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Alternative splicing of RAGE: roles in biology and disease

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1. ABSTRACT

The Receptor for Advanced Glycation End-products (RAGE) is a complex, multi-ligand signaling system implicated in the pathogenesis of diabetes, cardiovascular disease and various cancers. RAGE undergoes extensive alternative splicing to produce a variety of transcripts with diverse functions, including soluble antagonists and variants with altered ligand binding domains. Studies focused on the major soluble variant (RAGEv1/esRAGE) have revealed this to function by binding RAGE-ligands and preventing activation of RAGE signaling in vascular and tumor cells. Furthermore, measurement of this variant in human serum has revealed that RAGEv1/esRAGE levels may represent a novel biomarker for RAGE-ligand related pathogenic states. Understanding the full plethora of RAGE alternative splicing and its regulation is central to elucidating the role of RAGE in biology and disease.

2. INTRODUCTION

Alternative splicing has emerged as a central mechanism in the regulation of gene expression through producing multiple transcripts from a single gene (1,2). The importance of alternative splicing in generating proteomic complexity was reinforced from the results of the human genome project which revealed the existence of only ~25,000 genes, whereas ~100,000 different proteins are generated (3). Furthermore, current estimates suggest <90% of human genes undergo alternative splicing (4).

The alternative splicing of mRNA occurs through the utilization of different exons, introns, promoters and polyadenylation sites (1). These changes result in the production of different protein isoforms including changes in protein domains, truncated protein products and entirely novel isoforms (1). However, these changes can also affect

mRNA bioavailability through changing stability, localization and translational efficiency (1). In particular, the Receptor for Advanced Glycation End-products (RAGE) undergoes extensive alternative splicing and its multiple variants represent a model of many of these alternative splicing mechanisms. In the current review, we aim to discuss the repertoire and function of RAGE splice variants, their conservation between species, and their potential role in disease settings.

3. RAGE PROTEIN STRUCTURE AND DOMAINS

RAGE was initially purified from bovine lung as an AGE-binding protein and characterized as a member of the immunoglobulin superfamily of receptors (5,6). Subsequent studies revealed RAGE to possess a multi-ligand nature and has been termed a "pattern recognition" receptor due to the diverse variety of the ligands that bind and transmit cellular signaling through RAGE (7). These ligands in addition to AGEs, include members of the s100/calgranulin family (8-12), HMGB1 (high-mobility group box-1) (13), amyloid fibrils (14) and the counter receptor for beta-integrins, Mac-1 (7). Although high basal levels of RAGE are found in lung, most tissues including the liver, brain, heart, kidney and skeletal muscle express moderately low levels (15). At the cellular level, RAGE is expressed in the vasculature by endothelial, smooth muscle, and most peripheral blood mononuclear cells, in the lung by alveolar epithelial cells and in the kidney by podocytes (15-19). However, upregulation of RAGE is seen in a wide range of pathological states including diabetes, cardiovascular disease, cancer and neuronal dysfunction (15,16,20,21).

The canonical full-length isoform of RAGE exists as a cell surface 45-50kDa protein and consists of 404 amino acids (6). RAGE protein consists of an extracellular ligand-binding domain (amino acids 1-339), a single transmembrane domain (amino acids 340-361) and a highly-charged, hydrophobic intracellular cytoplasmic domain (amino acids 362-404) (Figure 1). The extracellular domain of RAGE consists of an N-terminal signal peptide (aa 1-22), a V-type immunoglobulin domain (aa 23-116) and two C-type immunoglobulin domains (aa 124-221 and aa 227-317) (Figure 1). Initial studies identified RAGE-ligand binding to occur within the V-type domain (22), however, recent data suggests certain RAGE-ligands are able to bind to the C-type domains (23).

The cytoplasmic portion of RAGE is the critical domain for RAGE-ligand signaling inside the cell. Deletion of this domain renders RAGE in a "dominant-negative" state, blocking RAGE-ligand signaling (22,24,25). Ligand binding to RAGE induces a diverse array of signaling cascades, depending on the cell-type and pathophysiological state. These include numerous members of the MAPK family, JAK/STAT, Rho GTPases and various transcription factors including NF-kB and Egr-1 (8,26-30). Homology analysis of the cytoplasmic domain has revealed that no known tyrosine or serine-threonine kinase motifs are contained within this region, suggesting alternative signaling mechanism(s) may function through RAGE. Although initial studies suggested that the MAPK, ERK 1/2 may bind directly to the RAGE cytoplasmic domain (31), a recent study suggests the interaction with the formin homology protein, diaphanous-1, to be a more likely candidate for transmitting RAGE-ligand signaling (29). Together these signaling pathways link RAGE to the induction of various proinflammatory genes and of cellular phenotypic changes including migration, invasion, proliferation and apoptosis (17,29,32).

Recent data suggests that not only do RAGE-ligands form oligomers, in order to induce cellular signaling (33,34), but also RAGE itself exists in a multimeric form at the cell surface (35). Furthermore, Zong and colleagues demonstrated that RAGE homodimerization occurs through the V-type domain and this dimerization is important for RAGE to activate MAPK signaling (35).

4. RAGE GENE STRUCTURE AND ORGANIZATION

The human RAGE gene (termed *AGER* by the HUGO nomenclature) is encoded on the short arm of chromosome 6 (6p21.3) in the major histocompatibility complex III (MHC) region (36). The MHC class III region contains numerous genes, many with an inflammatory function including components of the complement system and various cytokines (37). Furthermore, the class III region encodes many overlapping genes, with the RAGE gene itself overlapping at the 5' end with the PBX2 gene (38). The RAGE gene consists of 11 exons and 10 introns of variable length and a 3'UTR region that spans over 4 kbp (Figure 2). Studies have shown that extensive genetic variability occurs within the RAGE gene, with ~30 SNPs characterized to-date (39-41). Particular focus has occurred on a number of functional SNPs including a codon variant resulting in a Glycine to Serine substitution at amino acid number 82 of the V-domain (Gly82Ser), and two promoter region variants (-374 T/A and -429 T/C) (39,40). Various studies have demonstrated associations of these variants with a range of vascular disease states. In particular, numerous studies have demonstrated a relationship between the -374 AA genotype and lower incidence of cardiovascular disease (42-45)(46,47). Meta-analysis of the -374 T/A SNP in studies of diabetic vascular disease, confirmed the association of the -374 AA genotype (48). Most recently, genome-wide association studies (GWAS) have revealed an association of the Gly82Ser polymorphism with pulmonary function (49,50).

5. MECHANISMS OF MRNA DIVERSITY OF RAGE

5.1. Alternative promoters

The use of alternative promoters is a mechanism to increase transcript diversity by use of an alternative transcriptional initiation site (51). Initiation from a different start site may be a means to differentially control gene expression and / or the generation of a different amino terminus of the gene of interest. Initial studies on the human RAGE gene identified a single transcriptional initiation site at -13 nucleotides proximal to the protein initiation codon (52). Subsequently, Schraml and

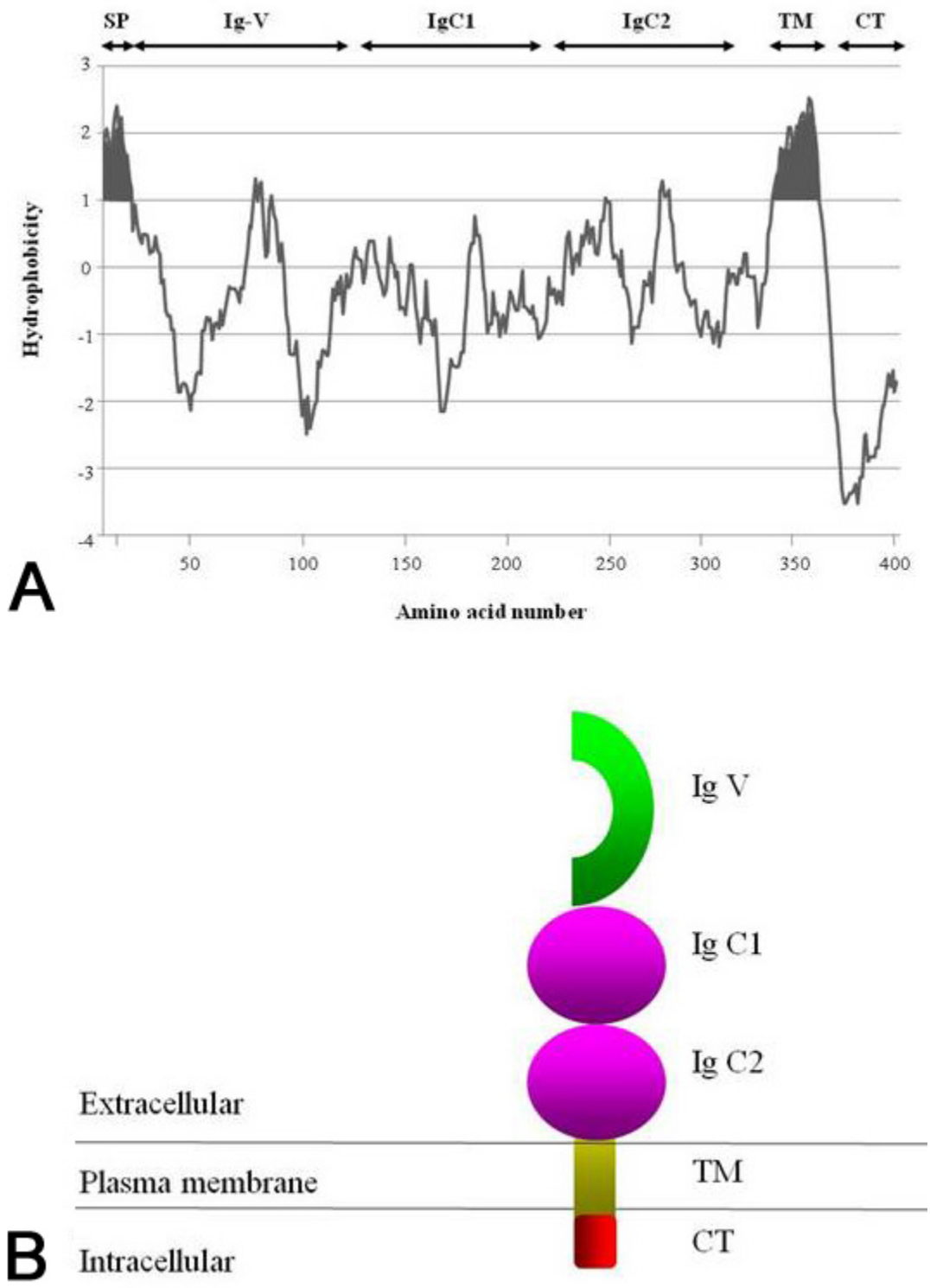

Figure 1. RAGE protein structure and organization. A. Hydropathicity plot of RAGE using the Kyte-Doolittle method (101). Hydrophobic domains are shaded and represent the signal peptide (SP) and transmembrane (TM) domains. Other regions of RAGE shown include the immunoglobulin variable (IgV), two immunoglobulin constant (IgC1 and IgC2) and the cytoplasmic tail (CT). B. Schematic drawing of the RAGE protein showing domains and extracellular, transmembrane and intracellular organization.

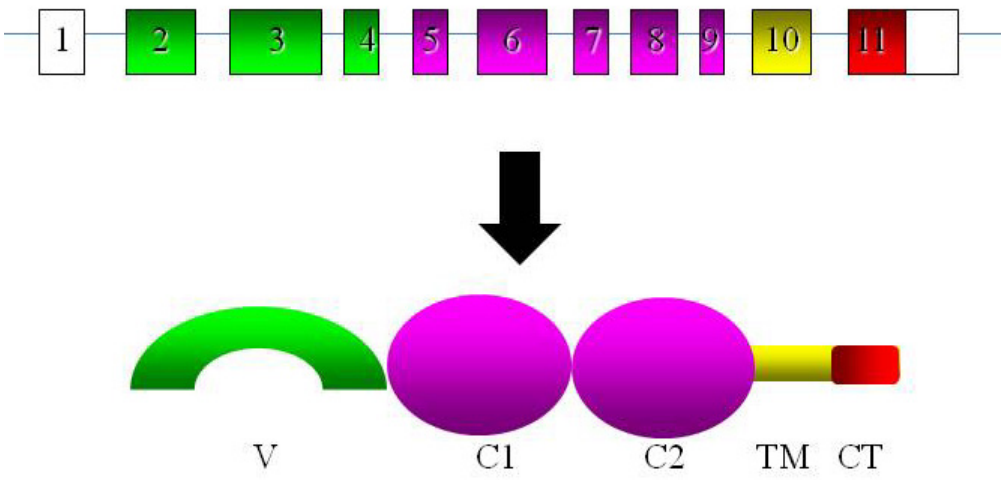

Figure 2. RAGE gene organization. RAGE is composed of 11 exons (indicated by colored boxes) and a 3'UTR. Color of exons correspond to the domains of RAGE for which they encode as shown in the RAGE protein schematic below. Domain of RAGE are as follows; IgV (green), IgC (purple), TM (yellow) and CT (red).

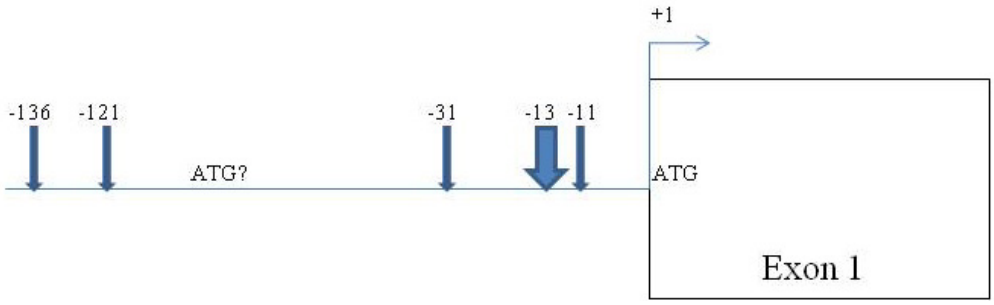

Figure 3. Alternative transcription of RAGE. Alternative identified transcriptional start sites are shown by down arrows, with a wide arrow demonstrating the major start site. RAGE protein initiation codons are shown (ATG) in exon 1 and a putative upstream initiation site (ATG?).

colleagues were able to clone a transcript from lung that initiated at -136 nucleotides and additionally contained an alternative initiation codon, 93 nucleotides upstream of the originally identified ATG (53). However, no studies to-date have validated whether this alternative initiation codon is utilized. Recent extensive 5'RACE analysis of lung revealed the predominant initiation sites to occur at ~-13, with much rarer transcripts detected at -31 and -121 (Figure 3) (19). This would indicate that the major promoter start site for RAGE is as originally identified (52), and other sites may be used less preferentially. Future studies are required to validate these findings in other tissues and under pathological conditions. Furthermore, no studies to date have characterized the alternative transcription / promoter usage of RAGE in other species.

5.2. Alternative poly(A) sites

The use of alternative polyadenylation sites, is another mechanism used to generate mRNA diversity in the cell (54). This mechanism functions through affecting mRNA turnover by the presence of stability elements in the 3'UTR, in particular AU-rich elements (AREs) (55). Studies of human RAGE have not revealed any differences in the polyadenylation signal site of the 3'UTR (19). However, it appears in rats that mechanisms exist to alter the polyadenylation site of RAGE. Analysis of the 3'UTR in rats revealed the presence of two distinct transcripts with different polyadenylation sites (56). Within the longer novel transcript, an ARE was identified and found *in vitro* to reduce RAGE mRNA stability (56). Therefore, cellular changes in rat RAGE 3'UTR length could represent a major mechanism for regulating RAGE protein levels. Further studies *in vivo* and in other species are required to investigate these findings.

5.3. Alternative splicing of RAGE

Alternative splicing represents the major mechanism to produce RAGE mRNA diversity. Since 1999, numerous studies have identified a variety of splice variants of RAGE in multiple species including humans, mice and dogs (19,57-63). To-date the best characterized splice variants are those found in humans. Alternative splicing of RAGE consists of a number of different mechanisms including alternative exon/introns usage result in non-translated transcripts with premature stop codons that are targeted to nonsense-mediated decay (NMD),

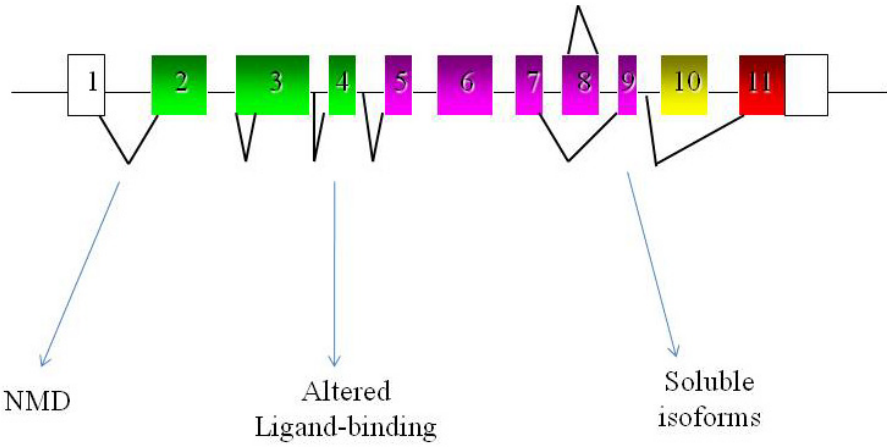

Figure 4. Major alternative splice forms of RAGE. Three classes of splice variants predominate in the RAGE gene indicated by lines between exon/exon, exon/intron to designate the splice changes. Alternative splicing involves intron 1 (NMD targeted transcripts), exons 3-5 (altered ligand binding site variants) and exons 7-11 (soluble isoforms lacking the transmembrane domain).

variants with altered domains of the extracellular domain, and truncated isoforms lacking the transmembrane domain (Figure 4). These latter variants result in the production of secreted isoforms and have been the focus of major research interest due to their potential therapeutic role as RAGE antagonists and as a biomarker due to their measurability in human blood.

5.3.1. Premature stop codons / NMD targeted variants

The presence of a premature stop or termination codons (PTCs), may result in the translation of a truncated protein or its targeting of the mRNA for degradation by NMD (64). Transcripts with a stop codon more than 50-55 nucleotides upstream of the final exon-exon splice junction, are considered to be targeted to NMD (64). Analysis of both human and mouse RAGE mRNA splicing revealed ~50% of all detected splice isoforms are potential targets for this pathway (19,61). The most abundant and conserved across species of these variants is a splice variant that retains intron 1 in the mature transcript (19). This variant was first identified as "N-truncated RAGE" (recently termed RAGE splice variant 2 or RAGE_v2) due a PTC resulting in translation from another downstream ATG codon, producing a variant lacking the V-domain (58). However, recent work revealed that this variant is most likely a target for NMD, as the original authors cloned N-truncated RAGE / RAGE_v2 starting from exon 3, skipping the segment of the transcript containing the important NMD signal in intron 1 (19). Transfection of both the full-length, intron 1 containing RAGEv2 form and shorter "N-truncated" RAGE revealed on the "N-truncated" form produced a detectable product (19). Therefore, the production of "N-truncated" RAGE is most likely due the proximity of the downstream ATG codon in exon 3 to strong transcriptional and translational signals present in mammalian expression vectors. Further evidence for this is supported by studies of murine splice variants of RAGE (61). In the mouse, an orthologue that includes intron 1 has been recently detected, which does not contain a potential initiating downstream ATG in exon 3 (61). This therefore suggests these conserved variants may be targeted to the NMD.

5.3.2. Altered extracellular domain variants

Alternative splicing can introduce or remove key domains of proteins to affect binding properties, cellular localization, activity and stability (1). Changes in receptor-ligand interaction can result in the abolishment of ligand binding or even create binding affinities for novel ligands (1). Investigation of human RAGE splice variants revealed a number of variants affecting the region around the exons encoding the V-domain (19). These included RAGE_v4 which led to the loss of 14 amino acids at position 53 and RAGE_v5 which led to the introduction of 17 novel amino acids at position 140 (19). Although no studies have been performed to determine their function, it is likely that these changes would affect ligand binding. It is possible that as RAGE is a multi-ligand receptor, alternative splicing of the V-domain may be a means to increase the ligand repertoire of RAGE. Interestingly, orthologues of these variants are rare in mice (61).

5.3.3. Secreted non-membranous variants

The formation of secreted isoforms variants is a common phenomenon amongst membrane receptors (65). The function of these secreted or soluble variants may be to act as an agonist or antagonist of their membrane bound version (65). Studies have identified a range of potential soluble spliced variants of RAGE in humans and mice (19,57-61,63). Malherbe and colleagues first described a potential soluble variant in 1999 which was termed hRAGEsec (57), which led to further studies identifying other soluble variants including sRAGE1/2/3 and endogenous soluble (es) RAGE (58,59). To understand the importance of these variants, a recent study classified all these soluble variants by *in vitro* methods (19). Most of these potential splice variants were predicted to by targeted to NMD and transfection of variants into cells did not produce a secreted protein for most variants (19). In particular the only detected soluble variant was RAGE_v1

(RAGE splice variant 1), previously termed esRAGE / sRAGE3 (19). RAGEv1 is produced due to the alternative splicing of intron 9 and the removal of exon 10. This altered exon/intron organization alters the reading frame of the transcript resulting in a premature stop codon in intron 9, and subsequently removing the transmembrane and cytoplasmic domain of RAGE (19). This alteration in reading frame results in the introduction of 16 novel amino acids at the C-terminus and has allowed the design of RAGE_v1/esRAGE specific antibodies that do not detect the membrane form of RAGE. Western blot experiments demonstrated as expected, RAGEv1 is secreted and migrates as ~48kDa protein (compared to 55kDa for full-length RAGE) (19). RAGEv1 was also determined to be the second most prevalent RAGE isoform after full-length RAGE (19).

5.3.4. Function of RAGEv1 / esRAGE

The notion of soluble receptors acting as decoys for their cell surface counterpart is a common biological feature. Before the discovery of an endogenously occurring soluble RAGE isoforms, a recombinant soluble RAGE (sRAGE) isoform was engineered and used as a RAGE inhibitor (66). Extensive work has revealed that this recombinant sRAGE acts as a decoy for RAGE-ligands and as a consequence blocks the development and progression of a wide-range of pathological states in animal models including cardiovascular disease, diabetes, neuronal dysfunction and cancer (32,67-69). Therefore, since RAGEv1/esRAGE exists as a soluble isoform of RAGE, it was therefore proposed that this may act in a similar manner to sRAGE and block RAGE-ligand signaling induced effects (19,58). Initial studies to characterize esRAGE, revealed that in a similar manner to full-length RAGE, RAGEv1/esRAGE bound to glyceraldehyde-derived AGE-BSA (58). Furthermore, in the endothelial-derived cell line ECV304, RAGEv1/esRAGE blocked AGE-induced cellular proliferation and tube-formation (58).

To fully explore the mechanism of RAGEv1 inhibition of RAGE-ligand signaling, its role in RAGE-mediated tumorigenesis was investigated (70). Previous studies had suggested recombinant sRAGE as a potent agent to prevent tumorigenesis and metastasis (32). Using tumor cells overexpressing RAGEv1, array analysis revealed that RAGEv1 affected expression of a wide-range of genes including those involved in angiogenesis, adhesion, apoptosis, invasion/metastasis, cell cycle control and signaling (70). Exploration of the *in vitro* mechanisms of tumorigenesis revealed that RAGEv1 inhibited RAGE-ligand induced angiogenesis and tumor cell invasion through direct binding to RAGE-ligand (S100B) (70). Analysis of RAGE-ligand signaling revealed that RAGEv1 inhibited MAPK signaling, including MEK 1/2, p38 and JNK (70). Pharmacological inhibition of MAPKs revealed JNK signaling to be the predominant mechanism of RAGE-ligand blockage of tumorigenic gene expression and cellular invasion (70). To test the RAGEv1 impact on tumor formation, *in vitro* (soft agar) and *in vivo* (ectopic tumor model) methods were tested (70). Both *in vitro* and *in vivo* tumor formation models demonstrated that RAGEv1 strikingly reduced tumorigenesis (70). Furthermore, other studies have confirmed an *in vivo* role for RAGEv1 in inhibiting RAGE-ligand signaling, with the restoration of diabetes-associated impairment of the angiogenic response (71). Together, these studies suggest that RAGEv1 acts as an endogenous decoy system for cell surface RAGE and therefore elucidating its regulation is essential.

5.3.5. Regulation of RAGEv1/esRAGE

Understanding the mechanisms underlying the production and regulation of RAGEv1 levels may lead to the development of novel therapeutics. Mechanisms to increase the production of RAGEv1 levels may be useful in inhibiting RAGE-ligands signaling in various pathological states. The precise mechanism(s) governing the production of RAGEv1 versus RAGE in the cell through alternative splicing is/are not fully understood. Most recently, Ohe and colleagues investigated the elements governing splicing of RAGE vs RAGEv1 at the mRNA level (72). Using RAGE minigene constructs spanning from exon 8 to exon 11, a G-rich cis-element in intron 9 was identified, which led to preferential splicing of full-length RAGE (72). Mutagenesis of this consensus sequence resulted in a shift towards producing increased RAGEv1/esRAGE, whilst inhibiting full-length RAGE production (72). Previous studies of other genes have revealed that G-rich stretches can act as binding sites for the hnRNP H family of proteins (48,73). Although it is not fully clear which the biological role of hnRNP proteins is, hnRNP complexes process pre-mRNA to mature mRNA species for nuclear export (74). Binding studies using HEK293 nuclear extracts confirmed the interaction between the G-rich intron 9 sequence and hnRNP H (72). Further investigation is required into the identification of other key elements involved in the control of RAGEv1 alternative splicing and the factors involved.

A number of studies have demonstrated that various commonly used therapeutics that have cardioprotective effects, may alter RAGEv1 levels. In the monocytic cell-line THP-1, treatment of cells with atorvastatin led to increased RAGEv1 production and secretion in a time and concentration dependent manner (75). Furthermore, treatment of diabetic subjects for 6 months with atorvastatin, revealed significant changes in esRAGE serum levels compared to controls (75). A similar increase in serum levels of esRAGE in response to the thiazolidinediones, pioglitazone (76). These data therefore suggest a key balance of RAGE/RAGEv1 levels and that the measurement of RAGEv1 levels in human serum may be a potential biomarker for RAGE-related pathologies.

5.3.6. RAGEv1/esRAGE as a novel biomarker for disease

By the virtue of their name, soluble receptors levels can be detected in serum and their concentrations have been associated with various pathological states (77). In human serum, soluble RAGE can exist as a number of different isoforms, which include RAGEv1/esRAGE formed through alternative splicing, and an isoform generated by ectodomain shedding of cell-surface RAGE (78,79). Ectodomain shedding of RAGE is less understood

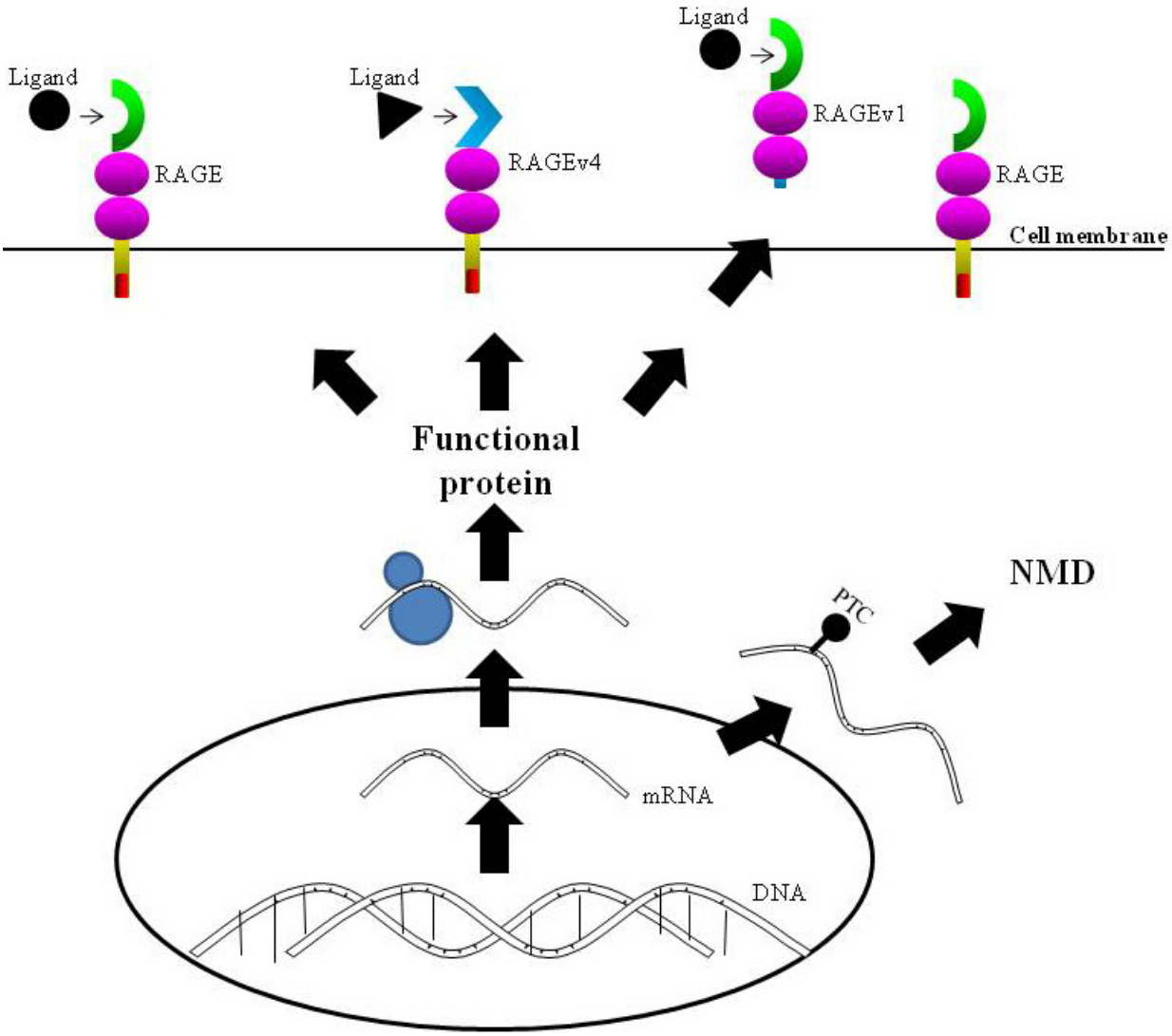

Figure 5. The repertoire of RAGE alternative splicing. RAGE mRNA splice variants are produced in the nucleus where they are then transported to the cytoplasm for translation on ribosomes. The presence of PTCs in mRNAs lead to termination of translation and targeting to the NMD. Mature RAGE protein is transported to the cell surface where it can bind to its ligands and transmit cellular signaling. Changes in the ligand binding domain (RAGEv4) may alter the structure of affinity towards RAGE-ligands. Removal of the transmembrane domain of RAGE through alternative splicing results in a soluble isoform (RAGEv1). Ligand binding by RAGEv1 sequesters these away from cell surface RAGE and blocks RAGE-ligand signaling.

than the formation of alternatively splicing RAGEv1/esRAGE, however, studies suggest cleavage is mediated by ADAM10 (a disintegrin and metalloprotease) or MMP-9 (matrix metalloproteinase) (79-81). Whether the shed ectodomain acts as an antagonist in a manner similar to RAGEv1, or just as a marker of cell surface RAGE levels is not currently clear. In mice, similar mechanisms exist to produce soluble isoforms of RAGE, including alternative splicing to form an orthologue of RAGEv1 (61,63), which can be detected at the protein level in tissue (63). However, studies have not clearly demonstrated whether murine RAGE undergoes ectodomain shedding to form soluble RAGE, although a "cleaved" form can be purified from murine lung (82). Interestingly, analysis of murine serum has revealed no detectable levels of any soluble RAGE isoform, within the range seen in human serum.

The measurement of soluble RAGE isoforms in human serum is facilitated by two distinct ELISA systems. The most commonly used sRAGE ELISA (Human RAGE Quantikine ELISA, R&D Systems) measures the total pool of soluble RAGE using antibodies that recognize both the spliced and cleaved forms of sRAGE. Whilst it is possible to specifically measure RAGEv1/esRAGE in serum by another sRAGE ELISA (esRAGE ELISA) due to its unique C-terminus sequence, there is no specific method to accurately quantify the cleaved form of RAGE alone.

Regardless of this information, a number of studies that have measured sRAGE levels with both ELISAs in human subjects, have attempted to correlate the ratio between these isoforms (83,84). Until a rigorous cross-comparison is performed between these ELISAs, caution should be taken before interpreting any ratio obtained from two independent ELISA systems.

The association of total soluble RAGE levels with a pathological state, was first reported by Falcone and colleagues, who demonstrated that lower sRAGE levels were associated with a increased risk of coronary artery disease (85). Subsequently, serum levels of total sRAGE have been shown to be associated with a range of pathologies including essential hypertension (86), coronary artery disease (85), intimal-medial thickening of the arteries (87), hypercholesterolemia (88), vascular dementia (89), non-alcoholic fatty liver disease (90), schizophrenia (91) and the diabetic state (92). Moreover, increased sRAGE levels have been associated with extreme longevity, suggesting a role for RAGE/sRAGE in the aging process (93). Studies to specifically focus on measurement of esRAGE levels have revealed similar associations. Lower esRAGE levels have been associated with carotid and intimal atherosclerosis in both diabetic and non-diabetic subjects (84,87,94,95), the metabolic syndrome (94), cardiovascular mortality (96), anemia (97), autism (98) and various tumorigenic states (70,99). However, in addition to the finding that various therapeutics may raise esRAGE levels, other factors have been identified that may confound any associations in population studies. In particular, the strongest factor affecting both sRAGE and esRAGE levels is renal function, with an inverse relationship seen between decrease in renal capacity and increasing esRAGE levels (100). Whether this increase reflects a decrease in clearance mechanisms for esRAGE or whether esRAGE levels are increased to compensate for kidney damage is not currently known. Furthermore, to-date the majority of studies on sRAGE / esRAGE in human subjects have been of a cross-sectional nature, in relatively small populations and without serial measurements. Therefore, future studies should be performed on large, prospective cohorts to assess how sRAGE / esRAGE levels change over time, and to critically assess its utility as a biomarker for RAGE-related pathologies.

6. CONCLUSIONS

Understanding both the function and regulation of alternative splicing of the RAGE gene is critical for elucidating the physiological functions of RAGE in health and disease. Analysis of RAGE in humans and mice have revealed RAGE undergoes extensive alternative splicing to form a range of variants with different biological properties. These include non-translated transcript, isoforms with altered ligand binding sites and secreted non-membranous isoforms (Figure 5). In particular focus has centered on these secreted forms both as potential therapeutic agonists to interrupt RAGE-ligand signaling and as potential biomarkers for vascular and inflammatory disease states. Further in-depth studies of these isoforms will help in the understanding of the biological regulation of RAGE.

7. ACKNOWLEDGEMENTS

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Abbreviations: ADAM: A Disintegrin And Metalloprotease, AGE: Advanced Glycation End-products, ARE: AU-rich element, DN-RAGE: dominant negative Receptor for Advanced Glycation End-products, esRAGE: endogenous secretory Receptor for Advanced Glycation End-products, GWAS: Genome-Wide Association Study, HMGB1: High-Mobility Group Box-1), JNK: c-Jun amino-terminal kinase, MAPK: mitogen activated protein kinase, MHC: major histocompatability complex, MMP: matrix metalloproteinase, NF-kB: nuclear factor k-beta, NMD: nonsense-mediated mRNA decay, PTC: premature termination codon, RAGE: Receptor for Advanced Glycation End-products, RAGEv1: RAGE splice variant 1, SNP: single nucleotide polymorphism, sRAGE: soluble RAGE.

Key Words: Alternative splicing, receptors, Advanced Glycation End-products, mRNA, Review

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