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Wound healing activity and mechanism of action of antimicrobial and lipopolysaccharide-neutralizing peptides from enzymatic hydrolysates of rice bran proteins

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In our previous study, we identified multifunctional cationic peptides from enzymatic hydrolysates of rice bran proteins (RBPs) that have antimicrobial and lipopolysaccharide-neutralizing activities. In this study, we investigated the potential of the peptides RBP-LRR, RBP-EKL, and RBP-SSF to promote proliferation, angiogenesis (tube formation), and migration in human umbilical vein endothelial cells (HUVECs). To determine mechanisms of wound healing actions, angiogenic and migration-promoting activities of these peptides were evaluated following pretreatments of HUVECs with specific inhibitors. In these experiments, the cationic peptides RBP-LRR, RBP-EKL, and RBP-SSF induced cell proliferation at low concentrations of 0.1 μM or 1 μM. Moreover, the three cationic peptides had angiogenic activities at concentrations more than 1 μM in tube formation assays, and their effects were similar to those of LL-37. Subsequent scratch migration assays exhibited that RBP-LRR, RBP-EKL, and RBP-SSF promote wound closure at optimum concentrations of 10, 10, and 0.1 μM, respectively. In further studies, we performed tube formation assays using HUVECs pretreated with SU5416, which inhibits vascular endothelial growth factor (VEGF) receptors, and suggested the possibility that the three cationic peptides induce angiogenesis by activating VEGF receptors. In corresponding scratch migration assays using HUVECs, pretreatment with the proliferation inhibitor mitomycin C did not alter the effects of RBP-LRR and RBP-EKL, and significant contribution to wound closure were mediated by cell migration regardless of proliferation rates. In contrast, RBP-SSF contributed to wound closure exclusively by promoting cell proliferation. The present data indicate that RBP-LRR, RBP-EKL, and RBP-SSF are candidates for use as wound healing agents.

[**Key words:** Angiogenic peptide; Cell migration-promoting peptide; Proliferation-promoting activity; Wound healing activity; Multifunctional peptide]

Among bioactive peptides, cationic peptides of 12–50 amino acid residues exhibit multifunctional effects, and have high isoelectric point (pI) values and amphipathic structures comprising approximately 50% hydrophobic residues (e.g., tryptophan, phenylalanine, and leucine) (1–5). Multifunctional cationic peptides are superior to other peptides that specifically promote health and/or have activities that can be exploited as treatments for diseases because they protect against infection and have additional anti-inflammatory and immunomodulatory activities (3–5). In humans, cathelicidin LL-37, which is derived from the C-terminal 37 amino acid residues of human cationic antimicrobial peptide 18 (hCAP-18), has antimicrobial activity against pathogens at relative high concentrations (10–50 μM) (6,7) and plays central anti-inflammatory roles in innate immune responses. LL-37 also contributes to cell migration, angiogenesis, cytokine induction, and apoptosis in various cell types at low concentrations (0.1–10 μM) (8,9). Human beta-defensins (hBDs) are another family of cationic antimicrobial peptides (AMPs), and are characterized by six cysteine residues with three intramolecular disulfide bridges (10,11). hBD-1 has demonstrated antimicrobial activity against laboratory and clinical strains of *Escherichia coli* at a concentration of 30 μM (12). Recent studies also show biological activities of hBDs that are unrelated to antimicrobial activities. In particular, hBD-2 stimulates proliferation, *in vitro* migration, and angiogenesis (capillary-like tube formation) of human umbilical vein endothelial cells (HUVECs) at a concentration of 0.12 μM (13).

Considering the high concentrations of cationic peptides that are necessary for antimicrobial effects (6,7,12), wound healing properties such as promotion of cell migration, and angiogenic and immunomodulatory activities of the cationic peptides LL-37 and hBDs may represent greater contributions to host defenses than their antimicrobial activities. Wound healing and the associated tissue remodeling follow cell migration, angiogenesis, and the formation of new extracellular matrices and blood vessels, requiring collaborative activities of multiple tissues and cell lineages (14,15).

Previously, we identified three peptides in enzymatic hydrolysates of rice bran proteins (RBPs) and five peptides in enzymatic hydrolysates of rice endosperm proteins as novel multifunctional peptides with cationic and hydrophobic amino acids (16,17). The three cationic peptides from enzymatic hydrolysates of RBPs LRRHASEGGHGPHW (RBP-LRR), EKLLGKQDKGVIIRA (RBP-EKL), and

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SSFSKGVQRAAF (RBP-SSF) were superior to the five cationic peptides from enzymatic hydrolysates of rice endosperm proteins, as indicated by higher antimicrobial and lipopolysaccharide (LPS)-neutralizing activities (16,17). In particular, RBP-LRR inhibited the growth of *Candida albicans*, an opportunistic fungal pathogen, with a 50% growth-inhibitory concentration (IC_{50}) of 289 μM. RBP-EKL and RBP-SSF also exhibited antimicrobial activity against *Porphyromonas gingivalis*, a major etiological agent in the onset and progression of chronic periodontitis, with IC_{50} values of 75.6 and 78.5 μM, respectively (16). In addition, these three cationic peptides had little or no hemolytic activity at significantly antimicrobial concentrations (16). To further investigate the functions of these three cationic peptides, we examined their abilities to neutralize the endotoxin LPS using *Limulus* amebocyte lysate assay kits. In these experiments, the endotoxic activity of LPS was abolished in a concentration-dependent manner, with 50% effective (50% neutralizing) concentrations (EC_{50}) of 0.86–1.41 μM (16). Moreover, these cationic peptides were angiogenic in tube formation assays using Matrigel, with significant increases in tube formation by HUVECs in the presence of peptides at 1 and 10 μM (16).

Similar to LL-37 and hBDs, the present multifunctional cationic peptides from enzymatic hydrolysates of RBPs have promise as agents that promote health and have efficacy in the treatment of diseases, particularly in the prevention of endotoxin shock and sepsis during gram-negative bacterial infections. Herein, we further characterized wound healing functions of RBP-LRR, RBP-EKL, and RBP-SSF as adjuncts to endogenous bioactive peptides, and demonstrated their effects on proliferation, angiogenesis (tube-like structures formation), and migration in HUVECs. To determine mechanisms of action, we investigated the effects of the three cationic peptides on angiogenesis and cell migration in assessments of wound closure in the absence and presence of specific inhibitors.

MATERIALS AND METHODS

Peptides and inhibitors Chemically synthesized RBP-LRR, RBP-EKL, RBP-SSF, and LL-37 were purchased from Eurofins Genomics Co. Ltd. (Tokyo, Japan). Synthetic peptides were purified to >95% purity using reversed-phase high-performance liquid chromatography and their molecular weights were confirmed using matrix-assisted laser/desorption ionization–time-of-flight mass spectroscopy. SU5416 (Sigma–Aldrich Co., St. Louis, MO, USA) was used as a vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitor (18,19). Mitomycin C was used as an inhibitor of proliferation (19,20) and was purchased from Wako Pure Chemicals Ltd., Osaka, Japan.

Cell proliferation assays HUVECs (Kurabo Industries, Osaka, Japan) were cultured in HuMedia-EG2 (Kurabo Industries) and were incubated at 30°C in a humidified atmosphere containing 5% CO_2. After reaching 90%–95% confluence, cells were harvested and counted using a hemocytometer as described previously (16,17,21) with a slight modification. Briefly, HUVECs were seeded at 1×10^4 cells/mL in HuMedia-EG2 containing cationic peptides at varying concentrations, and were then cultured for 72 h. Cell viability was determined every 24 h using Cell-Counting Kit-8 (Dojindo Co., Kumamoto, Japan) and a microplate reader (2030 ARVO X3; PerkinElmer, Waltham, MA, USA) at 450 nm as described by the manufacturer (22).

Tube formation assays HUVECs were cultured, harvested, and resuspended in HuMedia-EG2 and tube formation assays were performed using Matrigel (Becton Dickinson and Company, Santa Clara, CA, USA) as described by the manufacturer (16,17,21,23). Briefly, solid gels were prepared on 96-well plates and HUVECs in HuMedia-EG2 containing various concentrations of cationic peptide were seeded at 2×10^5 cells/mL onto solid Matrigel surfaces. After 15-h incubation, tube formation was observed at 40 × magnification using an inverted light microscope (TS100F, Nikon Instruments Inc., Tokyo, Japan) and random phase contrast images in each of five wells were procured using a digital camera (Nikon Instruments Inc.) as described previously (16,17,21). Tube-like structures were then analyzed using NIS-Elements BR Analysis software (Nikon Instruments Inc.) and average tube lengths per field were calculated. Relative tube lengths in the presence of peptides were expressed as percentages of those (100%) in controls without peptides. LL-37 was used as a positive control. In separate experiments, HUVEC suspensions were pretreated with the VEGF inhibitor SU5416 at 0.1 μM for 1 h and the cells were then seeded onto culture plates (21).

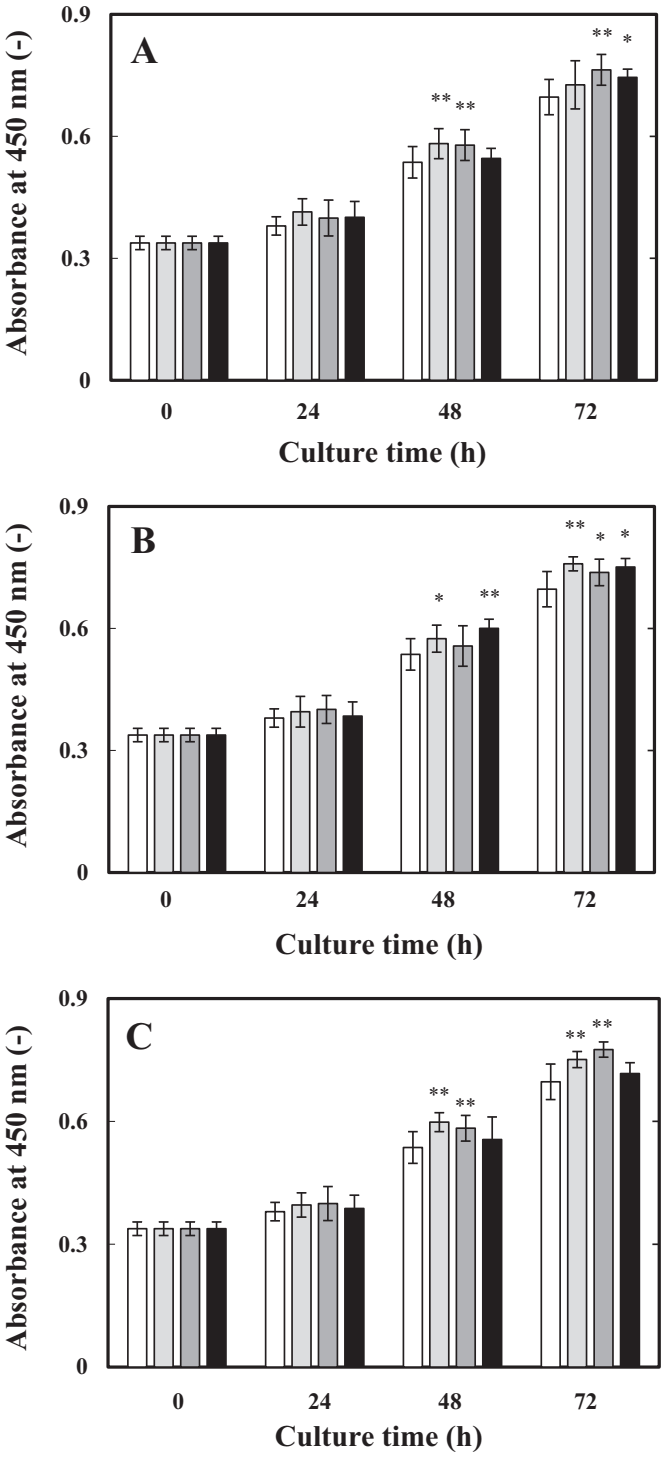

FIG. 1. Effects of RBP-LRR (A), RBP-EKL (B), and RBP-SSF (C) on proliferation of HUVECs. Numbers of viable HUVECs in the presence of RBP-LRR, RBP-EKL, or RBP-SSF at varying concentrations (open bars, 0 μM; light shaded bars, 0.1 μM; dark shaded bars, 1 μM; and closed bars, 10 μM) were determined using Cell-Counting Kit-8 every 24 h. At 0 h, only numbers of viable HUVECs in the absence of peptide were determined. Relative cell viability was determined according to absorbance at 450 nm. Assays were performed in triplicate and data are expressed as means ± standard deviations (SD) of three individual experiments. Differences were identified using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. untreated control cells.

Scratch migration assays HUVECs were cultured, harvested, and resuspended in HuMedia-EG2 as described for tube formation assays. *In vitro* wound closure was then assessed using scratch migration assays as reported previously (20,21,24,25) with slight modifications. Briefly, confluent monolayers of HUVECs

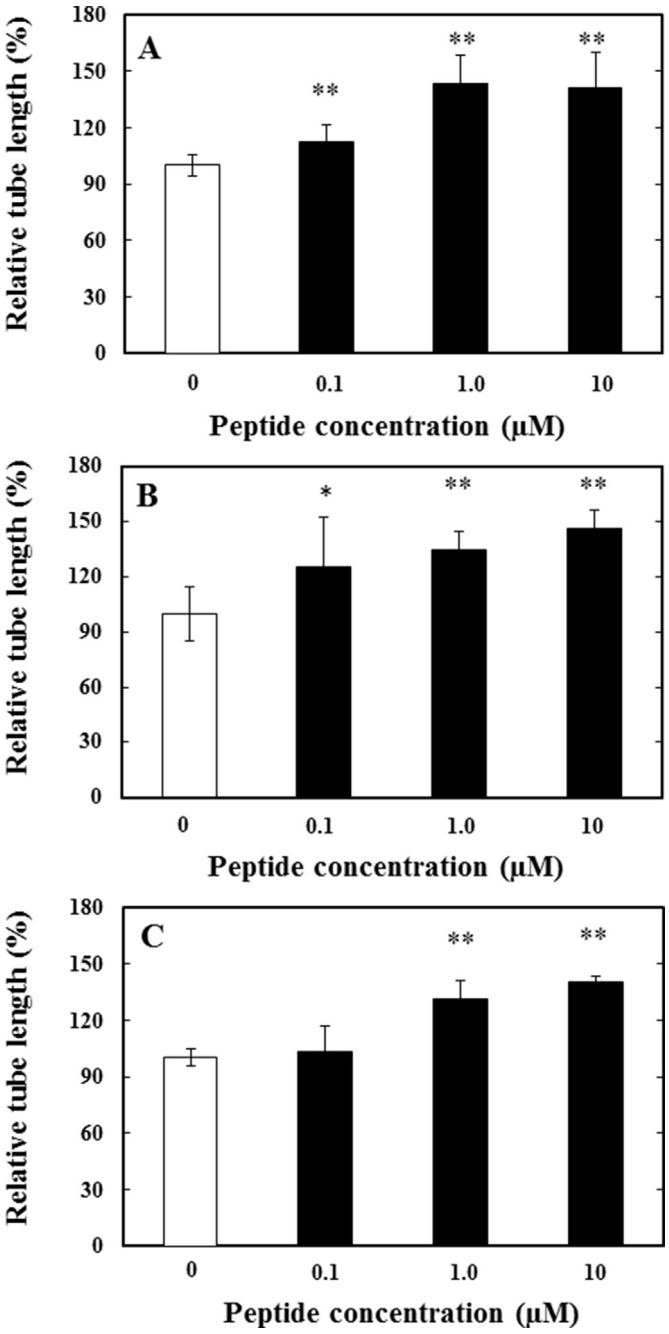

FIG. 2. Effects of RBP-LRR (A), RBP-EKL (B), and RBP-SSF (C) on tube formation of HUVECs. Microscope photographs of tube formation in Matrigel assays were obtained at 40 × magnification after 15-h treatments. Tube-like structures were then analyzed using NIS-Elements BR Analysis software (Nikon Instruments Inc.) and average tube lengths per field were calculated. Increases in tube formation in the presence of each peptide are expressed as percentages of those in untreated control cells (control; 100% tube formation). Supplementary Fig. S1 shows representative random phase contrast images. Assays were performed five times, and the data are expressed as means ± SD of three individual experiments. Differences were identified using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without RBP-LRR, RBP-EKL, or RBP-SSF.

were prepared on 24-well plates and were cultured in HuMedia-EG2 for 17–18 h. Cell monolayers were then wounded by creating uniform cell-free zones using a cell scratcher (AGC Techno Glass Co., Shizuoka, Japan). After removing cell debris, wounded monolayers were cultured in HuMedia-EG2 containing each cationic peptide at varying concentrations for 72 h. Culture media were replaced every 24 h with fresh media containing one of the cationic peptides. To characterize wound repair processes, wound areas were observed every 24 h at 40 × magnification using an inverted light microscope (TS100F, Nikon Instruments Inc.). Phase contrast images of three wells were recorded using a digital camera (Nikon Instruments Inc.). Average wound areas per well were calculated using NIS-Elements BR Analysis software. Reductions in cell-free areas in the presence of cationic peptides were expressed as percentages of the initial scratch areas at 0 h (100%). LL-37 was used as a positive control. Scratch migration assays were also performed after pretreating confluent cell monolayers with mitomycin C at 5 μM for 2 h (21).

Statistical analysis All cell proliferation, tube formation, and scratch migration assays were performed 3 or 5 times and data are presented as means ± standard deviations (SD). Differences were identified using Student's *t*-test.

RESULTS

Cell proliferation-promoting effects of cationic peptides To assess the effects of RBP-LRR, RBP-EKL, or RBP-SSF on cell proliferation, we determined numbers of viable HUVECs in the presence of the peptides at varying concentrations (Fig. 1). Cell numbers were significantly increased in the presence of 1 μM RBP-LRR (Fig. 1A), and although no concentration-dependent effects were identified, cell numbers were 1.1 fold higher than in the absence of peptide at 48 and 72 h. Similarly, cell proliferation was significantly increased after 48- and 72-h treatment with 0.1 μM RBP-EKL (Fig. 1B) or 0.1 μM RBP-SSF (Fig. 1C). At 72 h, the most efficient concentrations of RBP-LRR, RBP-EKL, and RBP-SSF were 1, 0.1, and 1 μM, respectively.

Tube formation-promoting effects of cationic peptides We assessed angiogenic effects of RBP-LRR, RBP-EKL, and RBP-SSF using tube formation assays with Matrigel (Fig. 2). In agreement with our previous study (16) and others, LL-37 (16,17,21), RBP-LRR (Fig. 2A), RBP-EKL (Fig. 2B), and RBP-SSF (Fig. 2C) promoted tube formation in HUVECs in a concentration-dependent manner, with 40%–46% increases at concentrations of 10 μM. Supplementary Fig. S1 shows photographs of tube formation in Matrigel assays in the presence of varying concentrations of RBP-LRR (Supplementary Fig. S1A), RBP-EKL (Supplementary Fig. S1B), and RBP-SSF (Supplementary Fig. S1C).

Effects of cationic peptides on wound closure To determine the effects of RBP-LRR, RBP-EKL, and RBP-SSF on wound closure, we performed scratch migration assays using HUVECs (Fig. 3) as reported previously (21). In these experiments, cell-free areas decreased more rapidly in the presence of 0.1 μM LL-37 than in controls (data not shown), as shown previously (21). As demonstrated in our previous study (21), LL-37 did not promote wound closure at 1 or 10 μM (data not shown), whereas in the presence of RBP-LRR (Fig. 3A) and RBP-EKL (Fig. 3B) at less than 10 μM, wound areas decreased in a concentration-dependent manner (Fig. 3A and B). Although RBP-SSF promoted migration of HUVECs into cell-free areas (Fig. 3C), decreases in wound areas did not vary with peptide concentrations, as observed with LL-37. Optimal concentrations of RBP-LRR, RBP-EKL, and RBP-SSF for wound closure were 10, 10, and 0.1 μM, respectively (Supplementary Fig. S2).

Tube formation by HUVECs pretreated with the VEGF receptor inhibitor SU5146 Members of the VEGF family have been widely associated with angiogenesis, and induce angiogenesis upon binding to VEGF receptors (13,19,26,27). Thus, we investigated interactions of RBP-LRR, RBP-EKL, and RBP-SSF with VEGF receptors in tube formation assays of HUVECs that were pretreated with the VEGF receptor inhibitor SU5416 (Fig. 4). SU5416 pretreatments led to significant decreases in tube lengths irrespective of subsequent treatments with RBP-LRR, RBP-EKL, or RBP-SSF, suggesting the possibility that these cationic peptides act via VEGF receptors. Supplementary Fig. S3 shows photographs of HUVECs tube formation in Matrigel assays in the absence and presence of RBP-LRR, RBP-EKL, or RBP-SSF following pretreatment with SU5416.

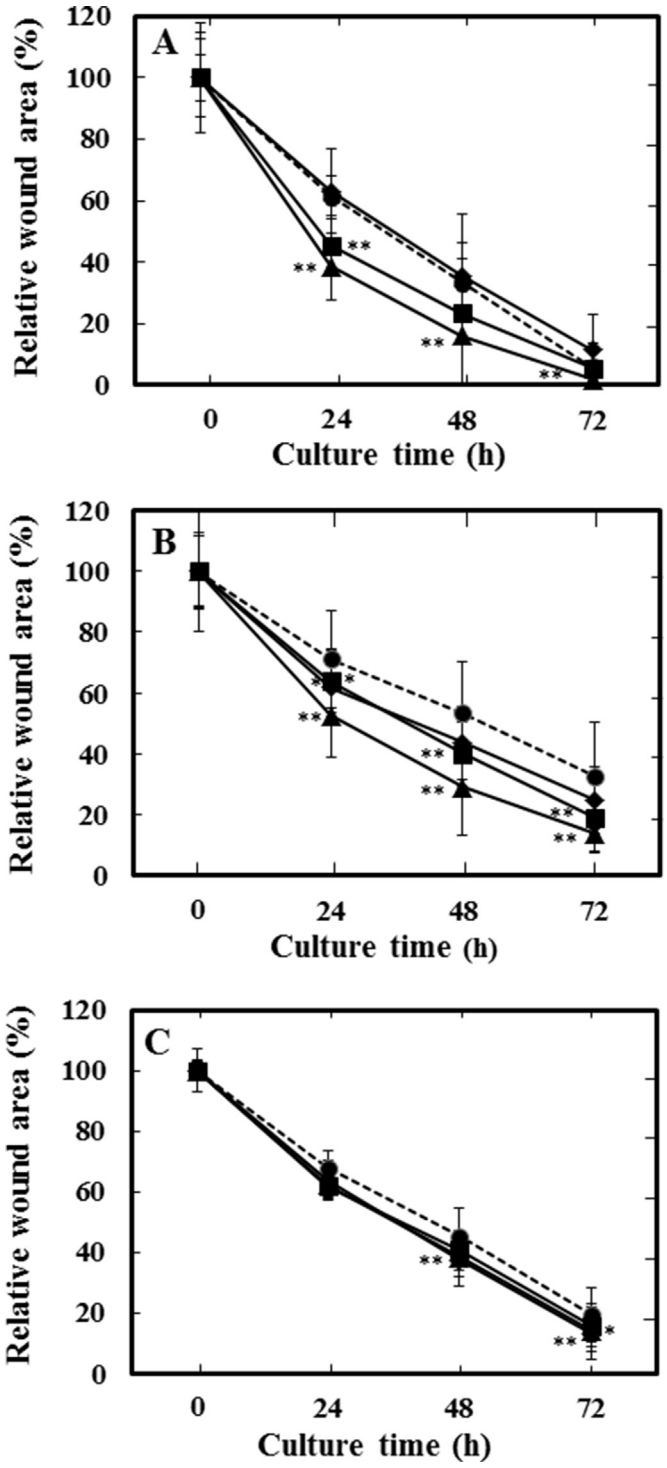

FIG. 3. Effects of RBP-LRR (A), RBP-EKL (B), and RBP-SSF (C) on wound closure. Cell monolayers were then wounded by creating uniform cell-free zones using a cell scratcher (AGC Techno Glass Co., Shizuoka, Japan). After removing cell debris, wounded monolayers were cultured in HuMedia-EG2 containing each cationic peptide at varying concentrations for 72 h. Culture media were replaced every 24 h with fresh media containing one of the cationic peptides. Wound areas were observed every 24 h at 40 × magnification using an inverted light microscope and phase contrast images in three wells were procured using a digital camera. Scratch areas were analyzed using NIS-Elements BR Analysis software and average wound areas per well were calculated. Decreases in cell-free areas in the presence of RBP-LRR, RBP-EKL, or RBP-SSF are expressed as percentages of the initial wound area at 0 h (100%). Symbols: circles, 0 μM (broken line); diamonds, 0.1 μM; squares, 1 μM; triangles, 10 μM. Supplementary Fig. S2 shows representative random phase contrast images. Assays were performed in triplicate, and the data are expressed as means REV±SD of three individual experiments. Differences were identified using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. untreated controls.

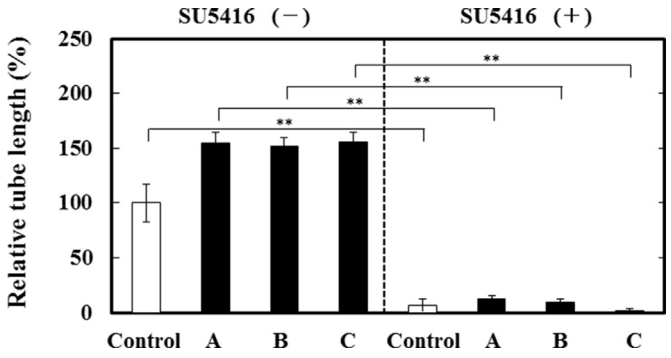

FIG. 4. Tube formation of HUVECs pretreated with the VEGF receptor inhibitor SU5416. HUVECs were pretreated with 0.1 μM SU5416 for 1 h before seeding into culture plates and the effects of RBP-LRR (A), RBP-EKL (B), and RBP-SSF (C) on tube formation were then examined. Microscope photographs of tube formation in Matrigel assays were obtained after 15 h. Tube-like structures were then analyzed using NIS-Elements BR Analysis software (Nikon Instruments Inc.) and average tube lengths per field were calculated. Relative tube lengths in the presence of RBP-LRR, RBP-EKL, or RBP-SSF are expressed as percentages of those in control cells that were not treated with SU5416 (control: left white bar, 100% tube length). Peptide concentrations were 10 μM. Supplementary Fig. S3 shows representative of three random phase contrast images. Assays were performed using five wells per test sample, and data are expressed as means ± SD of three individual experiments. Differences were identified using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without SU5416.

HUVEC migration following pretreatments with mitomycin C Wound closure is dependent on cell migration regardless of proliferation rates (20,28,29). Thus, to decipher relative contributions of proliferation to wound closure, we performed scratch migration assays of HUVECs after pretreatments with mitomycin C (Fig. 5). In these experiments, wound closure was inhibited in the absence (Fig. 5A) and presence of RBP-LRR (Fig. 5B), RBP-EKL (Fig. 5C), or RBP-SSF (Fig. 5D). These data show that pretreatments with mitomycin C abolished the wound healing properties of the peptides, indicating that RBP-LRR and RBP-EKL contribute to wound closure by promoting cell migration regardless of proliferation rates. In contrast, RBP-SSF exclusively contributed to wound closure by stimulating proliferation. Supplementary Fig. S4 shows photographs of scratch migration assays in the absence and presence of RBP-LRR, RBP-EKL, or RBP-SSF after pretreating HUVECs with mitomycin C.

DISCUSSION

Multiple peptides other than LL-37 and hBD-2 have been shown to induce proliferation, migration, and capillary-like tube formation in primary cultured human endothelial cells (18,24,30,31). For example, Erdogdu et al. (31) reported that exendin-4 is an analog of glucagon-like peptide (GLP)-1, and induces proliferation of human coronary artery endothelial cells. Kang et al. (24) also showed that exendin-4 promotes HUVEC migration in *in vitro* scratch wound assays and Albertin et al. (18) demonstrated that urotensin-II is expressed in cells of the cardio-vascular system, is a potent systemic vasoconstrictor, and induces self-organization of HUVECs into capillary-like structures *in vitro*. In addition, Song et al. (30) showed that the peptide irisin is released from skeletal muscles and induces proliferation in HUVECs. However, in comparison with peptides from humans and animals, few cationic peptides from plants have been characterized in terms of angiogenic, cell growth-promoting, and/or cell migration-promoting activities (2,21). Moreover, to our knowledge, multiple functions of cationic peptides from rice proteins have not been reported previously. Herein, we show that three multifunctional cationic peptides from enzymatic hydrolysates of RBPs (RBP-LRR, RBP-EKL, and RBP-SSF) have wound healing activity.

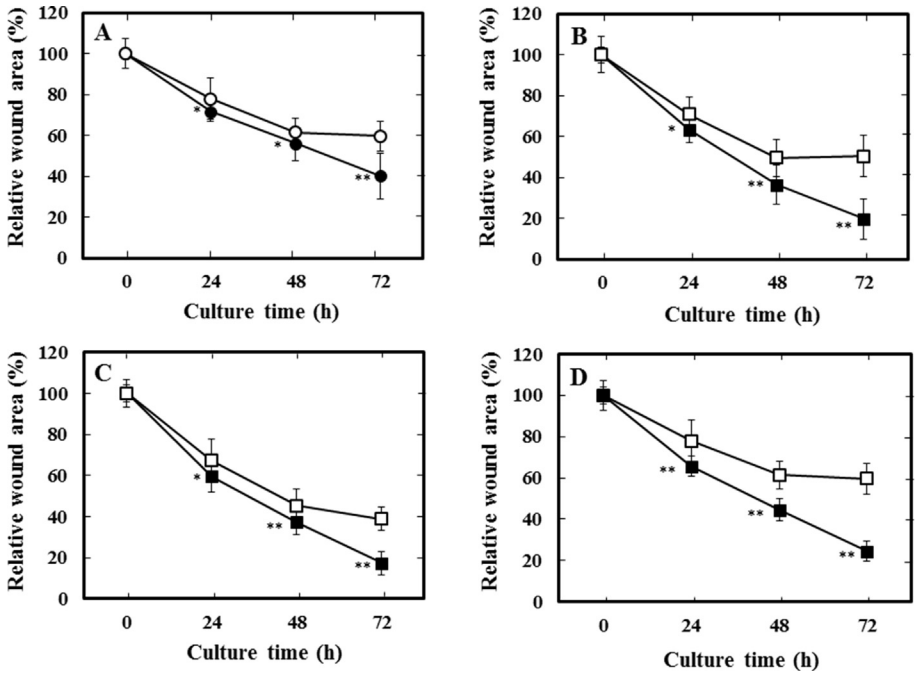

FIG. 5. Migration of HUVECs pretreated with the proliferation inhibitor mitomycin C (MMC). Confluent cell monolayers were pretreated with 5 μM mitomycin C for 2 h before scratching. Cell monolayers were then wounded by creating uniform cell-free zones using a cell scratcher (AGC Techno Glass Co., Shizuoka, Japan). After removing cell debris, wounded monolayers were cultured in HuMedia-EG2 containing each cationic peptide at varying concentrations. Culture media were replaced every 24 h with fresh media containing one of the cationic peptides. The effects of RBP-LRR (B), RBP-EKL (C), and RBP-SSF (D) on migration of mitomycin C-pretreated HUVECs were then examined over 72 h. Panel A shows cell migration into scratch areas in the absence of peptides. Scratch areas were analyzed every 24 h and average wound areas per well were calculated as described in the Materials and Methods. Relative cell-free areas in the presence of 10 μM RBP-LRR, 10 μM RBP-EKL, or 0.1 μM RBP-SSF are expressed as percentages of the initial wound area at 0 h (100%). Assays were performed in triplicate, and the data are expressed as means ±SD of three individual experiments. (A) MMC (−), closed circles; MMC (+), open circles; (B–D) MMC (−), closed squares; MMC (+), open squares. Supplementary Fig. S4 shows representative of three random phase contrast images. Differences were identified using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without RBP-LRR, RBP-EKL, or RBP-SSF.

In initial experiments, we evaluated wound healing activities of RBP-LRR, RBP-EKL, and RBP-SSF, and showed that RBP-LRR and RBP-SSF promote proliferation optimally at 1 μM. These proliferation experiments also showed that the optimal concentration for RBP-EKL was 0.1 μM (Fig. 1). In addition, these three cationic peptides were most proliferative at less than 1 μM, with lower effects at 10 μM (Fig. 1). These decreases in effect may reflect the hydrophobicity and low solubility of RBP-EKL. In fact, RBP-EKL has higher mean hydrophobicity (MH, 8.0), which was calculated using the hydrophobicity indices of amino acid residues reported by Shang et al. (32), than the other two cationic peptides (MH, 5.1–6.8).

In further experiments, RBP-LRR, RBP-EKL, and RBP-SSF exhibited concentration-dependent angiogenic activities in tube formation assays (Fig. 2), as reported previously (16). With 40%–46% increases in tube lengths at peptide concentrations of 10 μM, these data indicate comparable angiogenic activity (56% increases) to 10 μM LL-37 (16,17,21). Furthermore, RBP-SSF and LL-37 induced wound closure with similar efficacy at the same concentration (0.1 μM) in scratch migration assays. RBP-LRR and RBP-EKL treatments also decreased the sizes of wound area in a concentration-dependent manner, albeit at 100-fold higher optimal concentrations than RBP-SSF, indicating superior effects of RBP-SSF (Fig. 3). Although RBP-LRR, RBP-EKL, and RBP-SSF all promoted angiogenesis in concentration-responsive manners, peptide-mediated decreases in wound areas differed between these peptides. RBP-SSF differs in molecular weight (MW, 1283.6622 Da), isoelectric point (pI, 11.0), and MH (8.0) from RBP-LRR (MW, 1595.7818 Da; pI, 9.62; and MH, 5.1) and RBP-EKL (MW, 1667.0094 Da; pI, 9.70; and MH, 6.8) (16). Therefore, putative receptor interactions of these peptides likely differ, and further studies will be directed at identifying the mechanisms of these peptides in HUVECs.

Lee et al. (33) showed that treatments with the novel synthetic hexapeptide (SFKLRY-NH_2) resulted in 3-fold increases in VEGF concentrations in supernatants of HUVEC cultures. These investigators observed 50% decreases in angiogenic activities of these peptides in the presence of a VEGF neutralizing antibody (33), suggesting that angiogenesis of HUVECs is promoted following interactions of peptides with VEGF. In accordance, Finetti et al. (34) reported that a VEGF mimic peptide (acetyl-KLTWQELYQLKYKGI-NH_2) bound and directly activated VEGF receptors, and thus promoted angiogenesis of HUVECs. It is widely accepted that VEGF stimulates proliferation, angiogenesis, and migration of endothelial cells (33,34). Hence, we assessed contributions of VEGF receptors to the angiogenic activities of RBP-LRR, RBP-EKL, and RBP-SSF in tube formation assays with the tyrosine kinase inhibitor SU5416, which has been shown to affect VEGF receptor-mediated signaling (18,19). Pretreatments of HUVECs with SU5416 significantly reduced the effects of RBP-LRR, RBP-EKL, and RBP-SSF (Fig. 4), indicating the possibility that the three cationic peptides act via VEGF receptors. Further studies of peptide receptors on HUVECs are warranted.

Koczulla et al. (35) showed that LL-37 does not promote the release of VEGF from HUVECs, and a neutralizing antibody against VEGF did not reduce the effects of LL-37 on cell proliferation. Alternatively, Ramos et al. (36) reported that LL-37-induced wound healing was associated with endothelial cell migration and angiogenesis via direct effects on specific receptors, including formyl peptide receptor 2. Multiple studies indicate that the effects of LL-37 are mediated by various receptors, depending on the cell type and context (37–41). Taken together, these data warrant further studies to determine whether direct VEGF receptor interactions of the present three cationic peptides lead to angiogenesis, and to investigate the corresponding signaling via mitogen-activated

protein kinase, extracellular signal-regulating kinase, and phosphoinositide 3-kinase dependent pathways (18,31,42–44). In addition, it remains unknown if the peptides stimulate expression and secretion of VEGF via unknown HUVECs receptors.

Previous scratch migration assays show that wound closure is dependent on cell migration irrespective of cell growth (19,20). Herein, we evaluated the contributions of cell proliferation to wound closure by performing scratch migration assays after pretreatments of HUVECs with mitomycin C, which directly inhibits DNA replication and cell proliferation (19–21). Wound closure was significantly inhibited by mitomycin C pretreatments in the absence and presence of 10 μM RBP-LRR or 10 μM RBP-EKL. However, wound closure was not completely inhibited under these conditions, and remained enhanced in the presence of RBP-LRR or RBP-EKL (Fig. 5), suggesting that cell migration contributes predominantly to wound closure. In contrast, after pretreating HUVECs with mitomycin C, the effects of 0.1 μM RBP-SSF on wound closure were completely abolished (Fig. 5). These results indicated that RBP-LRR and RBP-EKL promote migration of HUVECs more potently than RBP-SSF, independently of proliferation effects. Further studies are required to relate the properties (e.g., size, pI, MH, amino acid composition) of these peptides with cell migration-promoting activities.

Previously we showed that RBP-LRR, RBP-EKL, and RBP-SSF have little or no hemolytic activity (16). In the present study, these peptides promoted proliferation, angiogenesis, and migration in HUVECs at low concentrations, suggesting potential as wound healing agents.

In conclusion, the effects of RBP-LRR, RBP-EKL, and RBP-SSF on proliferation, tube formation, and migration of HUVECs indicate wound healing properties of these peptides from RBP hydrolysates. The three cationic peptides induced cell proliferation most efficiently at 1, 0.1, and 1 μM, respectively. All three cationic peptides also promoted tube formation in HUVECs in a concentration-dependent manner, with 40%–46% increases at a peptide concentration of 10 μM. In tube formation assays with the VEGF inhibitor SU5416, angiogenic activities of the three cationic peptides were significantly diminished, indicating the possibility that their effects are in part mediated by VEGF receptors. In scratch migration assays, the three cationic peptides induced cell migration of HUVECs, and pretreatments with the DNA replication inhibitor mitomycin C showed that the wound healing effects of the three cationic peptides, especially RBP-SSF, are significantly dependent on their proliferation-promoting effects. Collectively, the present data demonstrate that these peptides are potent and non-toxic agents with multiple functions and potential as wound healing treatments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.02.002.

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