the association of RHAMM with the mitotic spindle (82). Therefore an ancestoral RHAMM may have initially functioned as an ERK docking protein, which subsequently acquired mitotic spindle and HA binding properties in chordates. Interestingly, arthropods do not produce HA and HA synthase (HAS) although they produce chitin from chitin synthases (83, 84). The chitin polymer resembles HA in the presence of glucosamine (but not glucuronic acid) as well as in having wound healing properties.

Structurally, RHAMM is a coiled coil protein with about 35% protein sequence homology to KIF15, a kinesin family member (85, 86). Like KIF15, RHAMM lacks a signal peptide for export through the golgi/ER and does not contain a membrane spanning sequence. In spite of sharing these characteristics with intracellular motor and coiled coil proteins, RHAMM can be exported to the cell surface. It therefore functionally belongs to a growing number of intracellular proteins that are either secreted/released following programmed cell death or by novel, unconventional export mechanisms and that perform distinct extracellular and intracellular functions (1, 2, 80). Extracellular RHAMM partners with CD44 (4, 6) and other integral receptors such as RON (87), a member of the HGF receptor family, and PDGFR (88) to activate signaling cascades in particular RAS-ERK1,2 (2) that control the expression of cell cycle genes such as cdk1/cdc2 (89, 90) and cell motility related genes such as MMP9 (91). Short spurts of cell motility that do not require gene expression depend only upon extracellular RHAMM/CD44 interactions since motility defects exhibited by RHAMM-/- cells are rescued for up to 4 hours by exposure to extracellular recombinant RHAMM protein (4). As predicted by its ability to control expression of cell cycle dependent genes, extracellular RHAMM can also affect cell proliferation (2) and blocking either cell surface or mitotic spindle RHAMM results in a G2M block (85, 90).

Intracellular RHAMM is a microtubule and nuclear protein that binds to interphase and mitotic spindle microtubules as well as motor proteins such as dynein (85). It also directly binds to ERK1 (16), forms complexes with MEK1 and ERK2 (16, 88) and is required for both retention of active ERK1,2 in the cell nucleus and for the expression of a subset of ERK1,2-regulated genes through AP-1 signaling. Additionally, intracellular RHAMM is required for Ran-dependent mitotic spindle formation. Either overexpression or loss of RHAMM results in mitotic spindle deformities (17). At least part of these effects of

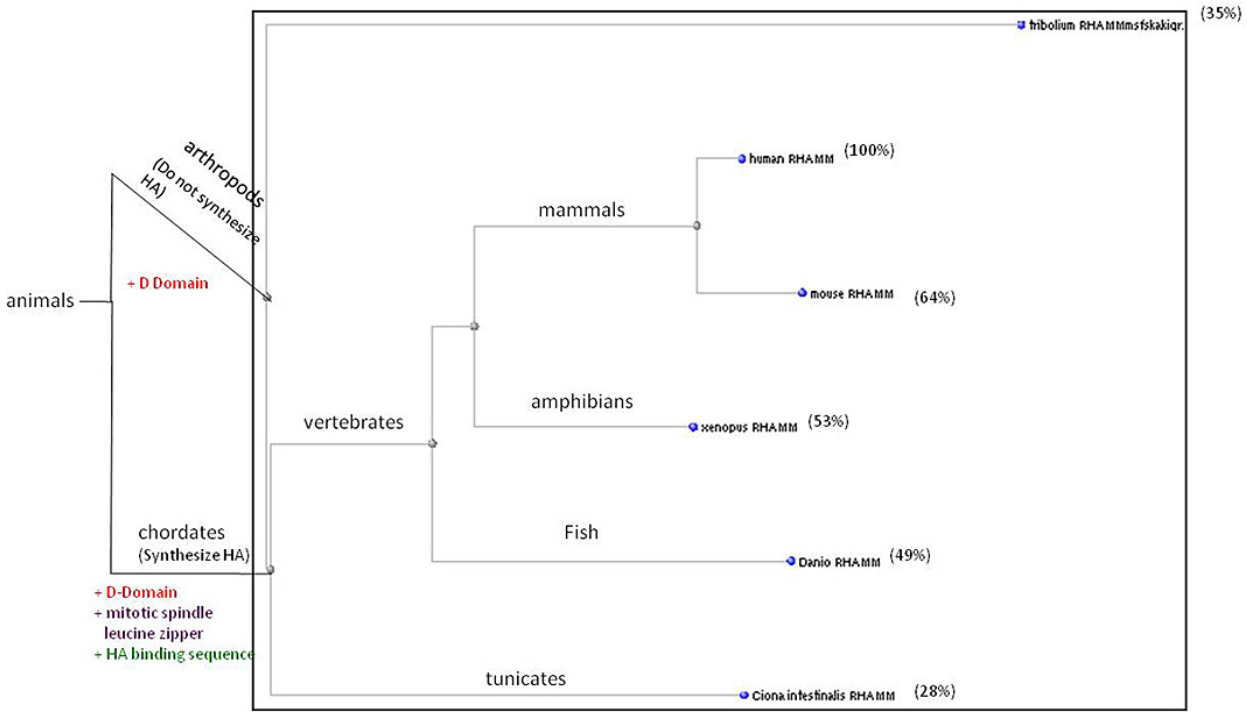

Figure 4. Evolutionary tree of RHAMM. RHAMM orthologs are present in animals but not plants and appear in both Arthropods, which generally do not synthesize HA but rather produce a related polymer, chitin, and in chordates, which Express HAS and produce HA. RHAMM orthologs are present throughout the chordate phyla, even in the ancient tunicates. Although tunicate homology to human RHAMM is low (28%), the D-domain for binding to ERK1, the carboxyl terminal leucine zipper for binding to tubulin (e.g. Mitotic spindle) and the HA binding sequence are highly conserved. Arthropod RHAMM Orthologs also have low homology to human RHAMM (35%) but only the D-domain is present as a highly conserved sequence. Both the carboxyl terminal leucine and HA binding sequence are absent. (from unigene (www.ncbi.gov)).

RHAMM are due to its functions as a MEK1/ERK1,2 docking site since aberrant mitotic spindles resulting from loss of RHAMM expression can be rescued by mutant active MEK1 activity (16). BRCA1/BARD1 complexes inhibit these mitotic spindle functions of RHAMM. Intracellular RHAMM may also play a role in directionality of cell motility. Recently, RHAMM, together with ARPC5 and PKC were shown to be necessary for the rearward localization of the microtubule-organizing center (MTOC), which is a characteristic of migrating cells that is necessary for maintaining polarized motility (92). The orientation of the centrosome, which gives rise to the MTOC, contributes to polarity by positioning the Golgi and ER recycling compartments so that directed trafficking of membrane components and exported proteins to the leading edge can be maintained. RHAMM and ARPC5 regulate rear MTOC polarization by functioning as organizers of the actin and microtubule cytoskeletons. Since dynein also plays a role in MTOC polarization, and ERK1,2 activity modifies dynein microtubule functions (93, 94), it is likely that the association of RHAMM with dynein and MAP kinases contributes to MTOC localization. Fascinating gaps in our knowledge of RHAMM function are whether or not its mitotic and migration functions require signaling input from both locations of RHAMM forms, and the identification of the molecular mechanisms for this novel type of inside outside signaling.

Like CD44, RHAMM is subject to alternative mRNA splicing, which generates isoform diversity (95, 96). In addition, RHAMM is post-translationally modified by phosphorylation particularly during G2M and cell polarization during migration (2). Implicated kinases include Src, PKC, ERK1,2 and AURKA. However, neither the functional consequences of phosphorylation nor the role of the multi-subcellular compartmentalization of RHAMM forms have been well defined.

Considering the clear roles for RHAMM in cell migration and cell cycle, it is perhaps surprising that RHAMM is poorly expressed in adult organisms during homeostasis even in organs such as intestinal crypts, which are sites of active proliferation and migration (97). There is some evidence that RHAMM/RON interactions may play a role in beating of the cilia lining the trachea and thus affect resistance of the homeostatic animal to infection (87). Genetic deletion of RHAMM does not impede normal embryogenesis and thus RHAMM is not essential for the massive amount of cell migration and proliferation that occurs during tissue morphogenesis (4, 98). RHAMM is transiently and highly expressed following severe tissue injury and genetic deletion or functional ablation of RHAMM results in aberrant tissue repair, in particular affecting mesenchymal responses to injury. For example, loss of RHAMM results in aberrant fibroplasia and mesenchymal differentiation following excisional but not

incisional injury of skin (4). Wounds lack robust fibroblast migration and myofibroblast differentation, and strangely exhibit aberrant differentiation into adipocytes. The timing of wound resolution is strongly impaired as a result.

In contrast to its limited homeostatic functions, RHAMM appears to play major roles in diseases that have an underlying inflammatory or wound component. Thus, RHAMM is involved in diabetes, arthritis and many types of cancers including solid tumors and leukemias (1, 2). It is likely that the HA binding functions of RHAMM contribute to its roles in wound repair and disease progression since HA accumulation and fragmentation are necessary to these processes as well (99). Peptides that mimic either RHAMM itself or the HA sizes that bind to RHAMM have been developed and these have been shown to affect inflammatory, wound repair and neoplastic processes.

9. RHAMM PEPTIDES USED AS VACCINES

While most proteins expressed by normal tissues are not antigenic and do not produce an immune response due to "self-tolerance", tumors have long been known to produce proteins that are antigenic and these are classified as tumor-associated antigens (TAA, 100). These are frequently protein products of mutant genes and oncogenic viruses but they can also be proteins that are either produced in very small amounts or have restricted expression profiles in normal tissues. Since their exposure to the immune system is restricted, they normally do not trigger an immune response. However TAA expression is dramatically increased in tumor cells and this increase triggers an immune response. TAA are thus important targets for immune-therapy in some cancers. RHAMM is an example of such a highly antigenic protein with restricted expression in normal tissues (101).

The first attempts to catalogue TAA utilized cytotoxic T lymphocyte cloning techniques (102, 103). Later serological screening of cDNA expression libraries (SEREX) was introduced and resulted in an increase in the number of TAA associated with both solid tumors and leukemias (104, 105). A SEREX screen identified RHAMM as a potential Leukemia associated antigen (LAA) in acute myeloid leukemia (106) and chronic myeloid leukemia (CML), and TAA in such solid tumors as breast, ovarian, renal and melanoma carcinomas and other cancers (107). High RHAMM mRNA and protein expression is associated with aggressive disease in many cancers but is also predictive of poor outcome in breast cancer (95, 108), multiple myeloma (95), subsets of colorectal cancer and other cancers (18, 109-111). In one study, 28% of AML patients were RHAMM protein-positive. Multi-variable analyses showed that RHAMM expression in >5% of leukemic blasts identified a subgroup of AML patients with adverse overall survival (112).

Although AML patients initially respond well to poly-chemotherapy and/or stem cell transplantation, they often relapse and their 5-year survival is relatively poor (113). The eradication of residual leukemic cells in these patients after allogeneic stem cell transplantation provides a strong rational for developing further immune-based therapies in this neoplastic disease. Cancer vaccines are an emerging therapeutic modality that may play a more prominent role in future AML treatment (114, 115). RHAMM was identified as one of the most promising TAA in AML and RHAMM peptides and tumor lysate-pulsed autologous dendritic cells are currently being developed as patient-nonspecific vaccines to induce an immune response AML and B-CLL blasts. RHAMM peptides that are naturally processed and that are specifically recognized by T cells in healthy volunteers and AML patients were identified (115). The RHAMM R3 peptide produced the highest T cell response, and an increase in cytotoxic T lymphocyte response against the R3 RHAMM peptide (Table 2) was observed following vaccination with dendritic cells pulsed with CLL cell lysate. Furthermore, the presence of CD8+ RHAMM-specific T cells were associated with selective elimination of a chemoresistant side-population of B-CLL cells (116). Phase I/II trials of AML, myelodysplastic syndrome, multiple myeloma patients receiving either 300 or 1000μg were initiated. Both doses were well tolerated in all cancer types. Patients receiving either dose demonstrated immunological and positive clinical responses to the R3 peptide vaccine suggesting that RHAMM structures are promising for further targeted immunotherapies in patients with hematological malignancies (117).

In addition to these hematological malignancies, a number of mouse tumor models have been used to explore efficacy of RHAMM-based immunotherapy. Xenopus (x) RHAMM was identified as a xenogenic target for immunotherapy using a cross-reactive SEREX assay (118). A DNA vaccine based on xRHAMM induced protective anti-tumor immunity against both local growth and metastasis of B16 melanoma mouse models. In these studies angiogenesis was inhibited and tumor cell apoptosis was promoted, consistent with previous reports for the role of RHAMM in these processes. Antitumor activity has also been reported for vaccination of dendritic cells transfected with RHAMM in a mouse glioma model (119).

10. RHAMM PEPTIDES THAT ALTER WOUND REPAIR

A number of HA-binding peptides that contain sequences resembling the HA binding region of RHAMM have been isolated by screening random phage libraries. These have been used as RHAMM antagonists to modulate repair of injured skin. In addition, peptides rationally designed to mimic the spacing of key basic amino acid residues within the RHAMM HA binding region and synthetic peptides of this RHAMM region both reduce inflammation and fibrosis in lung injury models (see Table 2 for summary).

The P-15 peptide was isolated by panning a 15mer random phage library with HA-Sepharose (120). Remarkably, clones of either of two sequences were obtained, one of which contained significant homology with the RHAMM HA binding sequence when aligned by COBALT multiple alignment tool (www.ncbi.gov) (121).

Table 2. Sequences of RHAMM-like peptides and their activity in experimental models and clinical phase I,II trials

Re	Source	Size (aa)	SEQUENCE	Effect of peptide
123,124 (Pep35)	Mouse	27	LKQKIKHVVKLKVVVKLRSQLVKRKQN	Reduces and prevents staphylococcal wound infection Modulates neutrophils by increasing CXCL1 and CXCL2; sequence in grey
123,124 (HABP42) (D aa), (P-15) (L aa)	Random phage library	15	STMMSRSHKTRSHHV	Prevents staphyloccal wound infection, reduces wound fibrosis; modulates neutrophils, M1 macrophages and dermal fibroblasts, acts as a RHAMM antagonist
125 (HABP)	Mouse and human	12	YKQKIKHVVKLK	Reduces lung inflammation and fibrosis Reduced alveolar macrophage accumulation in bleomycin-injured lung tissue and reduced lung collagen production; sequence in grey
125 (Peptide A)	Rational design	9	RGGGRGRRR	Same as HABP
116 (R3)	Human sequence	9	ILSLELMKL	R3 vaccine has anti-tumor effects in AML, B-CLL and MM; enhances antigen presentation, generation of central memory tumor specific cytotoxic T lymphocytes and their selective elimination of a chemoresistant side-population of B-CLL cells
81 (B2-A)	Biased peptide library	8	YDSeYeSe	Binds to RHAMM and acts as an HA peptide mimetic; binds to several of the basic residues in recombinant RHAMM protein ; required for HA/RHAMM interactions and competes with HA for binding to RHAMM
127 (12c, 14c)	Rational design	12	VEGEGEEEGEEY, FTEAESNMNDLV	Bind to recombinant RHAMM and inhibit OCA/PCA cell growth; binds to RHAMM but not to CD44 or TLR2,4; peptides compete with HA for binding to recombinant RHAMM and FITC-peptides are taken up by tumor cells in a RHAMM, but not CD44-dependent manner
90 (Soluble GST-Rhamm)	Mouse Rhamm sequence	aa^{217}-aa^{794}	Accession # NPO38580	Induces mitotic arrest in tumor cells; reduces expression of cdc2 and cyclin B1
5 (Soluble GST-Rhamm)	Mouse Rhamm sequence	aa^{217}-aa^{794}	Accession # NPO38580	Blocks collagen-induced arthritis in CD44-/- mice; blocks ability of RHAMM to compensate for CD44 loss in binding HA;promoting cell migration;up-regulating genes involved in inflammation and exacerbating collagen-induced arthritis

This peptide reduced parameters associated with scarring in excisional rat skin wounds, and inhibited migration and collagen production of rat skin fibroblasts in culture. Collectively these results indicated that P-15 antagonizes RHAMM/HA interactions and in particular blocks fibroblast functions during wound repair. These results are consistent with evidence that ablation of RHAMM expression or function also reduces fibrosis in excisional wounds and particularly affects mesenchymal cell migration and differentiation (4). This peptide had no discernable effect on incisional skin repair, which is consistent with its action as a RHAMM antagonist. Incisional wounds heal via very different mechanisms than excisional wounds. For example, RHAMM expression is induced by excisional but not incisional skin injury (122) and genetic deletion of RHAMM has marked effects on excisional but not incisional skin injury (4). When synthesized as a D-amino acid sequence, P-15 peptide (called HABP42) reduced staphylococcal aureus colonization of incisional wounds in mice (123), and this effect was related to reduced inflammatory cell infiltrate, in particular of neutrophils, 5 days post injury, rather than to the ability to directly block bacterial growth (124). Importantly, this effect was also obtained when antibiotic resistant strains of *S. aureus* were inoculated into incisional wounds.

Pep35, which encodes two RHAMM HA binding sequences bridged by 3 valine residues, also reduces Staphlococcal aureus colonization of incisional wounds (123). Detailed analysis revealed that Pep35 increased accumulation of CXCL1, CXCL2 and neutrophils at 6 and 24 hr post injury (124). These effects were shown to be NF kappa B and TLR2 dependent to the extent that Bay 11-7082, which blocks components of the NF kappa B signaling pathways, blocked increase in chemokine expression and the effect of Pep35 was blunted when TLR2-/- mice were used as the injury model. This study did not directly identify the HA/HA receptor interaction that Pep35 blocks and since Pep35 is in fact RHAMM HA binding sequence, it is most likely that Pep35 competes with RHAMM/HA interactions. The results of this study therefore raise the novel possibility that HA/RHAMM/TLR2 interactions are required for recruiting neutrophils to wounds infected with bacteria.

Short RHAMM (HABP) and RHAMM-like (Peptide A) peptides also reduce inflammation and fibrosis in lung injury models (Table 2). For example, elevated HA is associated with macrophage influx following intratracheal installation of bleomycin. The massive influx of macrophages in response to bleomycin results in sustained inflammation, tissue fibrosis and ultimately severe reduction in tissue function. Both peptides reduced macrophage migration *ex vivo* and accumulation within the lung 4 days post bleomycin exposure and significantly reduced lung hydroxyproline content (125).

Collectively, these results support targeting RHAMM/HA interactions for controlling inflammation and

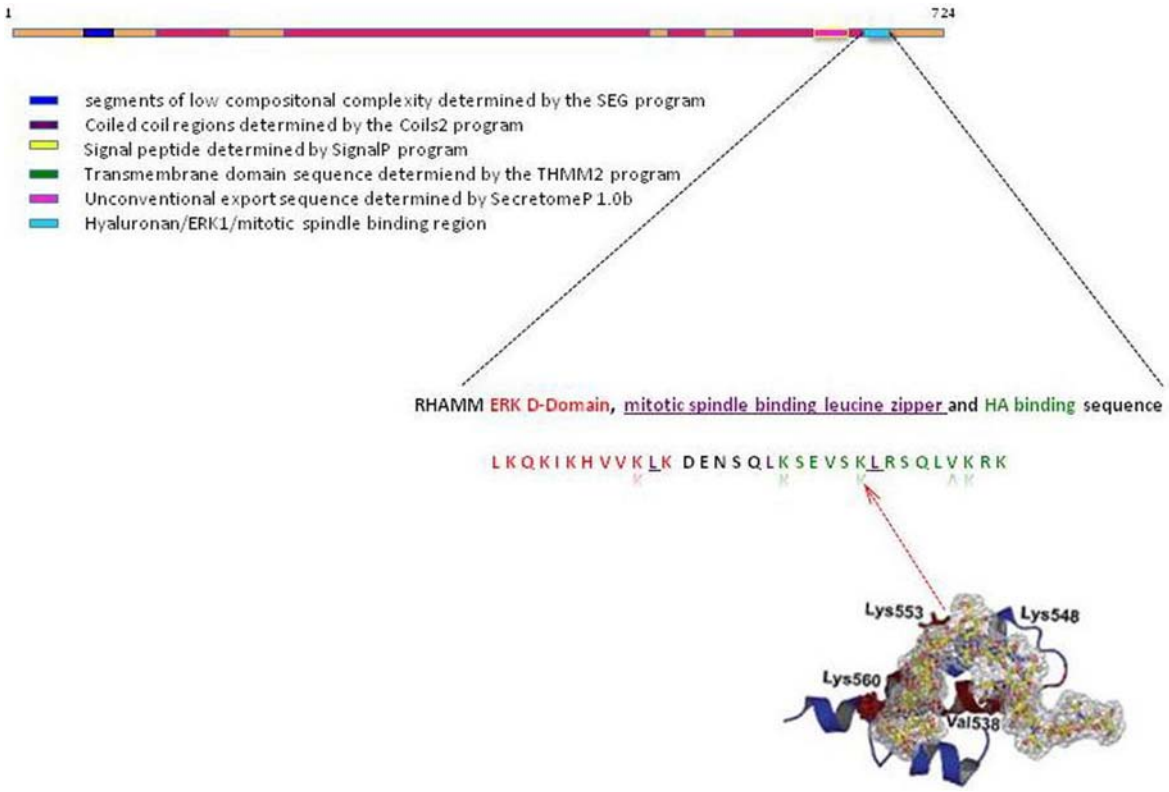

Figure 5. Human RHAMM domain and secondary structure.

fibrosis to prevent excessive scarring in large wounds, to reduce bacterial infection of wounds and to reduce tissue damage due to excessive inflammatory/fibrotic response to pro-fibrotic agents. These therapeutic effects of RHAMM peptides are consistent with the known role of HA/HA fragments in promoting inflammation following tissue injury.

11. USE OF RHAMM PEPTIDES IN CANCER AND ARTHRITIS

Recombinant RHAMM peptides injected subcutaneously together with H-RAS transformed 10T1/2 cells strongly reduced primary tumor formation and also metastasis following tail vein injections (90). This anti-tumor activity was related to inhibition of tumor cell motility and a block in G2M of the cell cycle. The block in cell cycle resulted from decreased expression of cdc2 and cyclinB1.

Collagen induced arthritis in mice is HA-dependent and blocking CD44 HA binding functions inhibits disease progression. Surprisingly, therefore, genetic loss of CD44 resulted in more not less severed disease (5). Disease severity was linked to increased accumulation of HA. Either treatment with hyaluronidase or blocking RHAMM function using competitive recombinant RHAMM peptides inhibited arthritis formation. These results indicted that RHAMM compensated for the loss of CD44 but likely because HA accumulation was increased, RHAMM/HA signaling was enhanced thus promoting disease severity. These particular results point to a need for identifying potential compensating HA receptors in disease processes when intervening with the functions of only one receptor and suggest that there are common functions of RHAMM in cancer and inflammatory diseases such as arthritis.

12. DEVELOPMENT OF RHAMM BINDING PEPTIDES AS HA PEPTIDE MIMETICS

Several studies have used random phage libraries (81, 126) as well as rational design to identify peptides that bind to RHAMM and that in particular block RHAMM/HA interactions as a novel approach to specifically ablating the HA binding properties of this protein. One of these used a biased phage library that contained high levels of negatively charged L and D amino acids and screens initially captured those peptides that bound to a recombinant HA binding fragment of RHAMM. Positive hits were tested for their ability to compete with HA in binding to recombinant RHAMM using ELISA assays. These peptides (e.g. B2-A) were predicted to bind closely to basic amino acids in RHAMM that were known to be involved in its interaction with HA. However, the biological consequences of these peptides have not yet been reported.

13. CONCLUSION

Biology-based peptide mimetics are now being developed as therapeutics for treatment of a number of diseases. Some of these are designed to target the diseased

tissue microenvironment. Here, we review the experimental evidence that peptide mimetics blocking specific functions of CD44 and RHAMM, are efficacious in arthritis, cancer and other diseases. CD44 is a complex protein that has many forms and many ligands. Several peptides derived from them, including those interacting with the polysaccharide HA, show promise in restricting tumor metastasis and inflammatory responses in culture and in animal models. CD44 interacts with a number of different ligands and receptors including RHAMM, which also binds to HA and which is over-expressed in many human tumors and is a tumor marker/antigen in acute myeloid leukemia, multiple myeloma, breast cancer and other cancers. Peptides that either mimic RHAMM sequences or HA are also effective in reducing tumorigenicity and chronic inflammation associated with experimental models of arthritis. Collectively, experimental data are promising and suggest that targeting receptors such as CD44 or its co-receptors/ligands modifyies signaling from the diseased tissue microenvironment, and may be an effective and novel approach to controlling disease.

14. ACKNOWLEDGEMENTS

Work was supported by a Canadian Breast Cancer Foundation and National Research Council of Canada (Discovery Grant) to ET and by baby seed grant to DN from Yissum, research and development company of the Hebrew University of Jerusalem.

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Key Words: RHAMM peptides, CD44 peptides, Peptide therapeutic potential, Arthritis, Polysaccharide hyaluronan, Review

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