RAGE: a novel biological and genetic marker for vascular disease

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ABSTRACT

RAGE [receptor for AGEs (advanced glycation end-products)] plays an important role in the development and progression of vascular disease. Studies in cultured cells and small animal models of disease have clearly demonstrated that RAGE is central to the pathogenesis of vascular disease of the macro- and micro-vessels in both the diabetic and non-diabetic state. Emerging results from human clinical studies have revealed that levels of circulating soluble RAGE in the plasma may reflect the presence and/or extent of vascular disease state. Additionally, genetic variants of the RAGE gene (AGER in HUGO nomenclature) have been associated with vascular disease risk. Combining RAGE circulating protein levels and the presence of particular RAGE polymorphisms may be a useful clinical tool for the prediction of individuals at risk for vascular disease. Therapeutic intervention targeted at the RAGE gene may therefore be a useful means of treating pathologies of the vasculature.

INTRODUCTION

RAGE [receptor for AGEs (advanced glycation end-products)] is a multi-ligand member of the immunoglobulin superfamily of cell-surface molecules. Since its isolation in 1992 [1], a growing body of scientific evidence has demonstrated a role for RAGE in the pathogenesis of vascular disease. Using a combination of in vitro cellular studies and rodent models of disease, it has been shown that RAGE plays a critical role in the development and progression of vascular disease [2–6]. Several comprehensive reviews have previously focused on presenting data from animal studies on RAGE [7–10]. Therefore the goal of the present review is to give a summary of these studies, and present in-depth the extensive data that have accumulated in humans on the role of circulating sRAGE (soluble RAGE) levels and genetic variants in human subjects as markers for disease. These exciting studies add further to the in vivo and in vitro studies by reinforcing the role of RAGE in the vasculature.

THE BIOLOGY OF RAGE

RAGE is highly expressed at the mRNA and protein levels in early developmental stages and in the lung under normal physiological conditions [11]. RAGE expression occurs in most tissues, including the heart, liver, brain and kidney [11]. The cell types which express RAGE include endothelium, monocytes/macrophages, T-lymphocytes,
neuronal cells and glomerular epithelial (podocyte) cells [11–13]. Under normal physiological states, RAGE is expressed to a relatively low degree in these cell types but, under pathological states, striking up-regulation of the RAGE gene (AGER in HUGO nomenclature) is seen with accumulation of its ligands [11,13–15].

**The multi-ligand nature of RAGE**

The hunt for the cellular receptor for the late products of the non-enzymatic glycation led to the isolation of RAGE [1]. These AGEs are different from enzymatically N-/O-linked glycosylation of proteins, since they occur spontaneously from the reaction between aldose sugars and macromolecules of cells and tissue, rendering them into cross-linked products which alter the structure/conformation of the protein and subsequently its cellular function [7]. AGEs occur under physiological conditions such as aging, but importantly at an accelerated rate in pathological states including hyperglycaemia of diabetes, oxidative stress and inflammation [7,16]. The consequence of their increased accumulation is the binding to cellular receptors to induce signal transduction and cellular dysfunction [7,16]. Although *in vivo* AGEs are composed of a heterogeneous pool, the best described and most abundantly seen to-date is the CML [Ne-(carboxymethyl)lysine] adduct [17]. This was the first AGE described in diabetic patients with renal and vascular complications and, importantly, it is a specific RAGE ligand [17,18]. A recent study challenged these findings by demonstrating that bacterial-produced RAGE was unable to bind to CML [19]. However, numerous studies have demonstrated that not only does RAGE specifically bind CML [17,18], but it also mediates its cellular effects [20–23]. In addition to CML, it has been demonstrated that RAGE can bind to and mediate the cellular effects of a variety of AGE ligands including pentosidine, pyrraline and numerous imidazolones [24]. RAGE has also been identified to bind to a variety of individual ligands that do not share any obvious structural characteristics, and has hence been termed a ‘pattern recognition receptor’ [25]. Among these additional ligands is amphoterin, more commonly known as HMGB1 (high-mobility group box-1) [26], which was originally demonstrated to function as a DNA-binding protein in the nucleus. Previous studies clearly show this molecule to act like a cytokine after its secretion by immune and necrotic cells [27]. Other RAGE ligands identified include amyloid fibrils (β-sheet fibrils), which accumulate in amyloidsoses and Alzheimer’s disease [28], Mac-1, the counter receptor for β integrins [25] and at least seven members of the S100 protein family, and a collection of small proteins with diverse cellular functions [29–33]. The common feature of the ligands of RAGE is their pro-inflammatory nature and their accumulation in tissues in disease settings in concert with increased RAGE expression [7]. It would appear that, rather than acting primarily as an initiator of the pathological state, RAGE–ligand interaction propagates and amplifies cellular dysfunction. Therefore, overall, the consequences of binding to a diverse group of ligands may implicate RAGE in a wide range of pathologies and suggest the induction of diverse cellular signalling cascades.

**The protein domains of RAGE**

Full-length human RAGE is a 45–50 kDa protein, depending on its N-linked glycosylation state, and is composed of 404 amino acids [1]. RAGE consists of distinct domains which include an extracellular domain (amino acids 1–339), a single transmembrane domain (amino acids 340–361) and a short intracellular cytoplasmic tail (amino acids 362–404) (Figure 1). The extracellular domain includes a short signalling sequence to target it to the cell membrane, a V-type immunoglobulin domain and two C-type immunoglobulin domains (C1 and C2). *In-vitro*-binding studies have, for the most part, revealed the ligand-binding site to occur within the V-domain [18]. This has been confirmed in protein structural studies which demonstrate that, for the most part, RAGE–ligand binding occurs within the V-domain [32,34–37]. It has recently been suggested however, that the V-domains may not act independently in ligand binding, but rather integrate into one structural unit with the C1-domain [37]. An additional intriguing finding from these studies is that RAGE does not act as a monomeric receptor, but forms dimers and multimers for ligand binding to occur [34,36]. The other domains of RAGE include the transmembrane domain which is a single hydrophobic helix and an intracellular domain which consists of the highly acidic short 42-amino-acid cytoplasmic tail, which is essential for RAGE-mediated signalling and overall RAGE function [4,18,38].

**Cellular signalling of RAGE**

In contrast with scavenger receptors for AGEs, RAGE acts as a cell signal receptor for ligands leading to diverse
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Figure 2  RAGE–ligand interaction induces cellular activation through intracellular signal transduction and altered gene expression

Binding of ligands of RAGE at the cell surface leads to rapid activation of intracellular signal transduction pathways which result in changes in gene expression. These pathways can be blocked by expression of the DN-RAGE isoform lacking the intracellular cytoplasmic domain or by using the sRAGE extracellular domain as a decoy. ERK1/2, extracellular-signal-regulated kinase 1/2; Jak2, Janus kinase 2; Stat3, signal transducer and activator of transcription 3.

Numerous studies have elucidated the downstream signalling cascades resulting from RAGE–ligand interaction and the importance of the cytoplasmic domain in their activation [4,18,29,38,39]. Deletion of the RAGE cytoplasmic domain both in vitro and in vivo imparts a DN (dominant-negative) effect on RAGE-dependent activation of cell signalling (Figure 2). The pathways induced by RAGE ligand signalling include numerous MAPKs (mitogen-activated protein kinases), various Rho GTPases and NF-κB (nuclear factor κB) [29,39,43,44]. These signalling pathways reprogramme cellular properties and functions, such as Ras pathways involved in apoptosis and stress responses, Rac/Cdc42 pathways involved in cell growth, motility and migration and JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathways involved in alteration of gene expression [38,39,43,45].

The possibility of differential effects of RAGE ligands on different cellular signalling pathways has been demonstrated [32,33]. Leclerc et al. [32] recently reported that two members of the calgranulin family, S100B and S100A6, at micromolar concentrations, possibly recognize different binding sites on RAGE and trigger diverse signalling pathways and opposite functional effects in glioblastoma cells in vitro. For instance, both S100B and S100A6 increase the formation of ROS (reactive oxygen species) in glioma cells; however, S100B activates PI3K (phosphoinositide 3-kinase)/Akt [also called PKB (protein kinase B)] and NF-κB, and affects cell proliferation and differentiation, whereas S100A6 affects JNK (c-Jun N-terminal kinase) phosphorylation/activation and
leads to cell apoptosis [32]. This suggests a heterogenous effect of RAGE signalling, dependent, perhaps, on the predominant ligand present and the pathological state.

**RAGE AND VASCULAR DISEASE**

The first evidence to implicate a role for RAGE in the pathogenesis of vascular disease was initial studies in rats which investigated the effects of the AGE–RAGE interaction on the endothelial cell barrier [46]. The RAGE–ligand interaction was shown to facilitate vascular permeability and to enhance vasculopathy in the diabetic state [46]. Furthermore, these effects could be blocked by the infusion of the recombinantly produced extracellular ligand-binding domain of RAGE [46]. By introducing a stop codon in the protein region proximal to the hydrophobic transmembrane domain by genetic engineering, this recombinant protein was termed sRAGE owing to its ability to circulate in the blood in injected rodents. sRAGE functions as a ‘decoy’ for the cell-surface receptor, acting by binding ligands in the circulation and preventing them from binding to the cell surface and hence blocking cell activation by RAGE (Figure 2). Recombinant sRAGE was subsequently used in a variety of rat and murine models of vascular disease and was demonstrated to suppress acceleration of atherosclerosis in the diabetic (Type 1 and Type 2) and non-diabetic state [2,3,6]. The effects of sRAGE administration on vascular disease progression was shown to decrease the aortic atherosclerotic lesion size, number and complexity in a dose-dependent manner [2]. Further studies have demonstrated sRAGE to block a range of vascular disorders including neointimal expansion after vascular injury (restenosis) [4,47], diabetic nephropathy [13], inflammatory disorders [48,49] and delayed wound healing [50].

However, it has been suggested that sRAGE, in addition to blocking the RAGE–ligand interaction, may also act as a decoy for these ligands binding to other receptors [25]. Experimental evidence to further confirm the role of RAGE in these pathways has been provided by using genetically engineered rodents which express either DN mutants of RAGE or complete genetic deletion of the RAGE gene [4,5,51]. In a murine model of restenosis, vascular injury was blocked in RAGE homozygous null mice or transgenic mice expressing the DN-RAGE isoform in VSMCs (vascular smooth muscle cells) [4]. Additionally, most recently, using RAGE-null mice, it was demonstrated that vascular inflammation, endothelial dysfunction and atherosclerotic plaque formation was blocked in mice deficient in apolipoprotein E [5]. Taken together, these data strongly support a role for RAGE in the development and progression of vascular disease in both the diabetic and non-diabetic state in animal models.

Emerging studies are now implicating RAGE in the pathogenesis of vascular disease in humans. Studies by Cipollone et al. [52] support that RAGE is overexpressed in atherosclerotic plaques of diabetic patients when compared with non-diabetic patients. This overexpression may lead to atherosclerotic plaque destabilization based on their findings that documented in human diabetic patients of an association of RAGE overexpression in macrophages with the expression of COX-2 (cyclo-oxygenase-2) and prostaglandin E2 synthase-1, two growth-factor-induced co-regulated enzymes that contribute to the clinical instability of plaques [52]. Furthermore, treatment of diabetic patients with simvastatin reduced plaque expression of RAGE and impaired mechanisms of atherosclerosis including cellular infiltration of macrophages and T-cells, and MPO (myeloperoxidase)-dependent AGE generation [53]. However, the majority of human studies implicating RAGE in the vascular disease process are emerging from the study of the measurement of endogenous circulating sRAGE in human blood. The development and mechanistic understanding of sRAGE levels as a biomarker for vascular disease is an emerging exciting field of research. How the sRAGE isoform is produced and whether this reflects total RAGE levels or acts as a decoy for RAGE ligands are all important questions.

**THE CIRCULATING ENDOGENOUS SOLUBLE ISOFORM OF RAGE**

A common feature of receptors of the immunoglobulin superfamily is the occurrence of a soluble circulating isoform [54]. Molecules with high homology with RAGE, including NCAM (neural cell adhesion molecule) and ALCAM (activated leucocyte cell adhesion molecule), have been demonstrated to contain, in addition to the membrane-bound isoform, a soluble truncated molecule, have been demonstrated to contain, in addition to the membrane-bound isoform, a soluble truncated isoform [55,56]. A number of mechanisms have been reported that lead to the production of soluble proteins. Among these are the alternative splicing of the mRNA to remove the transmembrane domain and the proteolytical cleavage from the cell surface. Biological studies of RAGE have shown that sRAGE can be formed by both alternative splicing and proteolytical cleavage (Figure 3).

**The formation of sRAGE by alternative splicing**

The existence of splice variants of RAGE and their potential ability to produce soluble isoforms was first described by Malherbe et al. [57] in 1999. Subsequent studies led to the identification of a distinct number of splice variants of RAGE which could potentially result in soluble isoforms, all with different nomenclatures including sRAGE1/2/3, eRAGE (endogenous sRAGE) and hRAGEsec (human RAGE secreted form) [57–60].
A recent publication contains a complete classification and identification of the splice variants and clarifies their biological relevance [61]. In this study, it was revealed that most of the splice variants of RAGE that led to removal of the transmembrane domain, to produce soluble variants, were most probably degraded at the mRNA level by the NMD (nonsense-mediated mRNA-decay) pathway (Figure 4) [61]. The only variant found to be secreted from cells and not a candidate for NMD was found to be the RAGEv1 (RAGE splice variant 1) isoform [61], classified according to the HGNC (Human Gene Nomenclature Committee) and previously termed esRAGE or sRAGE3 [58,59]. RAGEv1 was also determined to be the second most prevalent RAGE isoform after full-length RAGE and is formed from the alternative splicing of the inclusion of part of intron 9 and removal of exon 10, which changes the reading frame sequence of the protein at amino acid 332, leading to the loss of both the transmembrane and cytosolic domains [61]. Transfection of RAGEv1 produced a protein of a smaller size compared with the full-length RAGE (size approx. 48 kDa compared with 55 kDa respectively), which was detectable by Western blotting with a polyclonal antibody for RAGE (Figure 4) [61]. In vivo studies have confirmed a functional role for RAGEv1 in vivo, with the restoration of diabetes-associated impairment of the angiogenic response in murine models [51]. Further in vitro studies are underway to understand the molecular implications of RAGEv1 on the effect on the vasculature.

The formation of sRAGE by proteolytic cleavage

The alternative mechanism of forming soluble circulating protein from a receptor is the cleavage of the extracellular domain from the cell surface by proteolytic means. Although less well understood than the formation of sRAGE by alternative splicing, there are a few recent studies to suggest that sRAGE can be formed by cleavage from the cell-surface receptor isoform [62,63]. In HEK (human embryonic kidney)-293 cells transfected with full-length RAGE, sRAGE production could be induced by treatment with the Ca\(^{2+}\)-ionophore ionomycin, but not treatment with PMA or RAGE ligand stimulation [62]. In contrast, Raucci et al. [63], in studies using the same cell line and HeLa cells, were able to induce sRAGE production with PMA and the RAGE ligand HMGB1 [63]. However, both studies identified that ADAM10 (a disintegrin and metalloproteinase 10) was the protease responsible for sRAGE release from the cell [62,63]. Further studies are therefore required to clarify these findings and to elucidate the mechanisms of cellular release from the surface of distinct cell types in varied homoeostatic and pathological states.

Biochemical measurement of sRAGE

There are currently two distinctly different commercial methods available for the measurement of sRAGE levels in human blood. The first is the human RAGE QuantiKine ELISA from R&D Systems which...
Western blot of RAGE and splice forms from cell lysates and their cultured medium to demonstrate the expression of splice forms of RAGE

As seen in the conditioned medium of RAGEv1-transfected cells, only the RAGEv1 isoform readily produces detectable levels of secreted RAGE from the cell. The molecular mass in kDa is indicated on the left-hand side. WB, Western blot. Reproduced from [61] with permission © 2007 Federation of American Societies for Experimental Biology.

sRAGE as a biomarker for vascular disease

A variety of recent studies focused on the association between either total sRAGE or esRAGE levels and disease risk factors due to the availability of the aforementioned biochemical measurement kits (Table 1). It appears, mainly from these studies, that both sRAGE and esRAGE levels are lower in subjects with vascular disease and progression state and in pathogenic states where RAGE ligand accumulation is enhanced.

Total sRAGE levels and vascular disease

The first study to report total sRAGE levels in humans was published by Falcone et al. [65] in 2005 in non-diabetic male subjects [65]. In Italian patients with angiographically proven CAD (coronary artery disease), patients in the lowest quartile for sRAGE presented the higher risk for CAD, with the association being independent of other vascular disease risk factors [65]. Further to this, other vascular disorders/vascular risk factors have been associated with decreased sRAGE levels including essential hypertension [66], cardiovascular disease risk score [67], oxidative stress [68], hypercholesterolaemia [69], vascular dementia [70], atherothrombotic stroke [71] and the diabetic state (Type 1 and Type 2 diabetes) [67]. However, contrary data have been reported that suggest that sRAGE levels are increased in both the diabetic state and CAD in Japanese subjects [72–75].

RAGEv1/esRAGE levels and vascular disease

Koyama et al. [76] first described the development of an ELISA system specific for the human esRAGE protein. In the same study they additionally used it in a cross-sectional analysis of esRAGE levels and with atherosclerosis in the presence or absence of diabetes [76]. In the non-diabetic state, esRAGE levels were the third strongest predictor for carotid and femoral atherosclerosis as determined by ultrasound measurements of arterial IMT (intimal-medial thickness) [76]. In contrast, in Type 2 diabetic patients, esRAGE levels were increased compared with non-diabetic patients and there was no relationship with IMT [76]. Similarly, in a European Caucasian population, Humpert et al. [64] did not observe an association of esRAGE with IMT in Type 2 male diabetic patients. In contrast, Katakami et al. [77] reported an inverse correlation between esRAGE and carotid atherosclerosis both in Type 1 and Type 2 diabetic patients. In their study they reported inverse correlations of vascular disease risk factors such as BMI (body mass index) and HbA1c (glycated haemoglobin) with sRAGE levels [77]. In other diabetic vascular disorders, serum circulating levels of esRAGE were suggested to be a protective factor against diabetic microvascular complications [78]. The study included diabetic patients without clinical nephropathy, and the esRAGE levels in Type 1 diabetic patients without retinopathy were found to be significantly higher than in the Type 1 diabetic patients with simple and proliferative retinopathy [78]. Finally, esRAGE levels have very recently been shown to be related to the atherosclerotic state, reflecting the arterial stiffness and inflammatory state [79], and to be an independent factor for carotid IMT progression in Type 1 diabetic patients [80,81].

measures the total sRAGE pool in serum and plasma samples using a monoclonal antibody raised against the whole recombinant extracellular human sRAGE protein. The second kit (Human esRAGE ELISA; B-Bridge International) specifically measures the esRAGE/RAGEv1 protein only due to the use of an antibody directed against the unique C-terminus sequence of RAGEv1, and does not cross-react with other potential forms of sRAGE (Figure 5). It has been suggested from studies that the levels esRAGE/RAGEv1 only constitutes a fraction of the total sRAGE pool, as levels of RAGEv1/esRAGE measured with the esRAGE ELISA are 4- to 5-fold lower than levels measured in the generic sRAGE ELISA [64]. However, it is not clear what the ratio between RAGEv1/esRAGE and total sRAGE is and whether this may differ in distinct conditions, as a rigorous ELISA cross-comparison has yet to be performed.
Other factors affecting sRAGE/esRAGE levels

There are a number of other factors which may affect sRAGE levels, not always taken into account in these studies, which include gender, BMI, renal function and drug treatment [68,69,82–84]. Previous studies have revealed these factors to affect sRAGE levels, in particular the stage of renal disease, which has been demonstrated to lead to increased levels of sRAGE beyond the normal physiological range seen to date [83–85]. Therefore it is reasonable to conclude that there are limitations in the above studies in that they did not exclude diabetic patients with compromised renal function from their population sample. The inverse relationship of decreasing renal function with increasing sRAGE/esRAGE levels is a phenomenon observed with other biomarkers of disease and has been termed ‘reverse epidemiology’ [86]. The hypothesis of this reversal is that the risk factors are not different, but some may be more dominant and responsible for the reversal. Alternatively, the renal insufficiency associated with this syndrome may suppress handling and excretion of sRAGE, as the major site(s) where sRAGE is processed is currently not known.

Modulation of sRAGE levels by therapeutic intervention

There are a number of clinical studies reporting modulation of sRAGE/esRAGE levels by medication that is used to treat vascular disease and complications [69,87–89]. Nakamura et al. [87] documented that telmisartan, an ARB (AT1 [AngII (angiotensin II) type 1] receptor blocker) that is used to prevent progression of cardiovascular and renal or retinal disease complications in diabetic patients with hypertension, decreases the levels of sRAGE in treated and AngII-stimulated cultured endothelial cells, but also the serum levels of sRAGE in patients with essential hypertension. Other drugs reported to affect sRAGE/esRAGE levels include statins and glitazones, which both result in increases in both levels [69,88].

The relationship of sRAGE/esRAGE to RAGE ligand levels

If the biological function of sRAGE is to act as a decoy for the cell-surface RAGE protein, then it would be expected that an inverse relationship does exist between sRAGE and ligand levels. In the few studies that have investigated this phenomenon, an inverse relationship between sRAGE/esRAGE levels and S100A12 and AGEs (AGE pool and CML) has been shown [67,85,89,90].

The development of sRAGE/esRAGE as a biomarker for vascular disease

From the above-described studies, it seems that sRAGE/esRAGE levels are lower in subjects with vascular disease and more advanced clinical syndromes. However, there are obvious limitations to these studies; first, the sample population studied is in most cases < 100; secondly, since levels of sRAGE/esRAGE are affected by certain clinical states and drug treatments, these have to be taken into account or excluded; thirdly, all of these studies represent a specific snapshot in an individual and therefore a prospective study is needed to test the variability of sRAGE/esRAGE over time or disease status. It is also critical to determine what the absolute ratio is that exists between these two measurements. How much of the blood level is formed by splicing and by cleavage?
Table 1  A comparison of studies on plasma levels of sRAGE/esRAGE from human population studies in different vascular disease states

<table>
<thead>
<tr>
<th>RAGE form</th>
<th>Vascular disease</th>
<th>Ethnic group</th>
<th>Population size (n)</th>
<th>Gender (male/female)</th>
<th>Change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sRAGE</td>
<td>CAD, ND</td>
<td>Caucasians</td>
<td>656</td>
<td>Only males</td>
<td>↓</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>Essential hypertension</td>
<td>Caucasians</td>
<td>324</td>
<td>205/119</td>
<td>↓</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>ESRD</td>
<td>Caucasians</td>
<td>81</td>
<td>↑</td>
<td>[83]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AD, VaD</td>
<td>Caucasians</td>
<td>404; total AD = 152,</td>
<td>46/102 for AD, 39/52 for</td>
<td>↓</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VaD = 91, YaD = 51, 110 for control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Severeiy of nephropathy, T2DM</td>
<td>Asians</td>
<td>468</td>
<td>217/183</td>
<td>↑</td>
<td>[84]</td>
</tr>
<tr>
<td></td>
<td>Cardiovascular risk and T2DM</td>
<td>Asians</td>
<td>75</td>
<td>29/46 for both groups</td>
<td>↑</td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td>Atherosclerosis (IMT), T2DM</td>
<td>Caucasians</td>
<td>43</td>
<td>22/21</td>
<td>↓</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>Markers of oxidative stress (PGF2a) and endothelial</td>
<td></td>
<td>86</td>
<td>42/44</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>dysfunction (ADMA), T2DM</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Hypercholesterolaemia</td>
<td></td>
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<tr>
<td></td>
<td>Vascular inflammatory markers (MCP-1, TNFα), T2DM</td>
<td>Asians</td>
<td>86</td>
<td>36/50 for both groups</td>
<td>↑</td>
<td>[72,73]</td>
</tr>
<tr>
<td></td>
<td>Heart-failure-related cardiac events, T2DM</td>
<td>Asians</td>
<td>160</td>
<td>95/65</td>
<td>↑</td>
<td>[74]</td>
</tr>
<tr>
<td></td>
<td>Stroke (severity of neurological deficit)</td>
<td>Caucasians</td>
<td>707</td>
<td>355/352</td>
<td>↓</td>
<td>[71]</td>
</tr>
<tr>
<td>esRAGE</td>
<td>Carotid and intimal atherosclerosis, ND/T2DM</td>
<td>Asians</td>
<td>134 ND, 203 T2DM</td>
<td>↓/−</td>
<td></td>
<td>[76]</td>
</tr>
<tr>
<td></td>
<td>Carotid atherosclerosis, T2DM</td>
<td>Asians</td>
<td>67</td>
<td>22/45</td>
<td>↓</td>
<td>[133]</td>
</tr>
<tr>
<td></td>
<td>Diabetic retinopathy ND/T2DM</td>
<td>Asians</td>
<td>55</td>
<td>32/23</td>
<td>↓</td>
<td>[78]</td>
</tr>
<tr>
<td></td>
<td>(diabetic retinopathy −/+)</td>
<td></td>
<td>47</td>
<td>20/27</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atherosclerosis (IMT), T2DM</td>
<td>Caucasians</td>
<td>110</td>
<td>86/24</td>
<td>−</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>Carotid atherosclerosis, T2DM</td>
<td>Asians</td>
<td>179</td>
<td>119/60</td>
<td>↓</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>Increased cardiovascular mortality in ESRD patients</td>
<td>Asians</td>
<td>206</td>
<td>131 (ND), 75 (T2DM)</td>
<td>↓</td>
<td>[134]</td>
</tr>
<tr>
<td></td>
<td>Severity of renal dysfunction in T2DM</td>
<td>Asians</td>
<td>107</td>
<td>78/29</td>
<td>↑</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>Inflammatory markers and arterial stiffness</td>
<td>Asians</td>
<td>154</td>
<td></td>
<td>↓</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td>Carotid atherosclerosis, T1DM</td>
<td>Asians</td>
<td>47</td>
<td></td>
<td>↓</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td>Carotid atherosclerosis, T2DM</td>
<td>Asians</td>
<td>152</td>
<td></td>
<td>↓</td>
<td>[81]</td>
</tr>
</tbody>
</table>

Also, does this change with disease state? Finally, another factor that may affect sRAGE levels is genetic variability in the RAGE gene. In this case, a number of key polymorphic variants have been identified which have been demonstrated themselves to be markers for disease and to affect the function of the mature RAGE protein [91,92].

GENETIC VARIANTS OF RAGE AND THEIR ROLE IN VASCULAR DISEASE

The location of the human gene for RAGE in the MHC Class III region on chromosome 6, the most gene-dense region in the genome, makes the RAGE gene a strong candidate gene for genetic variation. Not only is the MHC the most gene-rich area of the genome, but it contains polymorphic markers for disease with some of the longest associations [93]. Flanked by the PBX2 (pre-B-cell leukaemia homebox 5) gene and Ring finger protein 3, the RAGE gene (also termed AGER) is composed of 11 exons, a 3′-UTR (untranslated region) and a 5′-flanking region which overlaps the 3′-UTR of the PBX2 gene. Genetic studies have identified that approx. 30 polymorphisms occur in the RAGE gene which result in changes in the amino acid sequence of RAGE, non-coding changes in exons, intronic and in the 5′-flanking region, as shown in Figure 6 [91,92,94].
Multiple studies have focused on a number of these variants including a glycine-to-serine substitution at amino acid number 82 [91], a −374T > A substitution and a −429T > C relative to the transcription start site [92].

Coding change polymorphisms in the RAGE gene: G82S

The G82S polymorphism was one of the first identified polymorphisms of the RAGE gene and occurs in the ligand-binding V-domain of RAGE and therefore has attracted considerable interest [91]. This polymorphism is the only frequent coding-change polymorphism in the RAGE gene, with all other polymorphisms identified to change the amino acid sequence occurring in less than 1% of subjects [91].

The glycine-to-serine change at position 82 occurs proximal to an N-glycosylation site (position 81) and within the ligand-binding domain for RAGE ligands and therefore strongly suggests that this variant may affect RAGE function. In cells expressing the Ser82 isoform of RAGE, increased binding occurred for the RAGE ligand S100A12, and this led to an increase in cytokine and MMP (matrix metalloproteinase) release from cells [95]. Other studies have confirmed these findings with other RAGE ligands including AGEs [96]. Most recently, Xie et al. [35] investigated the protein structural changes of this variant and revealed that, not only does the Ser82 substitution lead to local structural changes in the V-domain, but also it imparted more widespread overall tertiary structural alterations in the extracellular domain.

It is somewhat surprising that such biological changes seen with the Ser82 allele have not been identified in clinical studies of the G82S variant in population studies, including macrovascular disease of both diabetic and non-diabetic subjects and diabetic microvascular disease (retinopathy or nephropathy) [91, 97–99]. Although most of these studies are in relatively small study populations, even a large prospective study of the G82S polymorphism in the Framingham Offspring Study did not reveal any associations with disease [100]. This ‘common’ coding change variant only occurs with a prevalence of ∼5% in most studies, which may explain the lack of associations.

However, in Asian subjects where Ser82 occurs with ∼20% allele frequency, little or no associations have been seen with vascular disease [97,101]. There are only a few studies which have revealed an association of the G82S polymorphism with a disease state. This variant was associated with skin complications of Type 2 diabetes [94], and as an independent risk marker for diabetic nephropathy in the GENEDIAB (Genétique de la Néphropathie Diabétique) study [102].

Given the lack of strong associations and the incidence of coding-region polymorphisms of RAGE with vascular disease, other regions of the RAGE gene have been screened for variants. Most promising are the identification of two variants in the 5′-flanking region of RAGE in an area responsible for the transcriptional regulation of the RAGE gene [92].

Promoter region variants of the RAGE gene

The up-regulation of RAGE gene expression is its hallmark in vascular disease and therefore genetic variants affecting RAGE mRNA or protein levels may therefore be important disease markers. The RAGE gene is regulated by a number of key regions within the 1.5 kb region upstream of the transcription start site [103–105]. Within this region, numerous polymorphisms have been identified (Figure 6) which include a −374T > A substitution and a −429T > C substitution relative to the transcription start site [92].

Promoter region variants of the RAGE gene: −374T > A

Of all of the RAGE gene polymorphisms identified, the −374T > A variant has been shown to be the most strongly and consistently associated with vascular disease. The functional implications of this variant were revealed by reporter gene and transcription-factor-binding assays, which demonstrated that the −374T > A allele led to increased gene expression and completely abolished binding of transcription factor(s) in vitro [92]. This study prompted numerous groups to investigate the role of the −374T > A polymorphism in the risk and development of vascular disease.
of vascular disease (Table 2). Interestingly, case/controls studies have revealed a strong link between an individual’s possession of the −374A allele or AA genotype and protection against vascular disease. These include decreased risk of cardio- and cerebral-vascular disease [106–109], a decrease in restenosis after angioplasty [110], the number of affected vessels [111, 112], and mortality after myocardial infarction (Table 2) [113]. Studies have not identified an association with diabetic retinopathy [92, 114–116], which is not surprising since genetic studies of diabetic microvascular complications did not reveal a genetic component on the pathogenesis of retinopathy. Diabetic nephropathy on the other hand, is a highly genetically driven complication [117], and does reveal associations with the −374T > A polymorphism [106, 118]. If the −374A allele is protective, this may be in contrast with the in vitro data which suggest that this variant increases RAGE expression. It is however possible that this increase is a reflection of the cell types used for these in vitro studies and may give differing results in vascular cells. Further in vitro studies are required to reveal the role of the −374T > A variant in transcription.

Promoter region variants of the RAGE gene: −429T > C

Proximal to the −374T > A polymorphism is the −429T > C variant. Although this variant was shown to increase transcriptional levels in in vitro studies, unlike the −374T > A SNP (single nucleotide polymorphism), no effects were demonstrated on transcription-factor-binding assays [92]. Initial gene-association studies demonstrated a link between the −429T > C variant and retinopathy; however, subsequent studies have not revealed many disease associations [92]. In studies where the −374T > A variant has been associated with disease, there was no association of the −429T > C either [92, 106, 108, 109, 114–116]. A number of publications have perhaps revealed a role for the −429T > C variant as a marker for the diabetic/pre-diabetic state [119, 120]. In diabetic subjects, the −429C allele was associated

<table>
<thead>
<tr>
<th>Vascular disease</th>
<th>Effect</th>
<th>Population size (n)</th>
<th>Ethnicity</th>
<th>Allele/genotype frequency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 diabetic retinopathy</td>
<td>None</td>
<td>325</td>
<td>Caucasian</td>
<td>59% TT, 41% TA/AA</td>
<td>[92]</td>
</tr>
<tr>
<td>Type 2 diabetic retinopathy</td>
<td>None</td>
<td>571</td>
<td>Asian</td>
<td>86.4% T, 13.6% A</td>
<td>[114]</td>
</tr>
<tr>
<td>Type 2 diabetic retinopathy</td>
<td>None</td>
<td>258</td>
<td>Caucasian</td>
<td>41.4% TT, 42.9% TA, 11% AA</td>
<td>[115]</td>
</tr>
<tr>
<td>Type 2 diabetic retinopathy</td>
<td>None</td>
<td>536</td>
<td>South Asian</td>
<td>91% T, 9% A</td>
<td>[116]</td>
</tr>
<tr>
<td>Type 1 diabetic nephropathy</td>
<td>None</td>
<td>391</td>
<td>Caucasian</td>
<td>48.7% TT, 42.2% TA, 9.1% AA</td>
<td>[135]</td>
</tr>
<tr>
<td>Type 1 diabetic nephropathy/CAD</td>
<td>— 374AA genotype protective against nephropathy and CVD</td>
<td>996</td>
<td>Caucasian</td>
<td>87% T, 13% A</td>
<td>[106]</td>
</tr>
<tr>
<td>ESRD ↓ PSTR in haemodialysis patients with the −374TA genotype</td>
<td>30</td>
<td>Caucasian</td>
<td>66% TT, 34% TA, 0% AA</td>
<td>[118]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↓ Risk of CAD with the −374AA genotype</td>
<td>259</td>
<td>Caucasian</td>
<td>22.6% AA in CAD-free compared with 9.6% AA in CAD</td>
<td>[107]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↓ Number of diseased vessels in subjects with the −374AA genotype</td>
<td>234</td>
<td>Caucasian</td>
<td>39.7% TT, 51.3% AT, 9% AA</td>
<td>[112]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↓ Risk of IHD in African–Brazilian T2DM with the −374A allele</td>
<td>703</td>
<td>Caucasian–Brazilian and African–Brazilian</td>
<td>46.5% TT, 40.7% TA, 9.3% AA; 52.5% TT, 31.7% TA, 7.1% AA</td>
<td>[108]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↓ Number of diseased vessels in T2DM with the −374 AA genotype</td>
<td>246</td>
<td>Caucasian–Brazilian</td>
<td>40.8% TT, 50% TA, 9.2% AA</td>
<td>[111]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↓ Risk of restenosis in subjects with the −374AA genotype</td>
<td>267</td>
<td>Caucasian</td>
<td>88% T, 12% A</td>
<td>[110]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↑ Survival of ND after MI with the −374AA genotype</td>
<td>643</td>
<td>Caucasian</td>
<td>39.3% TT, 48% TA, 12.7% AA</td>
<td>[113]</td>
<td></td>
</tr>
<tr>
<td>CAD/CVD ↓ Risk of stroke in subjects with the −374A allele or the −374AA genotype</td>
<td>1200</td>
<td>Caucasian</td>
<td>74% T, 26% A</td>
<td>[109]</td>
<td></td>
</tr>
</tbody>
</table>
with higher HbA1c levels and occurred with an increased incidence in Type 1 diabetic patients [119]. Furthermore, the −429C allele has been linked to insulin resistance in healthy subjects [120].

**Other RAGE gene variants**

In the transcriptional region of the RAGE gene, the other functional variant identified was a 63 bp deletion which spans from −407 to −345 of the RAGE gene promoter [92]. The use of this variant as a genetic marker for disease is limited due to its incidence being <1% in populations studied to date [92]. Studies of other RAGE gene promoter variants are made complicated due to the overlapping 3′-UTR of the PBX2 gene 5′ from −505 having a pseudogene copy on chromosome 3 [121]. In essence, this means any variant amplified within this region is in fact amplifying both chromosome 6 and 3 and therefore renders it very difficult to genotype specific variants [121].

The other possible locations for genetic variation in the RAGE gene are within the intronic regions. These could theoretically affect RAGE mRNA splicing and therefore alter the ratio of splice variant, such as RAGE/RAGEv1 and hence the levels of full-length and sRAGE. Although no variants exist proximal to or within exon/intron boundaries, a number of variants have been identified within introns 7 and 8 [94]. These include the +1704G > T, +2184A > G and +2245G > A which were first identified in a Czech population and demonstrated to be associated with diabetic microvascular dermatitis [94]. Subsequent studies by these researchers demonstrated an association of the +1704G > T and +2184A > G with antioxidant status in Type 2 diabetes, but not with proliferative retinopathy [122]. However, limited studies of only the +1704G > T have been performed in other populations [123, 124]. Further studies in large numbers of subjects are required to thoroughly investigate these variants.

**Multi-genetic analysis of the RAGE gene**

Multi-factorial disease states including vascular disease in non-diabetic and diabetic patients have been shown to be polygenic [125]. The use of a single locus/disease association approach is thought to be limited. Therefore, in the case of the RAGE gene, the combination of multiple RAGE genotypes with other genetic variants in other genes may prove to be a better predictor for disease. To address this, researchers have performed a number of different complex analyses including whole-genome association studies, linkage and haplotype studies. Kankova et al. [126] were the first to approach this issue using the haplotype-based approach. In Type 2 diabetic patients with or without nephropathy, they constructed numerous haplotypes from single-locus genotyping including G82S, −374T > A, −429T > C, +1704G > T, +2184A > G and +2245G > A [126]. Interestingly, the ‘RAGE2 haplotype’ which comprised −429C, −374T, Gly82, 1704G, 2184G and 2245G, was enriched in the group with kidney disease and was a predictor of time of onset of nephropathy [126]. These results support findings published previously which demonstrate strong linkage disequilibrium between polymorphisms of the RAGE gene including the −429T > C and −374T > A [92, 106]. Further studies in patients with chronic kidney disease did not reveal the same results with RAGE gene haplotypes [127]. However, in this study of kidney disease, patients on haemodialysis were analysed in which diabetic and non-diabetic pathologies were responsible. Therefore these considerations may explain these results [127]. Using the −374T > A, −429T > C and G82S haplotypes in the Physicians Health Study, it was seen that the −429C, −374T and Gly82 haplotype and −429T, −374A and Gly82 were associated with reduced risk of myocardial infarction and ischaemic stroke respectively [109]. These haplotype-based approaches all confirm the previous results seen with the −374T > A and suggest this to be a potential marker for vascular disease.

The study of RAGE SNPs in combination with variants in other loci and genes is another possible means to predict disease outcome. In subjects with diabetic nephropathy, Kankova et al. [128] used a set-association approach combing 45 SNPs from 20 genes on eight different chromosomes and demonstrated that a number of SNPs [RAGE −429T > C and 2184A > G, LTA (lymphotoxin α) 252A > G and EDN1 (endothelin 1) 8002G > A] on chromosome 6p jointly associate with susceptibility to nephropathy. Most recently, Lindholm et al. [129] studied the chromosome 6p21 linkage effects of the RAGE gene, LTA and TNF (tumour necrosis factor) with susceptibility to diabetic vascular complications [129]. The authors found that a combination of the T60N (LTA) −308G > A (TNF) and −374T > A (RAGE) was associated with diabetic micro- and macro-vascular disease [129]. However, in this study the authors demonstrated a clear link between these effects and the HLA class II genotypes. The HLA class II contains the HLA-DQ and HLA-DR genes which function to present antigens to T-helper cells [130]. Within these genes have been identified a number of polymorphisms which have been demonstrated to be very strong predictors for numerous inflammatory diseases, including the development and pathogenesis of diabetes [130]. To date, it has been identified that RAGE gene polymorphisms exhibit strong linkage with HLA-DQ2/DR3 (−429T > C) [119], HLA-DRB1 (G82S) [131] and HLA-DQB1 (−374T > A) [132]. These results therefore suggest that a complex haplotype of multi-gene loci may be the best predictor for vascular disease when studying RAGE gene variants. Overall, larger studies, including multiple genotypes, are needed to discriminate the role of the RAGE polymorphisms in disease susceptibility and pathogenesis.
RAGE gene variation and sRAGE
With the availability of sRAGE ELISAs as a potential surrogate marker of RAGE and/or RAGE ligand expression levels, this raises the interesting premise that the effect of RAGE gene polymorphisms can be phenotypically investigated. Although multiple studies have been focused on sRAGE and esRAGE levels, to date only one study has investigated the association of RAGE gene variation and sRAGE levels in human blood [127]. In the previously mentioned study of RAGE gene variation and chronic kidney disease, the authors measured both sRAGE and esRAGE levels and studied the relationship with RAGE gene polymorphisms [127]. In these chronic haemodialysis patients, the RAGE –429C and +2184G alleles were associated with higher sRAGE and esRAGE levels [127]. However, as renal failure is associated with higher sRAGE levels, the opposite of most pathological states [127]. However, as renal failure is associated with higher sRAGE levels, this raises the interesting premise that the effect of RAGE gene polymorphisms can be phenotypically investigated. Although multiple studies have been focused on sRAGE and esRAGE levels, to date only one study has investigated the association of RAGE gene variation and sRAGE levels in human blood [127]. In the previously mentioned study of RAGE gene variation and chronic kidney disease, the authors measured both sRAGE and esRAGE levels and studied the relationship with RAGE gene polymorphisms [127].

CONCLUSIONS
The up-regulation and pathogenic effects of RAGE in vascular disease as well as the multiple genetic variants identified for RAGE, suggest the significant role of the RAGE gene as an important contributing mechanism. Intriguingly, what may place the RAGE gene as a potential protagonist in vascular disease pathogenesis is the relationship between levels of the full-length receptor and its soluble variant. The mechanisms underlying the regulation of these two distinct species, and their potential to be modulated by different factors such as the vascular environment or drugs, may highlight a novel strategy for therapeutic intervention. Future studies are required to fully elucidate the role of soluble isoforms of RAGE and the effects of RAGE gene allelic variants in these processes. With such studies, these could become powerful surrogate markers for identifying subjects at risk for vascular disease and identifying key targets for therapeutic intervention.

FUNDING
A. Z. K. is a recipient of a Postdoctoral Fellowship from the Juvenile Diabetes Research Foundation International. A. M. S. is a recipient of Scholar Award from the Juvenile Diabetes Research Foundation International. B. I. H. is a recipient of a Career Development Award from the Juvenile Diabetes Research Foundation International.

REFERENCES
7 Hudson, B. I., Wendt, T., Bucciarelli, L. G., Rong, L. L., Naka, Y., Yan, S. F. and Schmidt, A. M. (2005) Diabetic vascular disease: it's all the RAGE. Antioxid. Redox Signaling 7, 1588–1600


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Received 1 October 2008/5 November 2008; accepted 6 November 2008
Published on the Internet 16 March 2009, doi:10.1042/CS20080494