Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury

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The binding of advanced glycation end-products (AGE) to the receptor for AGE (RAGE) is known to deteriorate various cell functions and is implicated in the pathogenesis of diabetic vascular complications. In the present study, we show that the cellular constituents of small vessels, endothelial cells (EC) and pericytes express novel splice variants of RAGE mRNA coding for the isoforms that lack the N-terminal V-type immunoglobulin-like domain (N-truncated) or the C-terminal transmembrane domain (C-truncated), as well as the known full-length mRNA. The ratio of the expression of the three variants was different between EC and pericytes; the content of the C-truncated form was highest in EC, whereas the full-length form was the most abundant in pericytes. Transfection experiments with COS-7 cells demonstrated that those variant mRNAs were translated into proteins as deduced; C-truncated RAGE was efficiently secreted into the culture media, and N-truncated RAGE was located mainly on the plasma membrane. The three isoforms were also detected in primary cultured human EC and pericytes. Further, full-length and C-truncated forms of RAGE bound to an AGE-conjugated column, whereas N-truncated RAGE did not. The AGE induction of extracellular-signal-related kinase phosphorylation and vascular endothelial growth factor in EC and of the growth and cord-like structure formation of EC was abolished completely by C-truncated RAGE, indicating that this endogenous secretory receptor (endogenous secretory RAGE) is cytoprotective against AGE. The results may contribute to our understanding of the molecular basis for the diversity of cellular responses to AGE and for individual variations in the susceptibility to diabetic vascular complications.

Key words: alternative splicing, angiogenesis, diabetic vascular complication, non-enzymic glycation, soluble-type receptor.

INTRODUCTION

Reducing sugars such as glucose can react non-enzymically with the amino groups of proteins to form reversible Schiff bases followed by Amadori rearrangement. These early glycation products undergo further complex reactions such as rearrangement, dehydration and condensation to become irreversibly cross-linked, heterogeneous derivatives termed advanced glycation end-products (AGE). They are known to accumulate in circulating blood and in various tissues at an extremely accelerated rate under the diabetic state, and have been implicated in the development of diabetic vascular complications, e.g. retinopathy and nephropathy [1–3]. Receptor-dependent mechanisms are likely to work in the AGE-induced tissue dysfunction [2] and the best-characterized receptor for AGE is the receptor for AGE (RAGE) [3–5]. RAGE belongs to the immunoglobulin superfamily of cell-surface molecules and is composed of an extracellular region containing one 'V'-type and two 'C'-type immunoglobulin domains [3,5]. This portion of the receptor is followed by a hydrophobic transmembrane-spanning domain and then by a highly charged, short cytoplasmic domain that is essential for post-RAGE signalling [6,7]. RAGE is expressed in multiple tissues [8] and interacts with various ligands, including AGE [3]. We have shown previously [9–11] that engagement by AGE of RAGE leads to changes in endothelial cells (EC) and pericytes, the constituents of microvessels, which are characteristic of diabetic microangiopathy. AGE stimulate the growth of microvascular EC with an induction of vascular endothelial growth factor (VEGF), leading to angiogenesis on the one hand [9], and inhibit prostacyclin production and stimulate plasminogen activator inhibitor-1 synthesis by EC, thereby leading to thrombogenesis on the other [10]. AGE also exhibit a growth-inhibitory action on pericytes [11], which would lead to pericyte loss, the earliest histological hallmark of diabetic retinopathy [12]. The engagement of RAGE by AGE has been reported to induce cellular oxidant stress, activating nuclear factor-κB (NF-κB) [13,14], resulting in the perturbation of a variety of homoeostatic functions of the vasculature [3]. We have also demonstrated that AGE themselves up-regulate the *RAGE* expression in microvascular EC through the activation of NF-κB

Abbreviations used: AGE, advanced glycation end-products; EC, endothelial cell(s); ECD, extracellular domain; ERK, extracellular-signal-related kinase; esRAGE, endogenous secretory RAGE; FBS, foetal bovine serum; HA, haemagglutinin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NF-κB, nuclear factor-κB; poly(A)$^+$, polyadenylated; RAGE, receptor for AGE; RT, reverse transcriptase; VEGF, vascular endothelial growth factor.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession nos. AB036432 (full-length RAGE), AB061668 (endogenous secretory RAGE) and AB061669 (N-truncated RAGE).

[15]. Recently [16,17], we have demonstrated that glomerular changes resembling those seen in human diabetic nephropathy were significantly augmented in *RAGE* transgenic mice when they became diabetic. The AGE–RAGE system thus has been thought to play a central role in the development of diabetic vasculopathy and a promising target for the treatment of this disease. To deepen our understanding of the physiology and pathology of RAGE and to develop effective means for the prophylaxis and therapy of diabetic complications, it is important to elucidate the nature of RAGE proteins expressed in vascular cells.

In the present study, we isolated RAGE-encoding sequences from human EC and pericyte polysomal polyadenylated [poly(A)$^+$] RNA, which would be translatable to yield RAGE proteins under physiological conditions. They were composed of three major types: one was the known sequence, which encoded the full-length form, the other two were sequences previously not reported. One of them encoded an N-terminal-deleted, membrane-bound form lacking the ability to bind AGE. The other encoded an endogenous secretory receptor form, which was capable of capturing the ligand, thereby protecting against the AGE-induced vascular injury.

EXPERIMENTAL

Cells

Human microvascular EC isolated from neonatal dermis (Cascade Biologics Inc., Portland, OR, U.S.A.) were maintained in a Hu-Media MV2 medium, supplemented with 5% (v/v) foetal bovine serum (FBS), 5 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, 10 ng/ml epidermal growth factor, 1 μg/ml cortisol and 39.3 μg/ml dibutyryl cAMP according to the manufacturer's instructions (Kurabo Corp., Osaka, Japan). Human microvascular pericytes from cerebral cortex were from Applied Cell Biology Research Institute (Kirkland, WA, U.S.A.) and maintained in a CS-C complete medium (Cell Systems Corp., Kirkland, WA, U.S.A.) according to the manufacturer's instructions (Dainippon Pharmaceutical Co., Osaka, Japan). Cells at 5–10 passages were used for the experiments. The monkey kidney-derived cell line COS-7 was maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The human umbilical vein-EC-derived cell line ECV304 [18] was donated by Dr Yoshio Sawasaki (National Defense Medical College, Tokorozawa, Japan), and was maintained in medium 199, supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin; with this cell line, AGE have also been shown to cause VEGF induction [15].

Antibodies

A goat antibody against the C-terminal 20 amino acids (384–403) (C-20) of human RAGE protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit polyclonal antibodies raised against recombinant human RAGE extracellular domain (αRAGE-ECD) (amino acids 24–321) were kindly provided by the Institute of Biological Science, Mitsui Pharmaceutical Inc. (Mobara, Japan). Endogenous secretory RAGE (esRAGE)-specific antibody (αesRAGE) was raised in rabbits against the unique C-terminal 16-amino-acid peptide (amino acids 332–347; EGFDKVREAEDSPQHM) and immunoaffinity-purified with an antigen peptide-conjugated column. An anti-RAGE monoclonal antibody (13F11) was developed in collaboration with Daiichi Fine Chemicals Co. (Takaoka, Japan); it was raised against recombinant human esRAGE, and was confirmed to be capable of recognizing any of the three RAGE variant proteins under both reducing and non-reducing conditions. A mouse monoclonal antibody raised against phosphorylated extracellular-signal-related kinase (ERK) [p-ERK1(E-4)] and a rabbit polyclonal antibody raised against ERK [ERK1(K-23)] were from Santa Cruz Biotechnology.

Isolation and sequence determination of polysomal RAGE splice variants

Poly(A)$^+$ RNA was isolated from the total polysomal fraction of human microvascular EC or pericytes essentially as described previously [19]. Briefly, EC or pericytes, which had been scraped off in PBS and pelleted by centrifugation, were homogenized in 10 mM Tris/HCl buffer (pH 7.6), containing 0.25 M KCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.25 M sucrose (RNase-free), 0.1 mM dithiothreitol, 2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride and 1000 units/ml SUPERaseIn RNase inhibitor (Ambion, Austin, TX, U.S.A.) on ice with a Dounce-type glass homogenizer. The homogenates were centrifuged at 12000 ***g*** for 15 min to remove nuclei and mitochondria. The post-mitochondrial supernatant was further centrifuged at 100000 ***g*** for 60 min, and the resultant pellets (total polysomes) then underwent poly(A)$^+$ RNA isolation with Quickprep micro mRNA isolation kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Polysomal poly(A)$^+$ RNAs (50 ng) were reverse-transcribed with oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (RT), and RAGE cDNAs were amplified with 5′- and 3′-primers (5′-GCCAGGACCCTGGAAGGAAGCA-3′ and 5′-CTGATGGATGGGATCTGTCTGTG-3′) that correspond to exons 1 (nt 6641–6662) and 11 (nt 9752–9774) (GenBank® accession no. D28769) respectively using LA *Taq* polymerase (Takara, Kyoto, Japan). The thermal cycling parameters were 94 °C/30 s for denaturation, 60 °C/30 s for annealing and 72 °C/1.5 min for elongation. Amplified cDNAs were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.). Plasmid DNAs were purified with a Flexprep plasmid isolation kit (Amersham Pharmacia Biotech), and their nucleotide sequences were determined with an ABI377 sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.).

Construction of expression vectors

RAGE splice variant cDNAs were amplified with 5′- and 3′-primers having *Eco*RI and *Xba*I sites (indicated by underline) respectively. Full-length type cDNA was amplified with 5′-GAGAATTCGCCAGGACCCTGGAAGGAAGCA-3′ and 5′-GATCTAGACTGATGGATGGGATCTGTCTGTG-3′ (nt 1–22 and 1246–1268 of AB036432 respectively). C-truncated type cDNA was amplified with 5′-GAGAATTCGCCAGGACCCTGGAAGGAAGCA-3′ and 5′-GATCTAGAGCTTGTTGACCATCCCCCCAG-3′ (nt 1–22 and 1069–1088 of AB061668 respectively). N-truncated type cDNA was amplified with 5′-GAGAATTCTGAGGGGATTTTCCGGTGC-3′ and 5′-GATCTAGACTGATGGATGGGATCTGTCTGTG-3′ (nt 486–504 and 1429–1451 of AB061669 respectively). The amplified cDNAs were digested with *Eco*RI and *Xba*I, and inserted into a mammalian expression vector, pCI-*neo* (Promega, Madison, WI, U.S.A.) that had been digested with the same restriction enzymes. Recombinant plasmid DNAs were purified with a plasmid isolation kit (Qiagen, Valencia, CA, U.S.A.). For construction of expression vectors encoding C-terminal haemagglutinin (HA) epitope-tagged full-length and N-truncated type RAGE proteins, the cDNAs were amplified with a 3′-primer having the HA-tag

sequence and a *Not*I site (5′-GAGCGGCCGCTCA-AGCATAATCTGGAACATCATATGGATAAGGCCCTCC-AGTACTACTCT-3′; double underline and underline indicate HA-tag sequence and *Not*I site respectively). This primer corresponded to nucleotides 1217–1237 of AB036432 or nucleotides 1400–1420 of AB061669. The 5′-primers for full-length and N-truncated types of RAGE cDNA were 5′-GATCTAGAAT-GGCAGCCGGAACAGCAGTT-3′ and 5′-GATCTAGAATG-AACAGGAATGGAAAGGAGAC-3′ (underlines indicate *Xba*I site) respectively. They corresponded to nucleotides 24–45 and 511–533 of the respective cDNAs. The amplified cDNA was then inserted into *Xba*I and *Not*I sites of a pCI-*neo* vector and sequence-verified.

Expression in COS-7 and ECV304 cells

COS-7 cells (1×10^7 cells) were transfected with 20 μg of each expression vector or vector alone by electroporation. After incubation at 37 °C for 48 h, the expressions of RAGE isoforms were examined by Western-blot analysis. For purification of recombinant C-truncated RAGE (esRAGE), stable transformants were selected. Briefly, cells in a 25 cm^2 flask were transfected with 6 μg of the expression vector using Tfx-20 reagent (Promega). After incubation at 37 °C for 48 h, cells were cultured in the presence of G418 (Geneticin, 750 μg/ml) and G418-resistant colonies were picked up 2 weeks later. Cell clones were expanded individually, and clones expressing high levels of esRAGE were selected by Western-blot analysis with anti-RAGE antibodies. For stable expression in ECV304 cells, cells in 25 cm^2 flasks were transfected with 6 μg of each expression vector or the vector alone using Tfx-20 reagent, and selected for G418 resistance as described above.

Western-blot analysis

RAGE variant cDNA-transfected cells were washed with ice-cold PBS, scraped off in PBS and pelleted by centrifugation at 300 ***g*** for 5 min at 4 °C. The cells were lysed immediately by sonication in SDS/PAGE sample buffer [62.5 mM Tris/HCl (pH 6.8)/2 % (w/v) SDS/5 % (v/v) 2-mercaptoethanol/10 % (v/v) glycerol/0.002 % Bromophenol Blue] and boiled at 95 °C for 5 min. Protein concentrations were determined by the method of Bradford [20] using BSA as a standard. Cell lysates (25–100 μg of protein) were resolved by SDS/PAGE (12.5 %), and then transferred on to a PVDF membrane (Millipore, Bedford, MA, U.S.A.). The membranes were treated with one of the anti-RAGE antibodies described above, and the immunoreacted bands were visualized with an ECL® detection system (Amersham Pharmacia Biotech). For analyses of esRAGE secreted into culture media, confluent cultures of RAGE variant cDNA-transfected cells were incubated in serum-free medium at 37 °C for 24 h, and the conditioned media were collected and centrifuged at 10000 ***g*** for 10 min. The supernatants were directly analysed by Western blotting as described above.

Detection of the RAGE splice variant proteins in primary cultured human microvascular cells

RAGE variant proteins were partially purified from primary cultured human EC and pericytes by affinity chromatography on a pan-RAGE monoclonal antibody-conjugated column. The 13F11 monoclonal antibody (IgG) was coupled with HiTrap NHS-activated (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The concentration of the IgG immobilized was approx. 3 mg as protein/ml of gel. EC or pericytes (approx. 1.0×10^7 cells) were lysed by sonication in 10 ml of the extraction buffer [20 mM Tris/HCl (pH 7.4)/0.1 M NaCl/1 % 1-*O*-n-octyl β-D-glucopyranoside/1 mM PMSF] and centrifuged at 100000 ***g*** for 30 min at 4 °C. The supernatants (approx. 3 mg of proteins) were then applied to the antibody column previously equilibrated with 50 mM Tris/HCl (pH 7.4) containing 0.15 M NaCl and 0.5 % 1-*O*-n-octyl β-D-glucopyranoside. After washing with the same buffer, bound proteins were eluted with 100 mM Na_2PO_4/NaOH buffer (pH 12) containing 0.15 M NaCl and 0.5 % 1-*O*-n-octyl β-D-glucopyranoside. Eluates equivalent to 300 μg of the extracts were subjected to immunoblot analysis. For detection of esRAGE in conditioned media, confluent cultures of EC or pericytes (approx. 1.0×10^7 cells) were cultured in serum-free medium at 37 °C for 48 h. The conditioned media were then collected, supplemented by PMSF to a final concentration of 0.5 mM, and centrifuged at 10000 ***g*** for 10 min. The resultant supernatants (75 ml) were applied to the antibody column, and bound proteins were eluted with 100 mM glycine/HCl buffer (pH 2.5) containing 0.15 M NaCl and 0.5 % 1-*O*-n-octyl β-D-glucopyranoside. Eluates equivalent to 5 ml of conditioned media were subjected to immunoblot analysis.

Deglycosylation with glycopeptidase F

Cell extracts or conditioned media of RAGE variant cDNA-transfected COS-7 cells were treated with glycopeptidase F (Takara) under denaturing conditions at 37 °C for 24 h [21], and analysed by Western blotting as described above.

Immunocytochemistry

Immunostaining of HA-tagged RAGE was performed as described by Harada et al. [22]. Briefly, COS-7 cells expressing HA-tagged full-length or N-truncated RAGE were cultured on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.), fixed with PBS containing 3.7 % (v/v) formaldehyde at 25 °C for 20 min, and made permeable by incubating with PBS containing 0.2 % Triton X-100 at 25 °C for 3–5 min. After blocking with PBS containing 3 % (w/v) BSA (blocking solution) for 10 min, cells were incubated in blocking solution containing rat monoclonal anti-HA antibody (10 μg/ml; Roche Diagnostics, Basel, Switzerland) at 37 °C for 60 min, washed with PBS, and then incubated with blocking solution containing rhodamine-conjugated goat anti-rat IgG (1/500 dilution; Jackson ImmunoResearch Lab., West Grove, PA, U.S.A.) at 37 °C for 60 min. After washing with PBS, cells were observed under a confocal laser microscope (Carl Zeiss, Oberkochen, Germany).

AGE binding assay

The ability of the RAGE variant proteins to bind AGE was determined by affinity column chromatography. As an AGE ligand, we employed glyceraldehyde-derived AGE–BSA [23,24], which binds strongly to RAGE (Y. Yamamoto, H. Yonekura, S. Sakurai, R. G. Petrova, T. Watanabe, Md. J. Abedin, H. Li, K. Yasui, Z. Makita, M. Takeuchi and H. Yamamoto, unpublished work). Glyceraldehyde-derived AGE–BSA was prepared as described previously [24] and coupled with HiTrap NHS-activated (Amersham Pharmacia Biotech). The concentration of the ligand immobilized was approx. 20 mg of BSA/ml of gel. Full-length- and N-truncated-type RAGE proteins were extracted from membrane fractions of COS-7 cells transfected with the corresponding type of cDNA. Briefly, cells were homogenized in the homogenizing buffer [0.25 M sucrose/50 mM Tris/HCl (pH 7.4)/10 mM KCl/5 mM $MgCl_2$/1 mM PMSF]. The homogenates were centrifuged at 600 ***g*** for 5 min at 4 °C, and the supernatants were then centrifuged at 100000 ***g*** for 30 min at

4 °C. The precipitated membrane fractions were extracted with the extraction buffer and centrifuged. Supernatants were saved and used for the binding assay. For the binding assay of the C-truncated-type RAGE (esRAGE), esRAGE cDNA-transfected COS-7 cells were cultured in serum-free medium for 48 h, and the supernatant obtained by centrifugation at 10000 ***g*** for 15 min at 4 °C was used. The samples containing similar amounts of RAGE variant proteins as estimated by the immunoblot analysis were applied to the AGE column previously equilibrated with 20 mM Tris/HCl (pH 7.4) containing 0.15 M NaCl and 0.5 % 1-*O*-n-octyl β-D-glucopyranoside. The column was then washed with the same buffer and bound proteins were eluted with 20 mM Tris/HCl (pH 7.4) containing 2 M NaCl and 0.5 % 1-*O*-n-octyl β-D-glucopyranoside. The eluted fractions were then subjected to immunoblot analysis.

Purification of esRAGE protein

COS-7 cells stably transformed with pCI-*neo* carrying the esRAGE cDNA were cultured in serum-free media for 48 h, after which conditioned media were used for esRAGE purification with an AKTA purifier system (Amersham Pharmacia Biotech). Two litres of the conditioned media was first applied on a HiTrap-Heparin column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris/HCl buffer (pH 7.4). The column was washed with 20 mM Tris/HCl buffer (pH 7.4) containing 0.3 M NaCl, and the bound proteins were eluted with 20 mM Tris/HCl buffer (pH 7.4) containing 0.5 M NaCl. Eluted fractions were analysed by Western blotting with αesRAGE. Positive fractions were diluted with 50 mM acetate buffer (pH 4.5) and applied on a RESOURCE S column (Amersham Pharmacia Biotech) equilibrated with 50 mM acetate buffer (pH 4.5). After washing with 50 mM acetate buffer (pH 4.5) containing 0.2 M NaCl, the bound proteins were eluted with a linear gradient from 0.2 to 1 M NaCl. Eluted fractions were analysed by Western blotting, and the fractions that positively immunoreacted with αesRAGE were pooled. Finally, the pooled sample was loaded on a HiTrap desalting column (Amersham Pharmacia Biotech) equilibrated with PBS and the positive fractions were collected. The purified materials yielded a single band at 50 kDa when run on SDS/PAGE followed by silver staining. The concentration of esRAGE was determined by the method of Bradford [20], and the yield was approx. 100 μg.

Effect of esRAGE on AGE-induced expression of *VEGF* gene in EC

Subconfluent cultures of human microvascular EC in the medium lacking epidermal growth factor and cortisol were exposed for 4 h to glyceraldehyde-derived AGE–BSA at a final concentration of 10 μg/ml in the presence or absence of 25 μg/ml purified esRAGE. After the cells were washed with cold PBS, poly(A)$^+$ RNA was isolated with a Quickprep micro mRNA purification kit (Amersham Pharmacia Biotech), and analysed by RT–PCR with a SuperScript One-Step RT–PCR kit with Platinum Taq (Invitrogen). Oligodeoxyribonucleotide primers and probes for human VEGF and β-actin mRNA, and PCR conditions were the same as described previously [25,26]. After amplification, aliquots of the reaction mixtures were electrophoresed on a 3 % (w/v) agarose gel and transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech). The membranes were then hybridized with the ^{32}P-end-labelled probes as described previously [25,26].

Real-time RT–PCR was also performed for the determination of the relative amounts of VEGF-A mRNA in AGE and esRAGE-treated cells using an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems) with TaqMan reagents and poly(A)$^+$ RNA samples described above. We used Relative Standard Curve Method (User Bulletin #2, ABI PRISM 7700 Sequence Detection System) for relative quantification. The primer/probe set was designed using the manufacturer's software; the sequences of VEGF-A sense primer, antisense primer and probe were 5′-CATCTTCAAGCCATCCTGTGTG-3′, 5′-CATCTCTCCTATGTGCTGGCCT-3′ and 5′-TGCAGATTATGCGGATCAAACCTCACC-3′ (nt 269–290, 395–416 and 367–393 of M32977 respectively). First, to account for differences in the mRNA amounts in the starting materials, the relative β-actin mRNA levels were determined using TaqMan β-actin Control Reagents (Applied Biosystems). VEGF-A expression was then analysed by generating six-point serial standard curves using poly(A)$^+$ RNA from human microvascular EC cultured under 5 % O_2 [25,26]. Reactions were performed in 25 μl volumes using the RNA samples containing the same amount of β-actin mRNA with 2 μM primers, 0.1 μM probe and TaqMan EZ RT–PCR Core Reagent (Applied Biosystems). The thermocycler conditions comprised an initial holding stage at 50 °C for 2 min, 60 °C for 30 min for RT and 95 °C for 10 min, and then a two-step TaqMan PCR programme consisting of 94 °C for 20 s and 61 °C for 2 min for 40 cycles.

ERK phosphorylation

Subconfluent cultures of human microvascular EC were incubated in serum-free Hu-Media MV2 for 2 h at 37 °C. The cells were then exposed for 10 min to glyceraldehyde-derived AGE–BSA at a final concentration of 5 μg/ml in the presence or absence of 25 μg/ml purified esRAGE. After washing with cold PBS containing 1 mM Na_3VO_4, the cells were solubilized with lysis buffer [2 % SDS, 62.5 mM Tris/HCl (pH 6.8), 1 mM Na_3VO_4, 5 mM sodium pyrophosphate, 2 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 10 units/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine, 1 mM EDTA]. Protein concentrations were determined with bicinchoninic acid Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.) using BSA as a standard. The cell lysates (5 μg of protein) were resolved by SDS/PAGE (12.5 % gel) under reducing conditions, and then transferred on to a PVDF membrane. The membrane was treated with anti-phosphoERK antibody, and the immunoreacted bands were visualized with an ECL® detection system (Amersham Pharmacia Biotech). The same membrane was reprobed with anti-ERK antibody.

Cell proliferation assay

ECV304 cells stably transformed with RAGE variant cDNAs or vector alone were seeded at a density of 2×10^3 cells/well in a 96-well plate, and incubated overnight at 37 °C in 0.1 ml of medium 199 supplemented with 10 % FBS. After cell attachment, the culture medium was replaced with 0.1 ml of the fresh medium supplemented with 1 % FBS and 50 μg/ml glyceraldehyde-derived AGE–BSA, and further incubated for 24 h. After incubation, cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method [27].

Assay for cord-like structure formation by ECV-304 cells

The tube formation assay was performed as described previously [28]. Briefly, ECV-304 transformants were seeded at a density of 2.5×10^4 cells/well in a 24-well plate in 0.5 ml of medium 199 supplemented with 10 % FBS, and were grown to confluence. The culture medium was replaced with 0.5 ml of fresh medium supplemented with 50 μg/ml type I collagen (Wako Pure

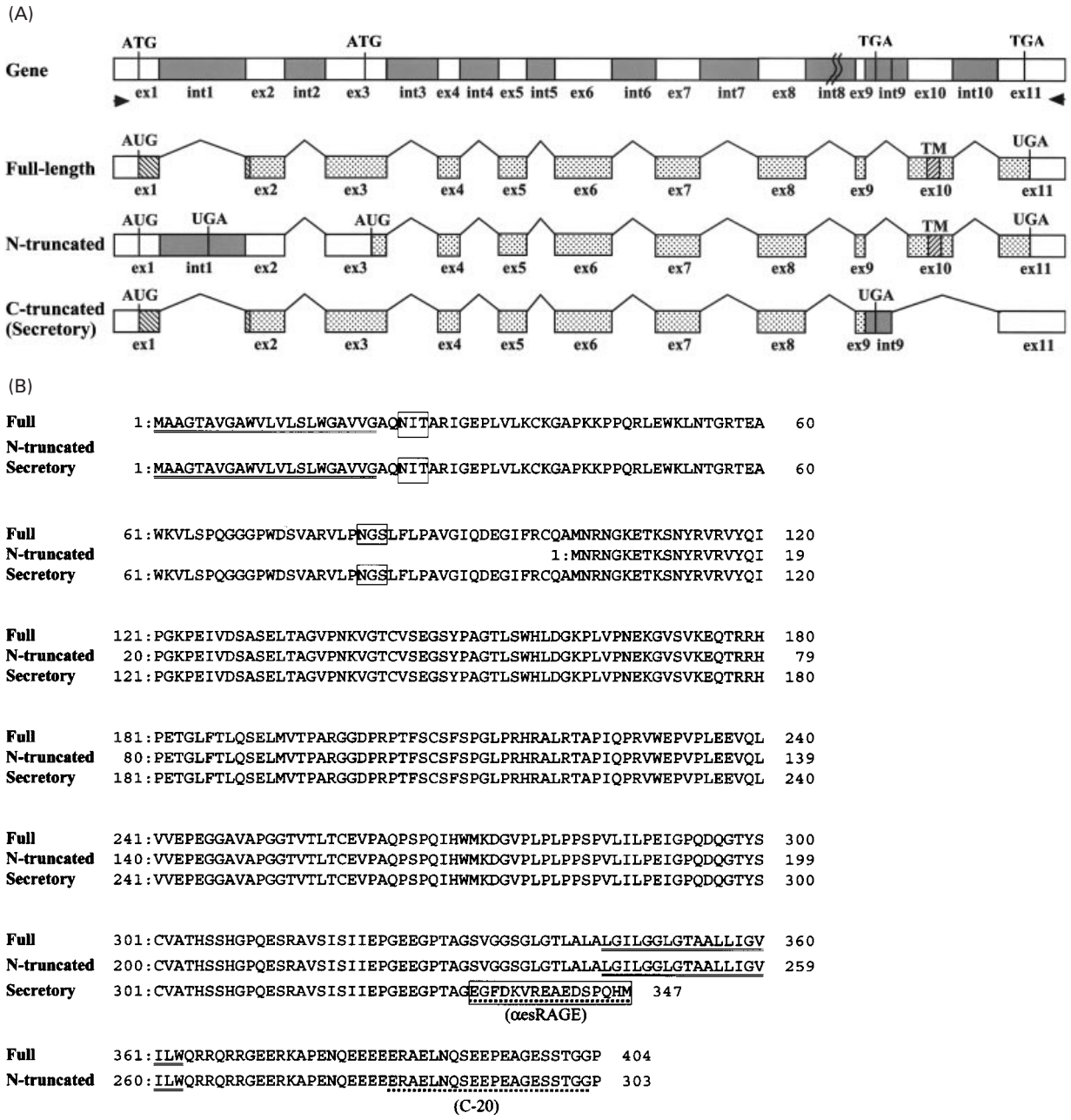

Figure 1 Schematic representation of the RAGE splice variants (A) and alignment of the amino acid sequences of the three RAGE isoforms (B)

(**A**) Open and shaded boxes indicate exons and introns of the human *RAGE* gene [42] respectively. Hatched boxes indicate the putative signal sequence and transmembrane region, and stippled boxes indicate other coding regions of the mRNAs. Arrows indicate the positions of primers used for RT–PCR cloning. (**B**) Amino acid residues are numbered beginning with the first methionine residue. Sequences of the putative signal peptide and transmembrane region are double underlined. Potential N-glycosylation sites and the sequence unique to the secretory C-truncated RAGE are boxed. Peptide sequences used for the preparation of anti-RAGE peptide antibodies are indicated by dotted lines.

Chemicals Industries, Osaka, Japan), and cells were further incubated for 24 h. After incubation, the formation of the network of cord-like structures was assessed under a microscope. In brief, the area (1.2 mm × 0.8 mm, approx. 1 mm^2) of the centre of each well was photographed and the photographs were scanned with a ScanJet 4c/t scanner (Hewlett Packard) on to a Macintosh computer. On the computer, cord-like structures were traced, and then quantification of their lengths was performed using the public domain NIH Image program (developed at the U.S. NIH and available from www.zippy.nimh.nih.gov). To examine the effects of AGE on the formation of the cord-like structures, glyceraldehyde-derived AGE–BSA was added to 50 μg/ml of the culture along with type I collagen.

Cell migration assay

Cell migration was assessed by a monolayer denudation assay as described previously [29]. Briefly, ECV304 cells stably transformed with N-truncated RAGE cDNA or vector alone (2×10^5 cells) were seeded and were grown to confluence in 6-well plates.

Cells were then wounded by denuding a strip of the monolayer approx. 1 mm in width with a 1000 μl pipette tip. Cultures were washed twice with serum-free medium 199 and incubated further in fresh medium supplemented with 2% FBS and 50 μg/ml type I collagen. Cultures were photographed over an 18 h period, and the rate of wound closure was assessed in six separate wells using NIH Image.

RESULTS

Isolation of RAGE splice variants from human microvascular EC and pericytes

To determine the structure of RAGE mRNAs that are actually translated in EC and pericytes, polysomal poly(A)$^+$ RNAs were isolated from these cells and used for RT–PCR cloning of RAGE cDNAs with primers corresponding to the first and last exonic segments. The recombinant plasmids were purified, and the entire region of each insert was sequenced. This screen revealed that EC and pericytes expressed three major RAGE mRNA variants, which were generated by alternative splicing events (Figure 1A). They encoded (1) the full-length RAGE (full-length type), (2) a variant protein lacking the N-terminal region (N-truncated type) and (3) another variant lacking the C-terminal region (C-truncated type). Figure 1(A) shows a schematic representation of the structure of these variants. Figure 1(B) shows the alignment of the amino acid sequences of the three RAGE isoforms. The full-length type mRNA encoded a protein of 404 amino acids with a 22-amino-acid signal sequence and 19-amino-acid transmembrane domain as reported [5]. The N-truncated-type mRNA contained the intron 1 sequence; this resulted in the occurrence of an in-frame stop codon in the intronic sequence, and the second methionine codon in exon 3 appeared to serve as the initiation codon of the largest open reading frame, which would generate a 303-amino-acid protein with the transmembrane domain but without the N-terminal signal sequence and the first immunoglobulin domain (V domain; Figure 1B). For the C-truncated type, the mRNA contained the 5′ part of intron 9 but not the exon 10 sequence that encodes the transmembrane domain (Figure 1A). The persistence of the intron 9 sequence resulted in a frame shift with a stop codon UGA within the 5′ region of the intronic segment, which would give rise to a variant protein having a unique C-terminal 16-amino-acid stretch (Figure 1B). This mRNA species thus coded for a 347-amino-acid protein with the signal sequence but lacking the transmembrane domain, and was therefore expected to be secreted extracellularly.

Relative abundance of RAGE splice variants in EC and pericytes

The number of clones in polysomal poly(A)$^+$ RNA-derived libraries should reflect the relative abundance of each isoform expressed in EC and pericytes. Accordingly, more than 30 independent clones were sequence-determined and classified. As shown in Figure 2, the occurrence of the three variants was similar in EC, but a higher incidence was noted in the C-truncated type (38%) when compared with that in the full-length (31%) and N-truncated (31%) types. In contrast, the full-length type was the predominant form in pericytes (61%) followed by the N-truncated (33%) and then the C-truncated (6%) types.

Expression of cDNA for the RAGE variants in COS-7 cells

To examine whether the N- and C-truncated types of mRNAs actually yield the RAGE protein products as deduced, we constructed expression vectors and transfected them into COS-7 cells. Cell lysates and conditioned media of each transfectant were then analysed by immunoblotting. As shown in Figure 3(A), when a polyclonal antibody against the RAGE (αRAGE-ECD) was employed, the immunoreacted bands were detected in the lysates of the three transfectants but at different positions: approx. 55 kDa in COS-7 cells transfected with the full-length type cDNA; two bands at approx. 50 kDa and approx. 46 kDa in cells transfected with the C-truncated type cDNA; approx. 42 kDa in cells transfected with the N-truncated type cDNA. When the antibody against the cytoplasmic region of human RAGE (C-20) was employed, only the approx. 55 kDa band in the full-length type cDNA transfectant and the approx. 42 kDa band in the N-truncated type cDNA transfectant were marked (Figure 3B). The results indicated that the full-length and N-truncated RAGE proteins had, but the C-truncated form lacked, the cytoplasmic domain. When the antibody against the peptide unique to the C-truncated type RAGE (αesRAGE) was employed, immunoreacted bands were marked only in the C-truncated-type cDNA transfectant at approx. 50 kDa and approx. 46 kDa (Figure 3C), indicating that the C-truncated RAGE-encoding mRNA was translated as deduced.

We next examined culture media (Figures 3D and 3E). In contrast with the cell lysates, only the conditioned medium of the C-truncated type cDNA-transfected cells gave a strong signal immunoreacting with αRAGE-ECD, which migrated to approx. 50 kDa, and a weak immunoreactivity at approx. 46 kDa (Figure 3D). The major (approx. 50 kDa) and minor (approx. 46 kDa) bands were also recognized by αesRAGE (Figure 3E). The results thus indicated that the C-truncated type mRNA actually encodes a soluble, secretory form of RAGE protein (esRAGE).

Further, when human genomic RAGE DNA was forced-expressed in the bovine EC line GEN-T under the control of a cytomegalovirus promoter, the major products in the cell lysates were esRAGE, the full-length type and the N-truncated type, and esRAGE was recovered in the culture media (results not shown).

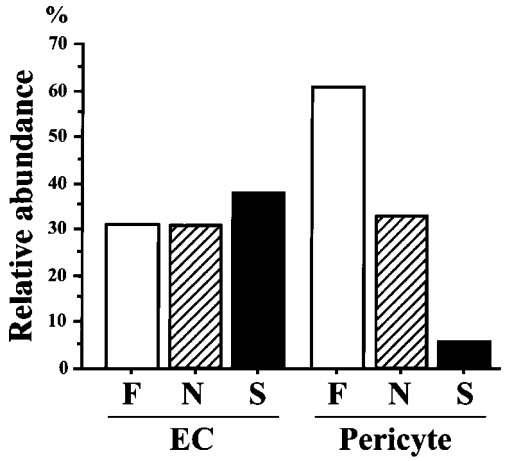

Figure 2 Relative abundance of RAGE splice variants in EC and pericytes

RAGE cDNAs were amplified and cloned from human EC- and pericyte-derived polysomal poly(A)$^+$ RNAs as described in the Experimental section. F, full-length type; N, N-truncated type; S, secretory C-truncated type. The contents are expressed as percentages of the sum of the three variant cDNA clones in each cell type.

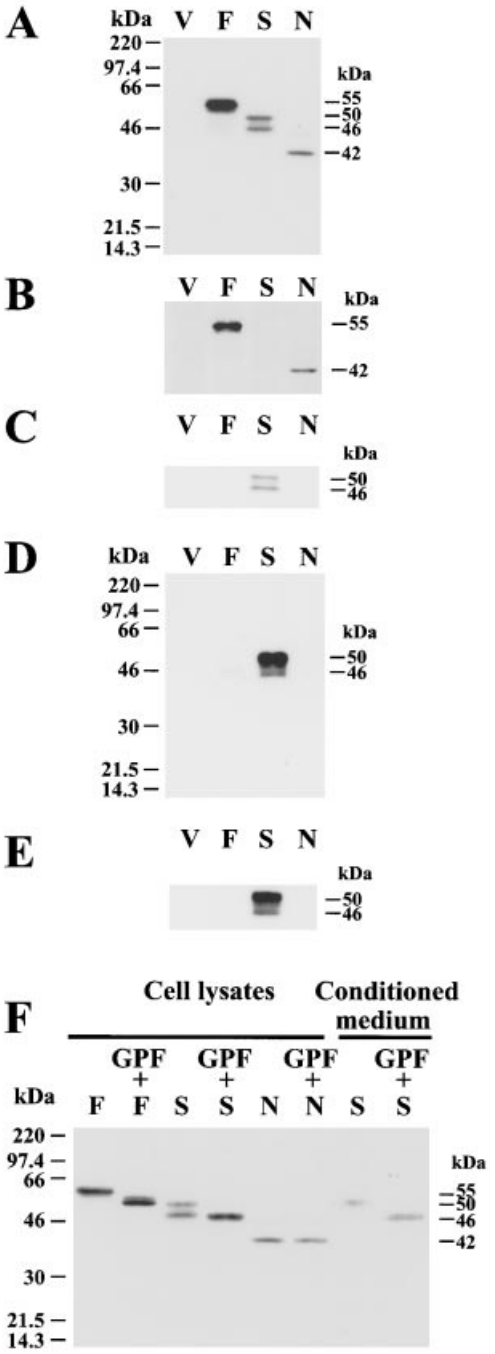

Figure 3 Expression of cDNA for each RAGE variant in COS-7 cells

(**A**) Lysates (25 μg) of COS-7 cells transfected with expression plasmids for the full-length (F), secretory C-truncated (S) or N-truncated (N) type of RAGE proteins or the vector alone (V) were run on SDS/12.5% polyacrylamide gels under reducing conditions, transferred to PVDF membranes, and probed with the antibody against recombinant human RAGE (αRAGE-ECD). (**B**) Western-blot analysis of COS-7 cell lysates using the C-20 antibody against the cytoplasmic domain. (**C**) Western-blot analysis of COS-7 cell lysates with the esRAGE-specific antibody (αesRAGE). (**D**, **E**), Conditioned media (10 μl) of COS-7 cells transfected with expression plasmids for the full-length (F), secretory C-truncated (S), or N-truncated (N) type of RAGE proteins or the vector alone (V) were analysed by immunoblotting with αRAGE-ECD (**D**) and with αesRAGE (**E**). (**F**) Glycopeptidase F digestion of RAGE variant proteins. Left (cell lysates): 5 μg of proteins from the full-length type-expressing COS-7 cells (F), 25 μg of proteins from the esRAGE-expressing cells (S) and 25 μg of proteins from the N-truncated type-expressing cells (N) were treated for 24 h (+GPF) with 0.5, 2.5 and 2.5 m-units of glycopeptidase F respectively or treated with the vehicle alone without the enzyme. Right (conditioned medium): 2 μl of the conditioned medium from the culture of the esRAGE-expressing COS-7 cells (S) was digested for 24 h (+GPF) or not digested with 0.5 m-unit of glycopeptidase F and then analysed by immunoblotting with αRAGE-ECD. Positions of molecular-mass markers (**A**, **D**, **F**) and/or the estimated sizes of immunoreactive bands (**A**–**F**) are shown on the right.

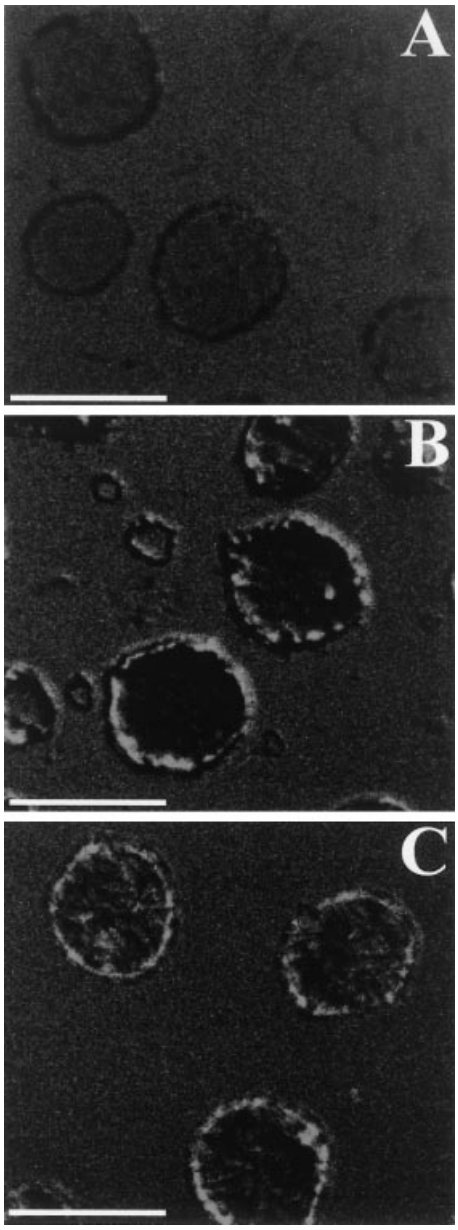

Figure 4 Localization of the N-truncated RAGE

(**A**) COS-7 cells transfected with vector alone, (**B**) HA-tagged full-length-type RAGE cDNA expression vector or (**C**) HA-tagged N-truncated RAGE cDNA expression vector, were stained with the anti-HA antibody and viewed under a confocal laser fluorescence microscope as described in the Experimental section. Scale bar = 20 μm.

Modification of RAGE isoforms with N-linked oligosaccharides

The full-length type RAGE and esRAGE, but not the N-truncated RAGE, had two potential N-glycosylation sites (Figure 1B). We examined whether the first two variants do have this type of modification by employing glycopeptidase F, which

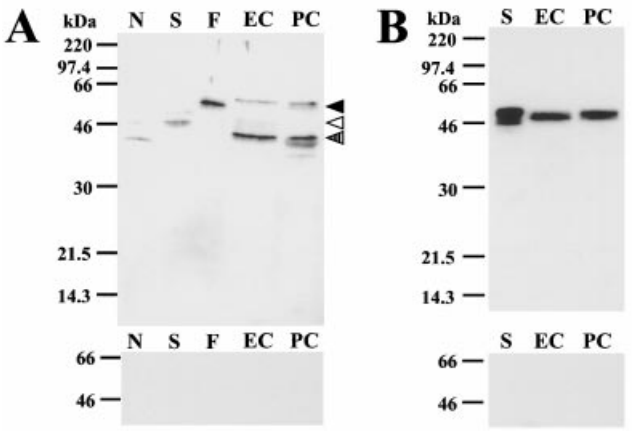

Figure 5 Expression of RAGE variant proteins in primary cultured human microvascular EC and pericytes

Cell extracts or conditioned media of primary cultured human microvascular EC (EC) and pericytes (PC) were applied to the RAGE antibody column, and bound proteins were eluted as described in the Experimental section. (**A**) The eluted fractions, which corresponded to 300 μg of proteins of the cell extracts that had been applied to the column, were subjected to immunoblot analysis with αRAGE-ECD. Lower panel shows the immunoblot of the same samples but without the first antibody. Lysates of COS-7 cells that had been transformed with expression plasmids for the full-length (F; 0.5 μg), secretory C-truncated (S; 2 μg) or N-truncated (N; 2 μg) RAGE proteins were also run on the gel as positive controls. Immunoreacting bands are indicated by triangles: the approx. 55 kDa (▲), approx. 46 kDa (△) and approx. 42 kDa (hatched triangle) bands would correspond to the full-length, secretory C-truncated and N-truncated RAGE proteins respectively. (**B**) The eluted fractions, which corresponded to 5 ml of the conditioned media that had been applied, were subjected to immunoblot analysis using αesRAGE. Lower panel shows the immunoblot of the same samples but without the first antibody. Conditioned medium of esRAGE cDNA-transfected COS-7 cells (2 μl) was loaded as a positive control (S). Positions to which molecular-mass markers migrated are shown on the left.

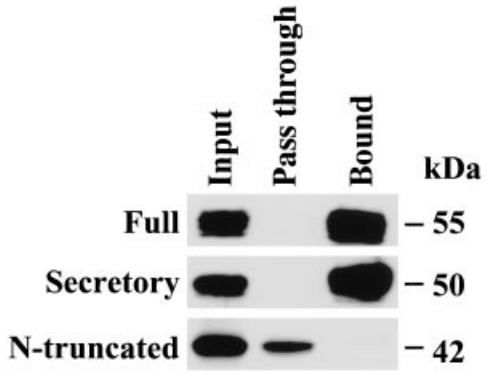

Figure 6 AGE binding of RAGE variant proteins

Similar amounts of the full-length (Full), N-truncated (N-truncated) and secretory C-truncated (Secretory) types of RAGE proteins expressed in COS-7 cells were applied on to the column to which glyceraldehyde-derived AGE–BSA was immobilized. The column was then washed with 10 bed volumes of the equilibration buffer, and bound proteins were eluted with the buffer containing 2 M NaCl. The same volume of the applied samples (Input), pass-through fractions (Pass through) and eluted fractions (Bound) was subjected to immunoblot analysis using 13F11 monoclonal anti-pan-RAGE antibody. The pass-through fractions became diluted about 2-fold during the passage through the column. Estimated sizes of the immunoreacting bands are shown on the right.

specifically cleaves off sugar chains attached to asparagine residues [21]. As shown in Figure 3(F), when the full RAGE was treated with glycopeptidase F, the approx. 55 kDa band disappeared and a new band appeared at approx. 50 kDa, indicating that the approx. 55 kDa full RAGE was actually modified with N-linked oligosaccharides. When the lysate of esRAGE cDNA-transfected cells was treated with the enzyme, the approx. 50 kDa band disappeared and the approx. 46 kDa band increased, indicating that the approx. 50 kDa and approx. 46 kDa esRAGE proteins were the N-glycosylated and non-glycosylated forms respectively. In contrast, the approx. 42 kDa N-truncated RAGE protein was not affected by glycopeptidase F, consistent with the fact that the sequence of this type has no N-linked glycosylation site. When the secreted esRAGE was treated with glycopeptidase F, the approx. 50 kDa band disappeared and the digests shifted to the position of approx. 46 kDa.

Cellular localization of the N-truncated RAGE

An algorithm by Tusnady and Simon [30] indicated that, even devoid of the N-terminal signal peptide, the deduced N-truncated RAGE protein should belong to type 1 membrane proteins. To address the issue of whether the N-truncated RAGE protein is transported to and localized on the plasma membrane, we examined the localization of C-terminally HA-tagged N-truncated or full-length RAGE proteins. No signal was detected in cells transfected with the vector alone (Figure 4A). On the other hand, clear immunoreactive signals were marked on the plasma membrane of cells expressing the N-truncated RAGE (Figure 4C) as well as that of cells expressing the full RAGE (Figure 4B). A weak signal was also seen in the cytoplasm of the N-truncated RAGE-expressing cells. The results indicated that the N-truncated RAGE resided mainly on the plasma membrane, as did the full-length RAGE.

Expression of RAGE variant proteins in human microvascular EC and pericytes

We next examined whether the three RAGE variant proteins were expressed in primary cultured human microvascular EC and pericytes. As shown in Figure 5(A), two major immunoreacting bands at approx. 55 kDa and approx. 42 kDa, and a faint approx. 46 kDa band were marked in EC and pericyte extracts. The three immunoreacting bands would correspond to the full RAGE, N-truncated RAGE and non-glycosylated esRAGE respectively. The results thus indicated that the three RAGE variant proteins were actually produced in EC and pericytes. As shown in Figure 5(B), conditioned media from EC and pericyte cultures gave bands that immunoreacted with αesRAGE at approx. 48 kDa and approx. 49 kDa respectively, whereas the conditioned medium of esRAGE cDNA-transfected COS-7 cells gave the major approx. 50 kDa glycosylated and minor 46 kDa non-glycosylated forms (see Figure 3). The results thus indicated that not only EC but also pericytes synthesize esRAGE and secrete it extracellularly, and that the extent of N-glycosylation may differ among cells.

AGE-binding ability of the RAGE variants

We next examined the ability of the three RAGE variant proteins to bind the AGE ligand by affinity chromatography. Partially purified RAGE variant proteins were applied to a column on which AGE was immobilized. As shown in Figure 6, esRAGE

bound to the AGE column and was eluted with high salts, as was the full-length RAGE. In contrast, the N-truncated RAGE did not bind to the column and was consistently recovered in the pass-through fraction. The results indicated that the site to engage the AGE ligand resides in the N-terminal V domain, which esRAGE and the full-length RAGE proteins possess, but the N-truncated RAGE lacks.

esRAGE neutralizes the AGE induction of *VEGF* expression in EC

esRAGE was proved to be capable of binding AGE (Figure 6). Thus the endogenous secretory receptor discovered in the present study was considered to have the potential to block the action of AGE on vascular cells. This issue was addressed by testing the effect of esRAGE on the AGE-induced expression of the *VEGF* gene by EC, one of the most outstanding cellular responses to AGE–RAGE interactions [9,15]. For this, we purified esRAGE proteins to homogeneity (Figure 7A). Human microvascular ECs were then exposed to AGE in the presence or absence of purified esRAGE. After the treatment, poly(A)$^+$ RNAs were isolated and analysed by semi-quantitative RT–PCR (Figure 7B) and by quantitative real-time RT–PCR (Figure 7C) using primers specific to human VEGF mRNA. As shown in Figures 7(B) and 7(C), the exposure to AGE increased the EC levels of VEGF mRNA, and this induction was completely abolished by esRAGE.

AGE has been reported to stimulate VEGF expression though an ERK-dependent pathway [31]. We thus examined the effect of esRAGE on the AGE induction of ERK phosphorylation. Human microvascular ECs were exposed to AGE in the presence or absence of esRAGE, and whole cell lysates were analysed by Western blotting using an antibody to phosphorylated forms of ERK1/2. As shown in Figure 7(D), AGE induced ERK1/2 phosphorylation, and this induction was also completely abolished by esRAGE.

Biological functions of the RAGE splice variant proteins in EC

We next looked into the biological functions of the RAGE variant proteins in EC. For this, we established ECV304 sublines which overexpress each variant protein. Western-blot analysis confirmed the overexpression of each RAGE variant protein in these sublines and the secretion of esRAGE only from the C-truncated RAGE cDNA-transfected cells (Figure 8A). RAGE-variant-overexpressing ECV304 cells were morphologically invariant from the parental ECV cells and grew at a similar rate without loss of viability (results not shown).

First, we examined the effects of the RAGE variants on the AGE-induced stimulation of ECV cell proliferation. Cells were grown in the presence or absence of AGE and underwent the MTT assay. As shown in Figure 8(B), AGE significantly stimulated the growth of vector-, full RAGE cDNA- and N-truncated RAGE cDNA-transfected ECV304 cells; the extent of the growth stimulation appeared to be larger in the full RAGE-overexpressing cells (117%) than in the other sublines (114%, vector; 111%, N-truncated), although there was no statistically significant difference among the three (Figure 8B). On the other hand, AGE failed to stimulate the growth of esRAGE cDNA-overexpressing ECV304 cells.

ECV304 cells have been reported to assemble cord-like structures when their confluent cultures are stimulated by type I collagen, and this has been regarded as a hallmark of the EC phenotype [28]. Accordingly, we next examined the effect of each

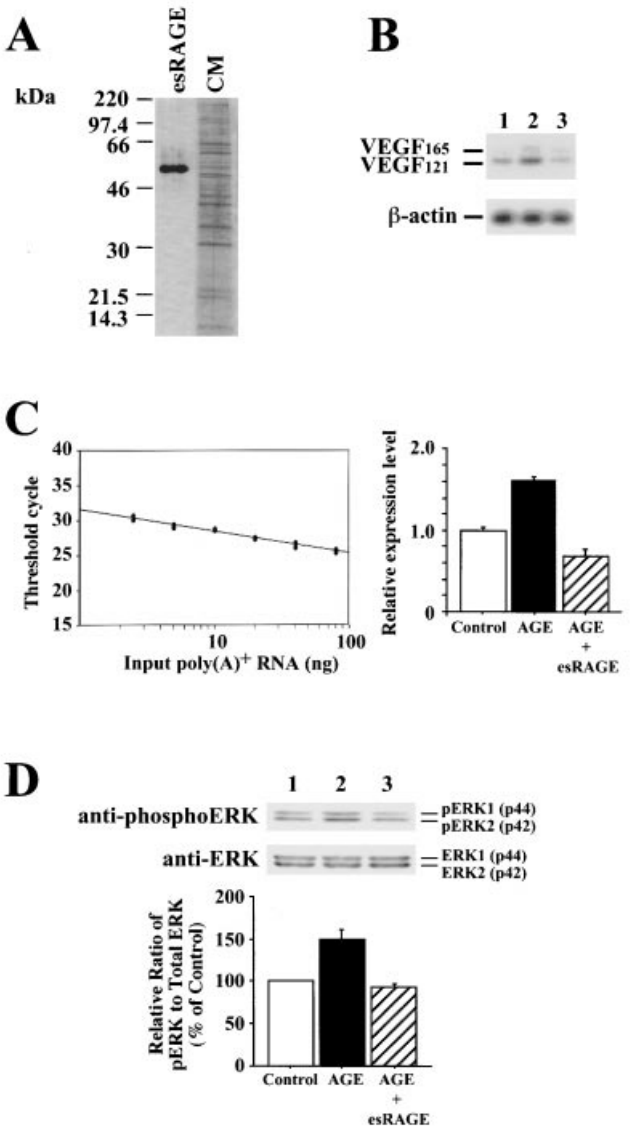

Figure 7 Effect of esRAGE on AGE-induced VEGF expression

(**A**) Characterization of purified esRAGE. The purified esRAGE protein (esRAGE; 500 ng) and the conditioned medium of COS-7 cells overexpressing esRAGE (1 μg as proteins) (CM) were run on SDS/12.5% polyacrylamide gels under reducing conditions and silver-stained. Positions of molecular-mass markers are indicated on the left. (**B**) Semiquantitative RT–PCR analysis of VEGF mRNA in AGE and esRAGE-treated EC. Human dermal microvascular EC were incubated with 10 μg/ml glyceraldehyde-derived AGE–BSA in the presence or absence of 25 μg/ml esRAGE or without additives. Poly(A)$^+$ RNA was then isolated and analysed by RT–PCR as described in the Experimental section. Lane 1, control without additives; lane 2, AGE; lane 3, AGE + esRAGE. (**C**) Quantification of the VEGF mRNA levels by real-time RT–PCR. Real-time RT–PCR was performed as described in the Experimental section. All reactions were performed in triplicate. Left panel: the relative standard curve for the amplification of VEGF mRNA. Measured threshold cycle values (i.e. the cycle number at which the measured fluorescence emission that reflects the amount of amplified products reaches an arbitrary threshold value; *y*-axis) were plotted against input poly(A)$^+$ RNA amount (ng, *x*-axis). Note that the *x*-axis is a logarithmic scale. Right panel: the expression level is indicated relative to the expression level in control cells. Columns and bars indicate the means and S.E. respectively ($n = 3$). (**D**) ERK phosphorylation in AGE and esRAGE-treated EC. Human dermal microvascular EC were incubated with 5 μg/ml glyceraldehyde-derived AGE–BSA in the presence or absence of 25 μg/ml esRAGE or without additives for 10 min. Cell lysates were analysed by Western blotting with anti-phosphoERK and anti-ERK antibodies as described in the Experimental section. Upper panel: typical results of the Western-blot analyses. Lane 1, control without additives; lane 2, AGE; lane 3, AGE + esRAGE. Lower panel: densitometric analyses. Intensities of the phosphoERK signals were normalized with those of total ERK signals, and are related to the value of the control. Columns and bars indicate the means and S.E. respectively ($n = 3$).

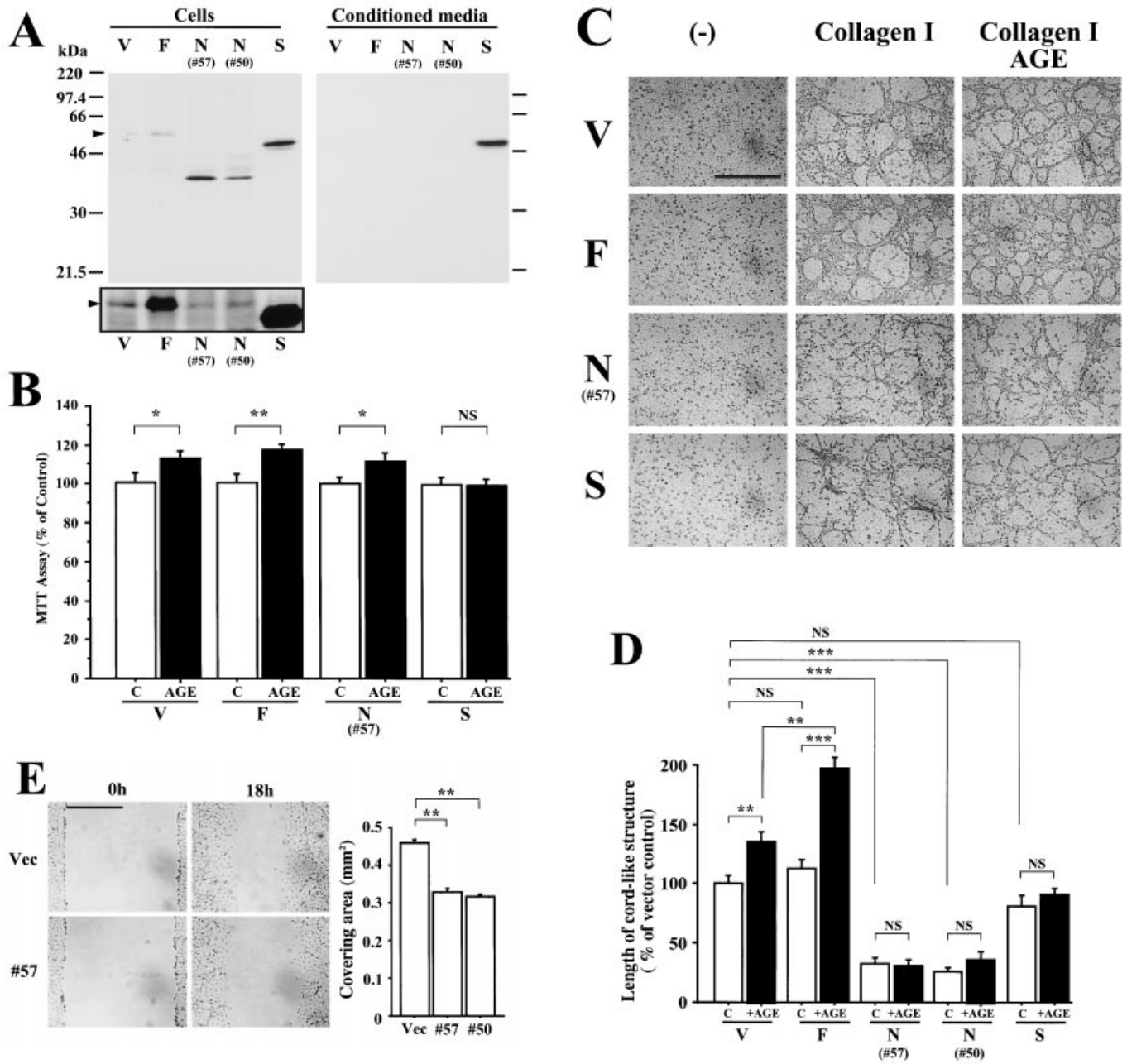

Figure 8 Effects of overexpression of RAGE variant proteins on growth and cord-like structure formation of ECV304 cells

(**A**) Western-blot analysis of ECV cells overexpressing RAGE variants. Left panel: lysates of cells stably transformed by expression plasmids for the full-length (F; 80 μg), N-truncated (N; 80 μg), or secretory C-truncated (S; 20 μg) type of RAGE proteins or by the vector alone (V; 80 μg) were run on an SDS/12.5% polyacrylamide gel, transferred to PVDF membrane, and probed with αRAGE-ECD. Inset: longer exposure of the same blot, by which endogenous full-length-type RAGE was detected at the position indicated by closed triangle. Right panel: conditioned media (5 μl) from ECV cells overexpressing the full-length (F), N-truncated (N), or secretory C-truncated (S)-type of RAGE proteins or from the vector-transfected cells (V) were run on an SDS/12.5% polyacrylamide gel, transferred to PVDF membrane, and probed with αesRAGE. #57 and #50 indicate independent ECV304 clones overexpressing the N-truncated type of RAGE protein. (**B**) Effect of RAGE variants on AGE-induced ECV cell growth. Cell proliferation was assessed by the MTT method as described in the Experimental section. Columns and bars indicate the means and S.E. respectively ($n = 10$). $^{*}P < 0.05$; $^{**}P < 0.01$ (versus control); NS, not significant. Essentially the same results were obtained by three independent experiments. (**C**, **D**) Effects of RAGE variants on the formation of the collagen-induced cord-like structure formation by ECV304 cells. (**C**) Confluent monolayer cultures of ECV304 cells stably transformed by expression plasmids for the full-length (F), secretory C-truncated (S) or N-truncated (N) type of RAGE proteins or by the vector alone (V) were incubated in the absence (—) or presence (Collagen I) of 50 μg/ml type I collagen for 24 h to induce the cord formation. To examine AGE effects on the cord-like structure formation, glyceraldehyde-derived AGE–BSA was added to the confluent culture at a concentration of 50 μg/ml (AGE) in addition to type I collagen. Scale bar = 0.5 mm. (**D**) Graph indicates the total length of cord-like structures. The length of the cord-like structures was quantified with NIH Image, and related to the control value with the vector-transfected ECV304 cells. The mean length of the tubes in the control vector-transfected cells was 2.59 ± 0.18 mm/mm^2. Columns and bars indicate means and S.D. respectively ($n = 4$). $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant. Essentially the same results were obtained by four independent experiments. (**E**) Effect of N-truncated RAGE on EC migration. Left: ECV cells stably transformed with N-truncated cDNA (#57) or vector alone (Vec) were plated in 6-well plates and cell migration was quantified by the scratch method after 18 h as described in the Experimental section. Scale bar = 0.5 mm. Right: graph indicates the areas that were covered with migrating cells. The areas were quantified with NIH Image. Columns and bars indicate means and S.D. respectively ($n = 6$). $^{**}P < 0.01$. #57 and #50 indicate independent ECV304 clones overexpressing the N-truncated RAGE protein. Essentially the same results were obtained by three independent experiments.

RAGE variant. As shown in Figure 8(C), ECV304 cells overexpressing full RAGE as well as vector-transfected cells actively formed cord-like structures when stimulated with type I collagen. The collagen-dependent cord formation of esRAGE-overexpressing cells appeared to be less active and the resultant cords looked thinner than those formed by full RAGE cDNA- and vector-transfected ECV304 cells (Figure 8C), although the total length of the cord-like structures was not statistically significantly different from the control (Figure 8D). AGE stimulated the cord formation in vector- and full RAGE cDNA-transfected cells, but not in esRAGE-overexpressing cells (Figures 8C and 8D). The cord formation of the ECV304 line overexpressing N-truncated RAGE (#57) was dramatically prevented both under basal conditions and in the presence of AGE (Figures 8C and 8D). Essentially, the same results were obtained with another N-truncated RAGE-overexpressing subline, #50 (Figure 8D). Thus N-truncated RAGE appeared to inhibit tube formation, but without inhibiting EC growth (Figure 8B). Therefore we then assessed the effect of N-truncated RAGE on EC migration, on which tube formation is thought to depend, by employing a denudation injury model [29]. Confluent scrape-wounded monolayers of ECV304 cells stably transformed with N-truncated RAGE cDNA or vector alone were incubated for 24 h, and the closure rate was estimated (Figure 8E). The migration of cells overexpressing N-truncated RAGE (#50 and #57) into the wounded area was significantly retarded compared with that of vector-transfected cells.

DISCUSSION

RAGE was first isolated as an AGE-binding protein [4,5]. In addition to AGE, endogenous RAGE ligands have been identified previously, including amphoterin [32], EN-RAGE [6] and Alzheimer amyloid β-proteins [33]. The interaction between amphoterin and RAGE has been suggested to participate in the network formation of cerebral cortex neurons [32]. The binding of EN-RAGE to RAGE appears to mediate pro-inflammatory reactions [6]. Such endogenous RAGE ligands probably have evolved to regulate various physiological processes. Our earlier studies [9–11,34] and by others [35–37] have shown that interactions between AGE and RAGE cause phenotypic changes in microvascular EC, pericytes and renal mesangial cells which are characteristic of diabetic vasculopathy. Obviously, diabetes abuses the molecular devices for the RAGE signalling pathway primarily evolved for other physiological processes, leading to the development and progression of diabetic complications. Further, AGE–RAGE interactions are related not only to diabetic retinopathy and nephropathy but also to diabetic macroangiopathies [38,39]. Therefore it is important both biologically and medically to clarify the nature of RAGE proteins in each cell type involved.

In the present study, we have determined the structures of RAGE mRNAs expressed in microvascular EC and pericytes, the very cell types whose derangement gives rise to diabetic vasculopathy, and demonstrated the presence of novel RAGE mRNA splice variants coding for C- (endogenous secretory) and N-truncated forms of RAGE proteins (Figures 1 and 2).

The mRNA for the C-truncated type contained the 5′ part of intron 9, and encoded the soluble, secretory form of the receptor protein (esRAGE) that has 347 amino acids with a 22-amino-acid signal sequence and a unique 16-amino-acid stretch (Figure 1). Transfection experiments demonstrated that this variant mRNA yielded an N-glycosylated approx. 50 kDa esRAGE and an unmodified approx. 46 kDa esRAGE (Figure 3). Both forms were detected in the lysates of esRAGE-expressing COS-7 cells, but the former N-glycosylated form predominated in the media (Figure 3). This suggests that the latter approx. 46 kDa protein species represented newly synthesized esRAGE that has not yet received the modification at the Golgi apparatus, and that most of the esRAGE was secreted after the full conversion into the approx. 50 kDa protein species. We also demonstrated that esRAGE is actually produced by human microvascular EC and pericytes, and liberated extracellularly (Figure 5). The results suggest the possibility that the endogenous secretory receptor is present in the circulating blood and extracellular fluids in the vascular walls of our bodies. Actually, we have detected the 48–50 kDa esRAGE in human sera, and found that the levels varied among individuals (S. Sakurai, Y. Yamamoto, H. Yonekura, T. Watanabe, R. G. Petrova, Md. J. Abedin, K. Yasui, H. Li, H. Tamei, K. Obata and H. Yamamoto, unpublished work).

Malherbe et al. [40] isolated from a human lung cDNA library a clone encoding another type of secretory RAGE as a minor RAGE species. In this case, intron 2 was excised at an alternative 3′ splice site within exon 3, resulting in a deletion of 42 nucleotides from exon 3. Intron 7 was also excised at an alternative 5′ splice site within this intron, resulting in a 29-nucleotides extension of exon 7, a total skipping of exon 8 and a connection of the altered exon 7 to exon 9; this caused a frame shift different from the present one, thereby yielding a soluble protein with a significantly altered C-terminal domain. Transfection experiments showed that this type of secretory RAGE protein was unstable and partially degraded in the culture medium [40]. In the present study, we did not encounter the same variant in the screening of EC- or pericyte-derived RAGE cDNA. The newly discovered esRAGE was found to be quite stable in the culture medium (Figures 3, 5 and 8A).

It has been reported that the ECD of RAGE proteolytically released from cells or soluble RAGE artificially produced by recombinant gene technology can bind AGE and prevent the progression of diabetic vasculopathy and atherosclerosis in experimental animals [35,36,38]. The esRAGE discovered in the present study binds to an AGE ligand (Figure 6) and has an activity that neutralizes the AGE action (Figures 7 and 8). Therefore the production of esRAGE by vascular cells themselves would be physiologically significant, since esRAGE in serum and/or the vasculature would capture AGE and would protect vascular cells against the activation of the cell-surface receptors, which would otherwise lead to vascular injury. Further, ligand engagement of the cell-surface RAGE is known to activate NF-κB, which in turn enhances the expression of its own gene, thus forming a positive loop of regulation [15]. The present study has suggested the presence of a negative-feedback mechanism in which esRAGE can serve to prevent the activation of the post-RAGE signalling.

Another new RAGE mRNA variant isolated in the present study has the largest open reading frame coding for a protein of 303 amino acids and has the transmembrane domain, but lacks the N-terminal signal sequence and 'V'-type immunoglobulin domain (Figure 1). The transfection experiment revealed that the mRNA directed the synthesis of the protein as predicted; it yielded an approx. 42 kDa protein without N-linked oligosaccharides (Figure 3), which was localized mainly on the plasma membrane as was the full-length type RAGE (Figure 4). Because the V domain of RAGE has been shown to be the binding site for AGE ligands [3,41], the N-truncated RAGE was considered not to bind AGE. This also was found to be true; the N-truncated RAGE failed to bind to an AGE-conjugated affinity column to which both the full-length RAGE and esRAGE efficiently bound

(Figure 6). Overexpression of N-truncated RAGE in ECV304 cells did not affect the growth stimulation by AGE, which probably was mediated by endogenous full-type RAGE (Figure 8B), but prevented their cord-like structure formation regardless of the presence or absence of AGE (Figures 8C and 8D). Overexpression of N-truncated RAGE significantly reduced the cell migration compared with those of the vector-transfected cells (Figure 8E). From these results, the N-truncated RAGE protein may have a new role in the regulation of angiogenesis, at least in part, by regulating EC migration, which may be independent of the AGE signalling pathway. It has been reported that RAGE regulates cytoskeleton organization though activation of Cdc42 and/or Rac in neuronal cells [7].

The relative abundance of the three RAGE mRNA variants was different between EC and pericytes (Figure 2). We have shown previously that the engagement of RAGE by AGE causes a decrease in retinal pericytes [11], whereas it causes an increase of EC [9,33]. The difference in the relative abundance of the RAGE variants in these cells could be a cause for the different responses to AGE. Further, preliminary RT–PCR cloning revealed that the contents of the three RAGE isoforms vary among cells and tissues (results not shown). The significance of this difference remains to be determined. The levels of RAGE variant expressions may also vary among individuals and/or conditions. We assume that such diversity could be a factor that endows diabetic patients with different susceptibility or resistance to the development of diabetic vascular complications. We are obtaining results suggesting the possibility that diabetic patients with higher serum esRAGE levels are more resistant to AGE than those having lower esRAGE levels (S. Sakurai, Y. Yamamoto, H. Yonekura, T. Watanabe, R. G. Petrova, Md. J. Abedin, K. Yasui, H. Li, H. Tamei, K. Obata and H. Yamamoto, unpublished work).

In conclusion, the present study has unveiled the molecular heterogeneity of the multiligand receptor-RAGE. The novel RAGE variants can modify ligand actions and receptor engagement on the cell surface, and can cause different post-receptor signalling events and subsequent cellular responses. Although more studies are needed to clarify better the significance of the co-expression of full type RAGE and the antagonistic RAGE variants in microvascular cells, the present findings have revealed new regulatory features in the expression and function of RAGE, which may provide new clues for clarifying the pathogenesis of diabetic vascular complications and other RAGE-related diseases, and for developing preventive measures against them.

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