Published in final edited form as:

Shock. 2009 November ; 32(5): . doi:10.1097/SHK.0b013e3181a1cb2e.

ELEVATED GLUCOSE AND FATTY ACID LEVELS IMPAIR SUBSTANCE P-INDUCED DERMAL MICROVASCULAR ENDOTHELIAL CELL MIGRATION AND PROLIFERATION IN AN AGAROSE GEL MODEL SYSTEM

Qiang Wang, Lara A. Muffley, Kyla Hall, Marie Chase, and Nicole S. Gibran

Department of Surgery, University of Washington, Seattle, Washington

Abstract

Substance P (SP), a sensory nerve derived neuropeptide, has been implicated in wound repair. Our hypothesis was that oxidative effects of elevated glucose and fatty acid levels as seen with diabetes mellitus inhibit SP-mediated endothelial cell directional migration and proliferation. Using a 2% agarose gel, immortalized human microvascular endothelial cells (HMEC-1) were plated into a 1.5-mm well, and agonist (SP; 10^{-4} mol/L) was loaded into a 3-mm well; controls included NaCl, albumin (bovine serum albumin), and vascular endothelial cell growth factor. The SP receptor antagonist spantide 1 was used to confirm SP specificity. Elevated glucose (40 mmol/L) and fatty acids (40 μ mol/L) were added to the medium with and without vitamin E and vitamin C treatment to determine whether endothelial cell responses to SP were altered by metabolic perturbations and whether they could be recovered with antioxidant treatment. Using computer-assisted image analysis, migration distance was measured. Cells were counted using a hemocytometer. Human microvascular endothelial cell 1 migration toward the SP exceeded NaCl or bovine serum albumin; vascular endothelial cell growth factor had similar effects. The SP receptor antagonist, spantide, inhibited SP-induced HMEC-1 migration. Substance P treatment was associated with increased cell number. Ki-67 staining was increased in SP-treated cells compared with controls. Elevated glucose and fatty acid levels diminished cell migration toward SP. The antioxidants vitamins C and E significantly improved proliferation but only marginally improved migration. Our data suggest that glucose and fatty acids perturb SP-induced HMEC-1 migration and proliferation in an agarose gel migration model.

Keywords

Neuroinflammation; response to injury; angiogenesis; cell proliferation; neuropeptide

INTRODUCTION

Cell migration is a complex, multistep process that is essential for many aspects of morphogenesis, including wound repair (1–3). Microvascular endothelial cell (MEC) migration and proliferation after tissue injury result in wound neovascularization (4–6). Regulated by both chemotaxis and mechanotaxis, MEC migration involves dynamic, coordinated changes in cell adhesion, cytoskeleton organization, and signal transduction (7). Whereas much progress has been made in understanding angiogenesis and angioinhibition,

successful, clinically effective, therapeutic targets for wound neovascularization have been limited.

Substance P (SP), a neuroinflammatory peptide derived from sensory nerves, contributes to early responses to cutaneous injury (8–15). First recognized as a mediator of pain and proprioception to the central nervous system (16–18), SP also causes inflammatory reactions when released into local tissue (19–21). After noxious stimuli, sensory neuron release of neuropeptides such as SP causes erythema, edema, and pruritus, and humoral inflammatory effects, including neutrophil release of cytokines (22, 23). Important for our work is the beneficial effect SP has on wound healing kinetics in models of impaired wound healing (13). We have also demonstrated that endothelial cell responses to SP may be inhibited by activation of neutral endopeptidase, a membrane-bound enzyme responsible for regulating SP activity (24). Our previous work suggests that elevated levels of glucose and the oxidative fatty acids linoleic acid and oleic acid increase neutral endopeptidase activity (24); the antioxidants vitamin C and vitamin E reverse the effect even after long-term exposure to the metabolic perturbation (25). Neutral endopeptidase activity is increased in the neuropathic skin of patients with diabetic neuropathy (26), and inhibition of neutral endopeptidase in a diabetic murine model of wound repair improves wound healing kinetics (27).

To better understand mechanistic events leading to impaired endothelial cell responses to SP, we wanted to establish an *in vitro* model of SP-mediated endothelial cell migration and proliferation that could be used to perturb endothelial responses.

MATERIALS AND METHODS

Cell culture

Human microvascular endothelial cells (HMEC-1) (28) were generously provided by Dr. Edwin Ades and Francisco J. Candal of the Centers for Disease Control and Prevention and Dr. Thomas Lawley of Emory University. The cells were cultured and maintained on attachment factor-coated T75 flasks at 37°C with 5% CO₂ in MCDB-131 (Cascade Biologics, Portland, Ore) medium supplemented with L-glutamine, penicillin/streptomycin, and microvascular growth serum (Cascade Biologics). Cell culture medium was changed every 48 to h until monolayers reached confluence.

For experiments, 24-well tissue culture dishes were coated with 1.5 mL of 2% agarose (w/v) in water. After the gel solidified, sterile punch biopsies were used to create 1.5- and 3-mm holes in the gel spaced 1.5 mm apart (Fig. 1A). Human microvascular endothelial cells (5,000 cells per well) were added to the 1.5-mm well. Agonist (20 µL per well) was loaded into the 3-mm wells (n = 10; Fig. 1). Experimental conditions consisted of either 10⁻⁴ mol/L SP (Sarmet; cat no. H-9410; Bachem Torrance, Calif), 0.9% NaCl, 0.2% bovine serum albumin (BSA; cat no. 9048-46-8; Sigma, Saint Louis, Mo) in phosphate-buffered solution (PBS), or recombinant human vascular endothelial growth factor (100 ng/mL; VEGF 165 cat no. 293-VE; R&D Systems, Ind). After loading, cells and mediator were incubated for 30 min at 37°C before adding MCDB-131 growth medium (400 µL) to each well. For experiments with spantide 1 ([D-Arg¹, D-Trp^{1,9}, Leu¹¹] SP; cat. no. 118F01204; Sigma), cells were pretreated with either MCDB-131 growth medium or spantide 1 (10⁻⁴ mol/L) for 4 h before addition of substance P (10⁻⁴ mol/L). For all experiments, stimulant and growth media were changed daily. Each experiment was repeated at least twice.

For studies with elevated glucose and fatty acid levels, the HMEC-1 cells were plated as usual in the wells with SP in the adjacent well. For experimental conditions with elevated glucose and fatty acids levels, glucose (40 mmol/L), linoleic acid (40 µmol/L), and oleic

acid (40 $\mu\text{mol/L}$) were added to the medium as previously described (24). Likewise, for cells exposed to antioxidants, the cells were incubated with vitamin E (100 $\mu\text{mol/L}$) for 4 h, followed by vitamin E (10 $\mu\text{mol/L}$) and vitamin C (5 mmol/L) for 48 h as previously described (25).

Wright staining and migration measurement

Experiments were terminated on days 1, 2, and 3. After the medium and agarose were carefully removed, cells were washed with PBS, fixed with 4% formaldehyde in PBS for 24 h at 4°C, and stained with Wright stain for 30 min. Excess stain was washed away with PBS. Images were captured with a Nikon camera (Q-imaging QICAM12-bit Monochrome Fast 1394 cooled with RBG slider) attached to a Nikon inverted Microscope (ECLIPSE TE300) at 2× magnification (Fig. 1B).

We used two methods to quantify migration. The first approach was adapted from Chernyavsky et al. (29) (Fig. 1C). In short, to standardize measurements, a median segment (B1B4) bisected each cell colony directed toward the treatment well, and two additional segments at 30-degree intervals were drawn through the center of each cell colony in the direction of the treatment well (Fig. 1, A and B). Directional migration distance (DMD) was computed in millimeters:

$$\text{DMD} = \frac{(A1B1 + A2B2 + A6B6) - (A3B3 + A4B4 + A5B5)}{3}$$

The second method emphasized *x* axis and *y* axis migration (Fig. 1D). This method specifically quantifies the cell distance from the center of the well in all four directions. An advantage over the DMD is that it not only indicates the length of the segment between the center of the well and the agonist but also the length of the segment opposite the agonist. This can tell you whether there is overall migration in all directions and directional migration. *x* Axis migration distance (X1) toward the agonist is measured from X0 to X1 (30, 31). *x* Axis migration distance away from the agonist (X2) is from X0 to X2. *y* Axis upward migration distance (Y1) is from Y0 to Y1. *y* Axis downward migration (Y2) is from Y0 to Y2 (Fig. 2C). *x* Axis (or *y* axis) migration distance was computed in millimeters. We would not expect that the agonist would affect Y1 or Y2 migration distance.

Immunocytochemistry

For experiments intended for the immunocytochemistry, agarose wells were created in four-well chamber slides (Nunc, Naperville, Ill). At each end point (days 1, 2, or 3), the agarose gel was removed, and the cells were fixed with 4% paraformaldehyde in Sorenson buffer at room temperature. Cells were rinsed with PBS and permeabilized with 0.1% triton in PBS for 10 min. Nonspecific antibody binding was blocked using 2% normal goat serum with 0.1% BSA in PBS for 1 h at room temperature, followed by incubation with a polyclonal primary antibody against the proliferation marker KI-67 (1:100; rabbit anti-Ki-67; cat. no. 18-0191Z; Zymed Laboratories, San Francisco, Calif) for 1 h at room temperature. After PBS rinses and a second blocking step, cells were incubated with the secondary antibody goat-antirabbit antibody labeled with Rhodamine Red X (1:200; goat-antirabbit immunoglobulin G; cat no. 111-295-144, Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa) for 30 min. 4,6-Diamidino-2-phenylindole was used as the nuclear stain.

Cell counts

To determine whether the agonists stimulate proliferation, HMEC-1 cells were harvested on days 2 and 3 for cell counts; these studies were performed in triplicate and repeated twice.

After medium and agarose gel were removed, cells were washed with PBS and detached with $1\times$ trypsin-EDTA solution for 4 min at 37°C . Enzymatic detachment was terminated with trypsin neutralizing solution (Cascade Biologics), and cells were washed and resuspended in MCDB-131 medium. Cells were counted using a hemocytometer three times by two blinded readers.

Data analysis

Quantitative data are expressed here as mean $\pm$ SD. Determination of statistical significance on migration distances and cell counts was performed using ANOVA. A P value less than 0.05 was considered statistically significant and is presented here as the actual value unless it was less than 0.001.

RESULTS

SP effects on HMEC-1 cell migration

Cell migration patterns in response to each chemotactic factor on days 1, 2, and 3 are illustrated in Figure 2. The HMEC-1 cells treated with NaCl or BSA generally have a uniform “circle” shape (Fig. 2, A–F), and those cells stimulated with SP or VEGF display “rocket” shape migratory patterns (Fig. 2, G–L).

Directional distances are summarized in Table 1. Both methods for measuring migration demonstrate that cells migrate toward SP. The DMD for SP-treated cells was longer than for either NaCl or BSA (Fig. 3; $P < 0.001$) on both days 2 and 3. The DMD for the positive control VEGF is also longer ($P < 0.001$).

Likewise, measuring migration distances along the x and y axes indicates that HMEC-1 cells migrate toward SP or the positive control VEGF on both days 2 and 3 (Fig. 4; $P < 0.0001$). Migration along the x_1 , x_2 , y_1 , and y_2 axes was relatively equal for NaCl-treated cells ($P = 0.81$) or BSA-treated cells ($P = 0.75$). However, SP ($P < 0.001$) or VEGF ($P < 0.001$) markedly increased HMEC-1 cell migration along the x_1 axis. Interestingly for both of these agonists, not only was migration along x_1 longer ($P < 0.001$) than for the NaCl and BSA-treated cells, but x_2 was significantly shorter for SP and VEGF ($P < 0.001$). This suggests that not only the cells closest to the agonist but also the cells at the opposite side of the well migrated toward the agonist (Fig. 2). Collectively, these data suggest that cell migration with NaCl or BSA is random and not directional, but that SP has a chemotactic effect on HMEC-1 cells.

Spantide inhibits HMEC-1 locomotion toward SP

To determine whether SP binding to its neurokinin 1 receptor specifically mediated the chemotactic effect, experiments were repeated with spantide 1, the neurokinin 1 receptor antagonist. Spantide 1 decreased SP-mediated migration. Directional migration distance was shorter in cells treated with both spantide 1 and SP compared with SP alone (Fig. 5A; $P < 0.0001$). Likewise, migration along the x_1 axis (toward the SP) was significantly shorter in cells treated with spantide 1 than with SP alone (Fig. 5B; $P = 0.001$), and x_2 distance (away from the agonist) was longer in spantide 1–treated cells ($P = 0.006$).

SP enhances HMEC-1 cell proliferation

Immunocytochemistry with an antibody against the proliferation marker Ki-67 suggests that cells treated with NaCl or BSA were clumped together with very few Ki-67⁺ cells (Fig. 6, A and B). In contrast, in wells treated with SP, the cells were more dispersed with more Ki-67⁺ cells (Fig. 6C). Similarly, in wells treated with our positive control VEGF, the cells were more separated, and Ki-67⁺ cells were common (Fig. 6D).

Because quantification of the immunocytochemistry results was challenging, we confirmed our immunocytochemistry results with cell counts at each time point (Table 2). Cell number in SP-treated wells (13.5×10^3 ; $P < 0.001$) was significantly higher than either the NaCl-treated wells (1.53×10^3) or the BSA-treated wells (3.35×10^3) on day 2. Spantide 1 reduced this proliferative effect (5.15×10^3 ; $P < 0.001$). On day 3, cell counts in SP-treated wells were 8-fold higher than in NaCl-treated wells and 5-fold higher than in wells treated with BSA. Spantide inhibitory effect persisted at this time point.

Elevated glucose and fatty acid levels impair HMEC-1 cell migration and proliferation

Elevated glucose, linoleic acid, and oleic acid levels decrease migration of endothelial cells toward SP on day 2 as measured using DMD ($P = 0.001$; Fig. 7A) and using xy axis measurements ($P = 0.05$; Fig. 7B); effects on day 1 are similar. The antioxidants vitamin E and vitamin C did not normalize this inhibitory effect on HMEC migration based on DMD; migration along the x_1 axis (toward SP) by HMEC cells treated with vitamin E and C approaches statistical significance ($P = 0.06$; Fig. 7B). Elevated levels of glucose and fatty acids decreased proliferation in cells treated with and without SP (Table 3). Vitamin C and E increased cell proliferation under all conditions.

DISCUSSION

Our goal for this project was to develop an endothelial cell migration model that could be used to modulate SP-mediated effects on endothelial cell migration and proliferation. Commonly used cell migration models have advantages and disadvantages for studying chemotaxis. The Boyden chamber, a simple two-compartment chamber used to study leukocyte chemotaxis, has the major advantage that it can detect chemokinetic effects and chemotactic effects (32, 33). Although the Boyden chamber can be used to demonstrate directional migration and to quantify numbers of migrating cells, it cannot be easily adapted for image analysis and cannot be used to demonstrate cytoskeletal events, which may be essential for SP-mediated cellular responses.

An alternate model, the scratch assay, in which “wounds” are created on a confluent cell monolayer, can be used to take serial images or live video imaging and illustrate cytoskeletal changes (34); however, scratch assays cannot be used to show directional chemotaxis because the agonists is in the media bathing the entire monolayer. Similarly, the omnidirectional migration assay (35), in which cells are plated in a ring, stabilized with a glass ball until removal of the ring creates a round precise monolayer that can be serially photographed to study outward cellular migration cannot be used for directional migration studies.

Tracking assays such as the gold particle assay (36–38) in which cells are plated on colloidal gold-coated wells are suited for cell motility studies because they allow for serial photos or video imaging and can be used for directional motility studies. However, these assays do not allow for quantification; in addition, analysis of the area of the tracks made by the migrating cells in the colloidal gold generates an indirect measurement of migration. Depending on cell spreading, the denuded areas may represent gold displacement due to cell spread rather than migration.

For our studies, the modified agarose gel model allows us to quantify both directional and random cell migration with the added option of image analysis (29, 39). The agarose gel model allows a whole image of a cell colony migration during the course of the experiment (Fig. 1B) and can be immunostained to demonstrate cellular markers or architecture. To capture images of the entire cell monolayer, we decreased the well size to 1.5 mm (Fig. 1A). This modification has several advantages. First, we capture low-power images of the whole

cell monolayer even after cells have been in culture for 3 days. Second, the smaller wells require fewer cells and fewer reagents, which can be resource intense when a mediator is limited or expensive. Third, the 24-well plate allows the assay to be easily performed in triplicate with multiple experimental conditions. Fourth, we can easily adapt the model to tissue culture slides to perform immunocytochemistry or histology.

In studying mitogen-mediated cell migration, quantifying distance is complicated by the need to account for random migration and directional migration. Whereas most studies report on average directional migration distance (29), this measurement does not account for cell migration away from the agonist. By measuring cell migration along both the *x* and *y* axes, (Fig. 1), we were able to quantify cell migration both toward and away from the agonist; the *y* axis represents nondirectional movement.

Our data using the modified agarose gel model suggest that SP interaction with its receptor neurokinin 1 induces HMEC-1 migration and proliferation. These results support previous reports using a Boyden chamber migration model that SP induces a concentration dependent migration of capillary endothelial cells. Whereas we had optimal migration with a supra physiologic SP concentration, there is likely a strong gradient effect through the 1.5 mm of agarose that separates the cells from the agonist. This is likely much longer than the *in vivo* distance separating capillaries and nerves. Inhibition of HMEC-1 proliferation and migration with spantide supports evidence that the effects that we observe involve specific SP-mediated signaling rather than nonspecific cellular responses to the agarose.

Our novel observations regarding the inhibitory effect of elevated glucose and fatty acid levels on cell migration suggest that metabolic perturbations alter cell migration essential for cutaneous wound repair. We based our choice of linoleic acid and oleic acid for these studies on our previous data that indicated that these two fatty acids increased enzymatic activity of neutral endopeptidase, the membrane-bound metallopeptidase that regulates substance P (24). Because these two fatty acids are both oxidative, we verified that they increased cell hydrogen peroxide levels in the endothelial cells and hypothesized that oxidative stress increased the enzyme activity. We have now confirmed that SP-mediated HMEC-1 migration and proliferation are negatively impacted by metabolic perturbation with glucose and fatty acids. In our current set of studies, the antioxidants vitamins C and E strongly improved proliferation with a less robust effect on migration. A recent publication reports that another antioxidant, *N*-acetyl cysteine, reverses glucose-mediated inhibition of retinal endothelial cell migration (40). Therefore, we conclude that elevated levels of glucose, linoleic acid, and oleic acid impair dermal endothelial cell migration and proliferation by increasing oxidative stress.

CONCLUSIONS

In this study, we have modified an agarose gel migration assay that can be used to study both directional and random migration by endothelial cells. Using this model, we have confirmed previously reported observations that SP induces both migration and proliferation of HMECs and have demonstrated that glucose and fatty acids retard dermal microvascular endothelial cell migration and proliferation. We suggest that this is due to oxidative stress because the antioxidants vitamin C and E improved proliferation significantly and migration marginally. Our results have clinical implications because endothelial cell migration and proliferation are crucial for normal angiogenesis during wound healing.

Acknowledgments

The authors thank Anne Hocking, Richard N. Tamura, Bob Underwood, and Carina Morningstar for assistance with this project.

This study was supported by Grant RO1GM56483-07 from the National Institute of General Medical Sciences and Grant RO1DK508007-06 from the National Institute of Diabetes and Digestive and Kidney Diseases.

References

1. Smith KD, Wells A, Lauffenburger DA. Multiple signaling pathways mediate compaction of collagen matrices by EGF-stimulated fibroblasts. *Exp Cell Res*. 2006; 312:1970–1982. [PubMed: 16595133]
2. Zaman MH, Trapani LM, Sieminski AL, Mackellar D, Gong H, Kamm RD, Wells A, Lauffenburger DA, Matsudaira P. Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. *Proc Natl Acad Sci U S A*. 2006; 103:10889–10894. [PubMed: 16832052]
3. Wells A. Tumor invasion: role of growth factor-induced cell motility. *Adv Cancer Res*. 2000; 78:31–101. [PubMed: 10547668]
4. Clark RA. Cutaneous tissue repair: basic biologic considerations. I. *J Am Acad Dermatol*. 1985; 13:701–725. [PubMed: 2416789]
5. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. *J Invest Dermatol Symp Proc*. 2000; 5:40–46.
6. Schlingemann RO, Rietveld FJ, Kwaspen F, van de Kerkhof PC, de Waal RM, Ruiter DJ. Differential expression of markers for endothelial cells, pericytes, and basal lamina in the microvasculature of tumors and granulation tissue. *Am J Pathol*. 1991; 138:1335–1347. [PubMed: 1711288]
7. Li S, Huang NF, Hsu S. Mechanotransduction in endothelial cell migration. *J Cell Biochem*. 2005; 96:1110–1126. [PubMed: 16167340]
8. Wiedermann CJ, Auer B, Sitte B, Reinisch N, Schratzberger P, Kahler CM. Induction of endothelial cell differentiation into capillary-like structures by substance P. *Eur J Pharmacol*. 1996; 298:335–338. [PubMed: 8846835]
9. Walsh DA, Hu DE, Mapp PI, Polak JM, Blake DR, Fan TP. Innervation and neurokinin receptors during angiogenesis in the rat sponge granuloma. *Histochem J*. 1996; 28:759–769. [PubMed: 8968728]
10. Scott JR, Muangman PR, Tamura RN, Zhu KQ, Liang Z, Anthony J, Engrav LH, Gibran NS. Substance P levels and neutral endopeptidase activity in acute burn wounds and hypertrophic scar. *Plast Reconstr Surg*. 2005; 115:1095–1102. [PubMed: 15793451]
11. Schaffer M, Beiter T, Becker HD, Hunt TK. Neuropeptides: mediators of inflammation and tissue repair? *Arch Surg*. 1998; 133:1107–1116. [PubMed: 9790210]
12. Nilsson J, von Euler AM, Dalsgaard CJ. Stimulation of connective tissue cell growth by substance P and substance K. *Nature*. 1985; 315:61–63. [PubMed: 2581142]
13. Gibran NS, Jang YC, Isik FF, Greenhalgh DG, Muffley LA, Underwood RA, Usui ML, Larsen J, Smith DG, Bunnett N, et al. Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. *J Surg Res*. 2002; 108:122–128. [PubMed: 12443724]
14. Dunnick CA, Gibran NS, Heimbach DM. Substance P has a role in neurogenic mediation of human burn wound healing. *J Burn Care Rehabil*. 1996; 17:390–396. [PubMed: 8889861]
15. Ansel JC, Armstrong CA, Song I, Quinlan KL, Olerud JE, Caughman SW, Bunnett NW. Interactions of the skin and nervous system. *J Invest Dermatol Symp Proc*. 1997; 2:23–26.
16. Taylor BK, McCarson KE. Neurokinin-1 receptor gene expression in the mouse dorsal horn increases with neuropathic pain. *J Pain*. 2004; 5:71–76. [PubMed: 15042514]
17. Skerrett PJ. Substance P causes pain—but also heals [news]. *Science*. 1990; 249:625. [PubMed: 1696396]
18. Pedersen BU, Nielsen LB, Jensen K, Edvinsson L, Jansen I, Olesen J. Calcitonin gene-related peptide, neurokinin A and substance P: effects on nociception and neurogenic inflammation in human skin and temporal muscle. *Peptides*. 1991; 12:333–337. [PubMed: 1712469]
19. Payan DG. Neuropeptides and inflammation: the role of substance P. *Annu Rev Med*. 1989; 40:341–352. [PubMed: 2471450]

20. Scholzen TE, Steinhoff M, Bonaccorsi P, Klein R, Amadesi S, Geppetti P, Lu B, Gerard NP, Olerud JE, Luger TA, et al. Neutral endopeptidase terminates substance P-induced inflammation in allergic contact dermatitis. *J Immunol.* 2001; 166:1285–1291. [PubMed: 11145711]
21. Gibran NS, Tamura R, Tsou R, Isik FF. Human dermal microvascular endothelial cells produce nerve growth factor: implications for wound repair. *Shock.* 2003; 19:127–130. [PubMed: 12578120]
22. Ansel JC, Brown JR, Payan DG, Brown MA. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *J Immunol.* 1993; 150:4478–4485. [PubMed: 7683320]
23. Delgado AV, McManus AT, Chambers JP. Production of tumor necrosis factor–alpha, interleukin 1–beta, interleukin 2, and interleukin 6 by rat leukocyte sub-populations after exposure to substance P. *Neuropeptides.* 2003; 37:355–361. [PubMed: 14698678]
24. Muangman P, Spenny ML, Tamura RN, Gibran NS. Fatty acids and glucose increase neutral endopeptidase activity in human microvascular endothelial cells. *Shock.* 2003; 19:508–512. [PubMed: 12785004]
25. Muangman P, Tamura RN, Gibran NS. Antioxidants inhibit fatty acid and glucose-mediated induction of neutral endopeptidase gene expression in human microvascular endothelial cells. *J Am Coll Surg.* 2005; 200:208–215. [PubMed: 15664096]
26. Antezana M, Sullivan S, Usui M, Gibran N, Spenny M, Larsen J, Ansel J, Bunnett N, Olerud J. Neutral endopeptidase activity is increased in the skin of subjects with diabetic ulcers. *J Invest Dermatol.* 2002; 119:1400–1404. [PubMed: 12485446]
27. Spenny ML, Muangman P, Sullivan SR, Bunnett NW, Ansel JC, Olerud JE, Gibran NS. Neutral endopeptidase inhibition in diabetic wound repair. *Wound Repair Regen.* 2002; 10:295–301. [PubMed: 12406165]
28. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol.* 1992; 99:683–690. [PubMed: 1361507]
29. Chernyavsky AI, Arredondo J, Marubio LM, Grando SA. Differential regulation of keratinocyte chemokinesis and chemotaxis through distinct nicotinic receptor subtypes. *J Cell Sci.* 2004; 117:5665–5679. [PubMed: 15494367]
30. Foxman EF, Kunkel EJ, Butcher EC. Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation. *J Cell Biol.* 1999; 147:577–588. [PubMed: 10545501]
31. Smith JT, Elkin JT, Reichert WM. Directed cell migration on fibronectin gradients: effect of gradient slope. *Exp Cell Res.* 2006; 312:2424–2432. [PubMed: 16730349]
32. Terranova VP, DiFlorio R, Lyall RM, Hic S, Friesel R, Maciag T. Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. *J Cell Biol.* 1985; 101:2330–2334. [PubMed: 3905825]
33. Grotendorst GR. Spectrophotometric assay for the quantitation of cell migration in the Boyden chamber chemotaxis assay. *Methods Enzymol.* 1987; 147:144–152. [PubMed: 3670081]
34. Gabbiani G, Gabbiani F, Heimark RL, Schwartz SM. Organization of actin cytoskeleton during early endothelial regeneration in vitro. *J Cell Sci.* 1984; 66:39–50. [PubMed: 6540272]
35. Dolle JP, Rezvan A, Allen FD, Lazarovici P, Lelkes PI. Nerve growth factor-induced migration of endothelial cells. *J Pharmacol Exp Ther.* 2005; 315:1220–1227. [PubMed: 16123305]
36. Takaishi K, Sasaki T, Takai Y. Cell motility assay and inhibition by Rho-GDP dissociation inhibitor. *Methods Enzymol.* 1995; 256:336–347. [PubMed: 7476451]
37. Woodley DT, Bachmann PM, O'Keefe EJ. Laminin inhibits human keratinocyte migration. *J Cell Physiol.* 1988; 136:140–146. [PubMed: 2456291]
38. Albrecht-Buehler G. Phagokinetic tracks of 3T3 cells: parallels between the orientation of track segments and of cellular structures which contain actin or tubulin. *Cell.* 1977; 12:333–339. [PubMed: 334371]
39. Grando SA, Crosby AM, Zelickson BD, Dahl MV. Agarose gel keratinocyte outgrowth system as a model of skin re-epithelization: requirement of endogenous acetylcholine for outgrowth initiation. *J Invest Dermatol.* 1993; 101:804–810. [PubMed: 8245509]

40. Huang Q, Sheibani N. High glucose promotes retinal endothelial cell migration through activation of Src, PI3K/Akt1/eNOS, and ERKs. *Am J Physiol Cell Physiol*. 2008; 295:C1647–C1657. [PubMed: 18945941]

Fig. 1. A, Our model uses two wells in a solid agarose gel with the 1.5-mm well for the HMEC-1 cells and the 3.0-mm well for the stimulant

B, The 4× image of Wright-stained cells can be easily mapped for quantification of the x and y axes. C, The direction migration distance method for quantifying migration uses angles 30 degrees above and below the horizontal axis between the agonist and the cells to determine cell migration. The calculated value averages migration toward the agonist with random migration. Directional migration toward the agonist results in higher values. D, The axis model calculates length of cell migration toward and away from the agonist: x_1 represents migration toward the agonist along the x axis; x_2 represents migration away from the agonist along the x axis; y_1 represents upward along the y axis; y_2 represents downward migration along the y axis. Nondirectional cell migration would result in similar distances for each axis.

Fig. 2. Wright staining of cells harvested on days 1, 2, and 3 illustrate the typical uniform “circle” shape of the cells treated with NaCl or VEGF, suggesting that NaCl-treated cells (A, B, C) and BSA-treated cells (D, E, F) migrated equally in all directions. In contrast, the cells treated with SP (G, H, I) or VEGF (J, K, L) have a typical “rocket”-shaped pattern of migration, suggesting that those cells migrate toward the agonist.

Fig. 3. On both days 2 and 3, DMD (in millimeters) for the cells is significantly greater in SP-treated MECs than in NaCl or BSA-treated cells. The positive control VEGF also demonstrated significantly greater DMD. Error bars represent SD.

Fig. 4. A, On day 2, HMECs treated with NaCl or BSA migrated evenly in all directions ($P > 0.1$) Substance P-treated HMEC migration toward the treatment (x_1 ; $P < 0.001$) was significantly greater than away from the treatment (x_2) or in the y axes (y_1 and y_2). Likewise, cells migrated toward the VEGF (x_1) compared with the other directions (x_2 , y_1 , or y_2). B, On day 3, the directional migration toward the SP (x_1 ; $P < 0.001$) and the VEGF (x_1 ; $P < 0.001$) persisted. Values represent distance in millimeters. Error bars represent SD.

Fig. 5. A, Using DMD to calculate migration, we can demonstrate that cells migrate toward SP, and that the neurokinin 1 antagonist spantide 1 inhibits this migration (* $P = 0.001$), suggesting that the HMEC migration toward the agonist involves SP binding to its receptor

B, Measuring distance along x and y axes demonstrates that cells migrate toward the SP, reflected as longer x_1 distance and shorter x_2 distance. Migration along the y axis is not affected. Using ANOVA, there is a significant difference in migration along the x axis in SP-treated cells ($P = 0.00001$) compared with NaCl. These measurements confirm that spantide 1 inhibits HMEC migration toward SP with a shorter x_1 axis ($^{\dagger}P = 0.001$) and longer x_2 axis ($^{\ddagger}P = 0.006$). Neither SP nor spantide altered migration along the y axes ($P =$ not significant).

Fig. 6. Ki-67 and 4 ,6-diamidino-2-phenylindole DAPI staining indicate that NaCl-treated HMEC cells are crowded in the center of their well with few Ki-67⁺ cells (A)
 B, Bovine-serum albumin-treated HMEC cells are slightly more spread with few Ki-67–positive cells. C, In contrast, SP-treated HMEC cells were more spread toward SP and had numerous Ki-67⁺ cells. D, Human microvascular endothelial cells treated with the positive control VEGF also spread toward the direction of the agonist and had numerous Ki-67⁺ cells.

Fig. 7. Elevated glucose and fatty acid levels decrease HMEC migration on day 2

A, Using DMD to measure migration, glucose and fatty acids significantly decrease cell migration toward SP ($^{\dagger}P = 0.001$). Whereas vitamins C and E do increase migration toward the SP, alone ($^*P = 0.02$), they do not significantly reverse the inhibition by glucose and fatty acids. B, Measuring distance along the x and y axes confirms that glucose and fatty acids decrease HMEC migration along the x_1 axis ($^*P = 0.05$) with no effect on migration on the y axes. Using this measurement method, we can show that HMEC migration along the x_1 axis is improved with vitamins C and E, but the results do not reach significance ($^{\dagger}P = 0.06$).

Table 1

On day 2, directional migration distance (in millimeters) of SP-treated cells is longer than NaCl-treated cells or BSA-treated cells^{*}

	NaCl	BSA	SP	SP and spantide	VEGF
DMD	0.034 ± 0.140	0.001 ± 0.098	0.519 ± 0.138 [*]	0.060 ± 0.012 [*]	0.654 ± 0.950
x_1	0.777 ± 0.120	0.741 ± 0.149	0.964 ± 0.255 [†]	0.605 ± 0.111 [†]	1.142 ± 0.173
x_2	0.776 ± 0.110	0.751 ± 0.135	0.322 ± 0.221	0.584 ± 0.127	0.355 ± 0.144
y_1	0.729 ± 0.169	0.688 ± 0.256	0.567 ± 0.222	0.646 ± 0.143	0.504 ± 0.170
y_2	0.698 ± 0.148	0.671 ± 0.247	0.582 ± 0.247	0.613 ± 0.152	0.605 ± 0.168

^{*} $P < 0.001$.

[†] $P < 0.001$.

Table 2

Endothelial cell counts ($\times 10^3$; n = 4)

	NaCl	BSA	SP	SP and spanide	VEGF
Day 2	1.53 $\pm$ 0.44 [*]	3.25 $\pm$ 0.64 [*]	13.35 $\pm$ 1.84 ^{*†}	5.15 $\pm$ 1.04 [†]	18.16 $\pm$ 5.79
Day 3	3.00 $\pm$ 1.71 [†]	8.60 $\pm$ 1.61 [†]	31.00 $\pm$ 5.11 ^{†§}	15.1 $\pm$ 9.28 [§]	44.23 $\pm$ 5.91

Cell counts confirm that proliferation is increased with SP (13.35×10^3) and VEGF (18.16×10^3) compared with NaCl (1.53×10^3) and BSA (3.25×10^3). Spanide 1 (5.15×10^3) significantly decreased the proliferative effect of SP ($P < 0.0001$). This trend persisted on day 3. There is no significant difference in cell number with BSA treatment compared with NaCl treatment on either day 2 or day 3.

^{*} $P < 0.01$;
[†] $P < 0.001$;
[‡] $P < 0.001$;
[§] $P < 0.05$.

Table 3Endothelial cell counts ($\times 10^3$; n = 3) with and without metabolic perturbation for 48 h

	Medium alone	Vitamins C and E	Glucose and fatty acids	Glucose, fatty acids, and vitamins C and E
Control	$2.57 \pm 0.21^{*†‡}$	$4.97 \pm 0.15^*$	$1.8 \pm 0.2^{†§}$	$4.07 \pm 0.31^§$
SP	$10.7 \pm 0.26^{*†‡//}$	$14.27 \pm 0.42^{†//}$	$6.57 \pm 0.74^{//**}$	$8.23 \pm 0.6^{**}$

Cell counts on day 2 demonstrate that glucose and fatty acids significantly decrease cell proliferation in both NaCl-treated cells ($†P = 0.01$) and SP-treated cells ($//P = 0.005$). Vitamins C and E alone increase proliferation in cells treated with either NaCl ($*P = 0.0002$) or substance P ($†P = 0.001$). Vitamin C and E also significantly reverse the inhibitory effect of glucose and fatty acids on HMECs treated with either NaCl ($§P = 0.0009$) or SP ($**P = 0.04$).

$*P = 0.0002$;

$†P = 0.01$;

$‡P = 0.001$;

$§P = 0.0009$;

$†P = 0.0006$;

$//P = 0.005$;

$**P = 0.04$.