Soluble Receptor for Advanced Glycation End Products Improves Stromal Cell-Derived Factor-1 Activity in Model Diabetic Environments

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Objective: In diabetes, hyperglycemia causes the accumulation of advanced glycation end products (AGEs) that trigger reactive oxygen species (ROS) generation through binding the receptor for AGEs (RAGE). Because exogenous growth factors have had little success in enhancing chronic wound healing, we investigated whether hyperglycemia-induced AGEs interfere with cellular responses to extracellular signals. We used stromal cell-derived factor-1 (SDF-1), an angiogenic chemokine also known to promote stem cell recruitment in skin wounds.

Approach: Human leukemia-60 (HL-60) cells and mouse peripheral blood mononuclear cells (PBMCs), which express the SDF-1 receptor CXCR-4, were incubated for 24 h in medium supplemented with 25 mM D-glucose. Soluble RAGE (sRAGE) was used to block RAGE activation. Response to SDF-1 was measured in cellular migration and ROS assays. A diabetic murine excisional wound model measured SDF-1 liposome and sRAGE activity *in vivo*.

Results: Hyperglycemia led to significant accumulation of AGEs, decreased SDF-1-directed migration, and elevated baseline ROS levels; it suppressed the ROS spike normally triggered by SDF-1. sRAGE decreased the ROS baseline and restored both the SDF-1-mediated spike and cell migration. Topically applied sRAGE alone promoted healing and enhanced the effect of exogenous SDF-1 on diabetic murine wounds.

Innovation: While there is interest in using growth factors to improve wound healing, this strategy is largely ineffective in diabetic wounds. We show that sRAGE may restore signaling, thus potentiating the effect of exogenously applied growth factors.

Conclusion: Blocking RAGE with sRAGE restores SDF-1-mediated cellular responses in hyperglycemic environments and may potentiate the effectiveness of SDF-1 applied *in vivo*.

Keywords: hyperglycemia, advanced glycation end-products, HL60 cells, peripheral mononuclear white blood cells, cell migration, intracellular calcium

INTRODUCTION

ADVANCED GLYCATION END products (AGEs), which arise from proteins and lipids reacting with reactive sugars, accumulate in diabetes.¹ AGEs trigger proinflammatory sig-

nals through binding to the AGE receptor (RAGE), which results in persistent activation of the transcription factor NF- κ B and increased levels of reactive oxygen species (ROS) and matrix metalloproteinases.² These

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effects are believed to underlie many of the reported diabetic complications, including impaired wound healing.³

Diabetic wounds are also characterized by decreased growth factor levels that normally drive the wound healing cascade³; thus, several studies have explored adding exogenous growth factors to attempt to restore the wound healing process.⁴ One such depleted growth factor that is decreased in diabetes is stromal cell-derived factor-1 (SDF-1),^{5,6} a pleiotropic chemokine that promotes cellular migration,⁷ proliferation,⁸ and tissue vascularization.⁹ SDF-1 also enhances wound healing in wild-type mice but not in diabetic mice.^{10,11} Previously, increased AGEs *in vitro* have been shown to decrease SDF-1 secretion and, in turn, migratory ability of endothelial progenitor cells.¹²

The soluble form of RAGE (sRAGE) has been shown to prevent activation of the endogenous RAGE pathway even in the presence of elevated AGEs, thus suppressing NF- κ B activation *in vitro*,¹³ and to improve skin wound healing in diabetic mice.¹⁴ While these past reports establish the beneficial effects of sRAGE on the endogenous wound healing response, we inquired whether sRAGE could enhance the effectiveness of exogenously applied growth factors. For this purpose, we investigated the effect of AGEs on the biological activity of SDF-1 *in vitro* and *in vivo*. Our results suggest that AGEs inhibit SDF-1 signaling by elevating baseline ROS levels, thus preventing the ROS burst by SDF-1, which is a critical step in SDF-1-mediated signal transduction. sRAGE decreased the ROS baseline, thus restoring SDF-1 function *in vitro* as well as *in vivo* in a diabetic wound model.

CLINICAL PROBLEM ADDRESSED

Chronic nonhealing wounds are a common long-term complication of diabetes.^{15,16} In addition to being an economic burden with costs in billions per year, the estimates are that as high as 25% of all diabetics will develop a foot ulcer in their lifetime.¹⁷ While a variety of treatment approaches exist, some of the most technologically advanced options include skin substitutes; some products are tailored to chronic wounds, such as Dermagraft® and Apligraf®, with closure rates reaching up to 55%.^{18,19} Further attempts to enhance diabetic wound healing involve the use of growth factors, stem cells, and transgenes.²⁰ Regranex®, a platelet-derived growth factor gel, is the only growth factor-based product currently approved for clinical use, which typically needs multiple applications.⁴

MATERIALS AND METHODS

Human leukemia-60 cell line maintenance

Human leukemia-60 (HL-60) cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained at 10^5 – 10^6 cells/mL in the Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and 1% v/v penicillin–streptomycin (Life Technologies, Grand Island, NY).

Mouse mononuclear cell isolation and migration

Peripheral blood mononuclear cells (PBMCs) were isolated from C57Bl/6 wild-type mice (Charles River Laboratories, Wilmington, MA) and diabetic C57BLKS-Lepr^{db}-modified black mice (Jackson Laboratory, Bar Harbor, ME). The blood was collected into tubes with sodium citrate anticoagulant (Sigma-Aldrich, St. Louis, MO). For every 2 mL of blood, 2 mL of Roswell Park Memorial Institute (RPMI) medium (Life Technologies) was mixed, and the total volume was carefully pipetted onto 3 mL of Ficoll-Paque Premium 1.084 (GE Lifesciences, Pittsburgh, PA). After 60 min of centrifugation at 400 g, the plasma layer was removed, and the mononuclear cell layer was harvested and washed in preparation for migration studies.

High-glucose culture

HL-60 cells or PBMCs were transferred to 12-well plates (Becton Dickinson, Franklin Lakes, NJ) at 500,000 cells/mL in 1 mL of serum-containing IMDM or RPMI, respectively, to which 25 mM D-glucose or L-glucose (Sigma-Aldrich) had been added. Supplemental glucose was used to approximate diabetes-relevant conditions compared to nondiabetic levels of 5 mM.²¹

Transwell migration assay

The bottom of a 24-well plate was preloaded with 600 μ L/well of IMDM or IMDM supplemented with 100 ng/mL SDF-1 (R&D Systems, Minneapolis, MN). HL-60 cells or PBMCs preincubated for 24 h in normal or high glucose were suspended at 10^6 cells/mL in the IMDM, and a total of 100 μ L cell suspension/well was placed on top of Transwell porous inserts (transparent polyethylene terephthalate [PET] membrane, 8- μ m pores; Corning, Corning, NY). Additional study groups included the IMDM with or without SDF-1 plus 500 ng/mL sRAGE (Ann Marie Schmidt laboratory and Aviscera Bioscience, Santa Clara, CA). Plates were incubated at 37°C and harvested after 70 min. The number of cells that reached the bottom well were counted using a hemacytometer (Hausser, Horsham, PA) and normalized to the initial number of added cells.

HL-60 AGE content

After 24 h of culture in normal or high glucose, HL-60 cells were resuspended to 5×10^4 cells/mL in phosphate-buffered saline (PBS), and 1 mL was placed in each well of a 24-well plate. Cells were settled to the bottom of the wells by centrifugation for 2 min at 1,000 rpm. The cells were washed and fixed for 15 min at room temperature in 4% paraformaldehyde (Sigma-Aldrich), washed three times with PBS, and then incubated with 10% goat serum (Life Technologies) + 0.1% Triton-X (Sigma-Aldrich) in PBS for 45 min. Cells were incubated with 5 μ g/mL primary antibody (rabbit polyclonal anti-AGE; R&D Systems) or rabbit IgG isotype control (Millipore, Billerica, MA) overnight at 4°C. Cells were then brought to room temperature, washed three times with PBS, and incubated with 1.25 μ g/mL goat anti-rabbit IgG fluorescein secondary antibody (R&D Systems) for 1 h. The cells were washed twice with PBS, and their nuclei were counterstained with 1:5,000 4',6-diamino-2-phenylindole dihydrochloride (DAPI; Life Technologies) for 10 min and washed again. The cells were imaged under an Olympus IX81[®] microscope to visualize AGE-positive cells (green) and nuclei (blue). Five images were taken per replicate, and fluorescence per cell was determined using ImageJ software (NIH) after background subtraction. Mean fluorescence intensity of AGE-stained cells was normalized to that of isotype control-stained cells.

HL-60 viability and CXCR4 expression

After 24 h of culture in normal or high glucose, HL-60 cells were suspended in PBS at 2×10^6 cells/mL. Cell suspension (10 μ L) was placed in a small Petri dish, and 150 μ L of stain (2 μ M carboxyfluorescein diacetate and 4 μ M propidium iodide; Marker Gene Technologies, Eugene, OR) was placed over the cell suspension. After 30 min at 37°C, the cells were imaged under an Olympus IX81 microscope to visualize live (green) and dead (red) cells. Three images were taken per replicate, and percent viability was calculated as number of live (green) cells/total cells (red+green) $\times 100$. Cells were also viewed in Brightfield to confirm cell count accuracy.

To measure HL-60 surface expression of the primary SDF-1 receptor CXCR4, cells were washed three times with PBS + 0.1% bovine serum albumin (Sigma-Aldrich) and resuspended to 4×10^6 cells/mL in PBS. Aliquots of 10^5 cells were blocked with 2 μ L of mouse serum (Life Technologies) and incubated with 10 μ L of antibody (either mouse monoclonal anti-human CXCR4-allophycocyanin (APC) or mouse IgG2A isotype control-APC; R&D Systems) for 40 min at 4°C. Cells were then washed twice and

resuspended in 200 μ L of PBS/aliquot. Each aliquot was run on an FACSCalibur 2 laser flow cytometer (BD Biosciences, San Jose, CA) to capture APC fluorescence (FL-4) from at least 10^4 cells. CellQuest (Becton Dickinson) software was used to acquire and analyze the results, and percent-positive cells were calculated in relationship to isotype control-stained cells.

HL-60 RAGE and mDia1 expression

After 24 h of culture in normal or high glucose, HL-60 cells were cooled to 4°C and centrifuged at 400 *g* for 7 min at 4°C. The cells were washed with cold PBS and centrifuged in a microcentrifuge for 10 min at 1,500 rpm at 4°C. The pellets were resuspended in 100 μ L of radioimmunoprecipitation (RIPA) buffer with 1:100 ethylenediaminetetraacetic acid (EDTA) and 1:100 Halt protease inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). Cell suspensions were rotated for 30 min at 4°C and then centrifuged for 20 min at 16,000 *g* at 4°C. A bicinchoninic acid-based assay was performed to determine total protein content (ThermoFisher Scientific). To quantify RAGE and mDia1 content, protein was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Bio-Rad, Hercules, CA) and subsequently transferred to nitrocellulose membranes. Nonspecific binding was blocked by a 2-h incubation at room temperature with TBST, consisting of 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (Bio-Rad). Primary rabbit polyclonal antibody to RAGE, mDia1, and GAPDH used as internal control (Millipore) were incubated with the membrane for 16 h at 4°C in a blocking solution. After incubation, membranes were washed with TBST and then incubated for 1 h with secondary goat anti-rabbit IgG horseradish peroxidase (Abcam, Cambridge, MA) at room temperature. Binding sites were identified by the SuperSignal[™] west picochemiluminescent substrate (ThermoFisher Scientific) for 5 min. Bands were digitized with a Canon MX420 scanner (Canon, Melville, NY).

Baseline and SDF-1-induced superoxide measurements

Twelve-well plates were coated with 125 μ L of fibronectin solution (200 μ g/mL in PBS; Becton Dickinson) per well for 1 h at room temperature. HL-60 cells were washed and resuspended to 5×10^5 cells/mL in PBS. Suspensions were then incubated with dihydroethidium (DHE) 1:1,600 (Sigma-Aldrich) for 45 min at 37°C, washed in PBS, plated on the fibronectin-coated wells at a density of 5×10^5 cells/well, incubated for 15 min at 37°C to allow for cell attachment, and washed in PBS. Each

well was then visualized under an Olympus IX81 microscope to capture background images. The PBS was removed and replaced with 250 μ L of fresh PBS, after which images were taken every 30 s for 3.5 min. The wells were then replaced with 250 μ L of SDF-1 100 ng/mL in PBS after which images were taken every 30 s for 3.5 min. Images (five per replicate) were digitally captured and analyzed using ImageJ software to determine mean fluorescence intensity after subtracting the background.

Preparation of SDF-1-coated liposomes

Nanosized liposomes ($\sim$ 100 nm diameter) were made as previously described.¹¹ Briefly, 10 μ mol of total lipid containing a molar ratio of 7:2:1 of distearoylphosphatidylcholine (DSPC):cholesterol:distearoylphosphatidic acid (DSPA; Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform in a 25-mL round-bottom flask. Chloroform was evaporated in a rotavapor for 10 min at 55°C followed by exposure to nitrogen for 5 min. The lipid film was hydrated in 1 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–sucrose buffer (pH = 7.4; Sigma-Aldrich) for 2 h with a final lipid concentration of 10 mM. Liposomes were formed by extruding the lipid suspension 21 times through two stacked polycarbonate membranes of 200 nm pore size and then again through 100 nm membranes in an 80°C water bath. Liposomes were diluted to 100 μ M lipid, and recombinant SDF-1 (R&D Systems) in the HEPES–sucrose buffer was vortexed in at a molar ratio of 1:9 with respect to DSPA.

Diabetic murine excisional wound model

Animal studies were carried out in accordance with the National Research Council guidelines and approved by the Institutional Animal Care and Use Committee at the Rutgers University. C57BLKS-Lepr^{db} genetically diabetic mice (strain BKS.Cg-Dock7^m+/+ Lepr^{db}/J; Jackson Laboratory) were used at the age of 10 weeks. One day before surgery, mice were anesthetized by isoflurane (Henry Schein, Melville, NY) inhalation on a heating pad at 37°C. Anesthesia was deemed sufficient when the animal lacked response to a toe pinch. The eyes of the animal were covered with medical ointment (Henry Schein) to prevent dryness. Dorsal hair was clipped and completely removed using NairTM cream (Church & Dwight, Ewing, NJ). On the day of surgery, the mice were reanesthetized, and the dorsum was prepared by a three-fold alternating application of Betadine scrub and 70% alcohol. A 1 $\times$ 1 cm square area of skin was cut out on the back of the animal to generate a full-thickness skin defect. An equivalent area of Integra[®] collagen–glycosaminoglycan dermal skin substitute (Integra

LifeSciences, Plainsboro, NJ) was applied onto the wound with vehicle control, SDF-1 liposome, or sRAGE + SDF-1 liposome treatment. The vehicle control treatment consisted of 100 μ L of HEPES–sucrose buffer applied at 0, 2, 4, and 6 days post-wounding. The SDF-1 liposome treatment consisted of 0.88 μ g of SDF-1 within liposomes in 100 μ L of HEPES–sucrose buffer applied at day 0 and 100 μ L of plain buffer applied at days 2, 4, and 6. The sRAGE + SDF-1 liposome treatment consisted of 0.88 μ g of SDF-1 within the liposomes in 100 μ L of HEPES–sucrose buffer applied at day 0 and 20 μ g of sRAGE in 100 μ L of HEPES–sucrose buffer applied at days 0, 2, 4, and 6. The wound was covered by TegadermTM (3M, St. Paul, MN), a semioclusive silicone dressing, and secured with 6-0 black silk braided sutures. Gross wound morphology was monitored over 35 days, and digital images of the wounds were taken weekly. Wound area was quantified on ImageJ. The wound closure percentage was calculated using the following formula: (1–remaining open wound area/initial wound area) $\times$ 100. The number of animals per group was 5–7.

Statistics

All values are presented as mean $\pm$ standard error of the mean (SEM). Multiple comparisons were carried out using one-way ANOVA followed by *post hoc* analysis. *Post hoc* tests are indicated in the figure legends.

RESULTS

Transwell cell migration model

To optimize assay conditions and to characterize the migratory response to SDF-1, we used the promyelocytic HL-60 cell line, which expresses high levels of CXCR-4.²² A preliminary dose–response in the range of 0–200 ng/mL SDF-1 established that 100 ng/mL SDF-1 induced a robust, but non-saturating, migration response after 70 min (Fig. 1A). Furthermore, a dose–response for the effect of supplemental glucose in the range of 0–50 mM D-glucose added to the IMDM during preincubation established that 25 mM supplemental D-glucose, the dose typically used in other studies,²³ caused a significant, yet nonsaturating, inhibition of migration in this assay. Subsequently, a dose of 100 ng/mL SDF-1 was used to assess SDF-1–mediated biological responses, and 25 mM D-glucose supplementation was used to simulate hyperglycemic conditions.

sRAGE restores SDF-1-induced migration of hyperglycemic leukocytes

To investigate the impact of hyperglycemia on leukocyte migration to SDF-1, HL-60 cells were

Figure 1. SDF-1 induces directed HL-60 migration, and increased glucose decreases biological activity of SDF-1. **(A)** HL-60 cells in regular IMDM were transferred on top of the Transwell membrane to measure the migratory response to 0, 25, 50, 100, and 200 ng/mL SDF-1. **(B)** HL-60 cells were preincubated in regular IMDM, IMDM +12.5 mM D-glucose, IMDM +25 mM D-glucose, or IMDM +50 mM D-glucose for 24 h and then transferred on top of the Transwell membrane to measure the migratory response to 100 ng/mL SDF-1. Data shown are the number of cells that migrated across the membrane after 70 min, normalized to the initial number of cells added (10^5 /well). ** $p < 0.01$ compared to no-SDF-1 group, * $p < 0.05$ compared to no-SDF-1 group; one-way ANOVA, Fisher's LSD posttest. HL-60, human leukemia-60; IMDM, Iscove's modified Dulbecco's medium; SDF-1, stromal cell-derived factor-1.

preincubated for 24 h in regular IMDM (normal glucose group), IMDM with additional 25 mM D-glucose (high-glucose group), or IMDM with additional 25 mM L-glucose (glucose decoy group). L-glucose is an enantiomer that does not enter

central carbon metabolism.²³ Furthermore, cell viability postincubation was $93.4\% \pm 1.5\%$ in the normal glucose, $94.0\% \pm 3.0\%$ in the high-glucose, and $94.1\% \pm 1.0\%$ in the glucose decoy groups ($N=6$), indicating that these conditions caused no change in viability.

In the absence of SDF-1, ~5% HL-60 cells migrated across the Transwell membrane, regardless of glucose supplementation (Fig. 2A). With 100 ng/mL SDF-1 below the membrane, the migrating cell fraction increased threefold in normal IMDM; however, hyperglycemia (high-glucose group) completely prevented the chemotactic response to SDF-1. Conversely, migration to SDF-1 was unaffected in IMDM supplemented with L-glucose (glucose decoy group), suggesting that metabolic processing of the D-glucose is required for inhibition. The presence of sRAGE wholly restored the migratory response to SDF-1. The culture of HL-60 cells in sRAGE alone had no effect on SDF-1-directed HL-60 cell migration.

To verify that these observations also apply to primary cells, PBMCs were isolated from C57Bl/6 wild-type mice, subjected to the same preincubation conditions, and assessed for their migration to SDF-1. Baseline migration, in the absence of SDF-1, was higher than HL-60 cells, ~35% (Fig. 2B). The migrating fraction increased to 75% with 100 ng/mL SDF-1. Preincubation in the high-glucose group suppressed SDF-1-mediated migration, which was also reversed if sRAGE was present. The migratory responses were unaffected in the glucose decoy group or by sRAGE alone. Thus, these results mimic the trends observed with the HL-60 cells.

Since these data were obtained from *in vitro*-induced hyperglycemic conditions over a short timescale, we proceeded to determine whether PBMCs from diabetic mice would exhibit similar behavior. PBMCs isolated from 10-week-old *Lepr^{db}* diabetic mice exhibited a somewhat lower baseline migration (27%) compared to cells from wild-type mice and a marginal nonsignificant increase in migration in the presence of SDF-1 (Fig. 2C). Adding sRAGE increased the migration to ~45%, thus making it statistically significant over baseline. These results confirm the ability of sRAGE to enhance SDF-1-mediated migration of leukocytes that were chronically exposed to *in vivo* diabetic conditions.

Hyperglycemia increases AGEs without affecting CXCR-4, RAGE, or mDia-1 in HL-60 cells

We hypothesized that hyperglycemic culture may increase AGE levels based on prior knowledge

Figure 2. sRAGE reverses hyperglycemia-induced impairment of mononuclear leukocyte migration to SDF-1. **(A)** HL-60 cells or **(B)** peripheral mouse mononuclear cells from wild-type mice were preincubated in regular IMDM (normal glucose), IMDM +25 mM D-glucose (high glucose), or IMDM +25 mM L-glucose (glucose decoy) for 24 h. Then, cells were transferred on top of the Transwell membrane to measure the migratory response to 100 ng/mL SDF-1 and/or 500 ng/mL sRAGE. Data shown are the number of cells that migrated across the membrane after 70 min, normalized to the initial number of cells added (10^5 /well). **(C)** Migratory response of freshly isolated peripheral mouse mononuclear cells from *Lepr^{db}* mice to 100 ng/mL SDF-1 and/or 500 ng/mL sRAGE. * $p < 0.01$ compared to no-SDF-1 group, + $p < 0.05$ compared to SDF-1 group; one-way ANOVA, Fisher's LSD posttest. sRAGE, soluble receptor for advanced glycation end products.

that diabetes increases AGE levels¹⁴ and the observation that hyperglycemic inhibition of SDF-1-mediated migration was reversed by sRAGE. Thus, we measured the accumulation of AGEs in HL-60 cells after 24 h of culture in regular IMDM (normal glucose group), IMDM supplemented with 25 mM D-glucose (high-glucose group), or IMDM supplemented with 25 mM L-glucose (glucose decoy group). The normal glucose and glucose decoy

groups had very low levels of AGEs, while a dramatic increase occurred in the high-glucose group (Fig. 3A, B). These results confirm that elevated D-glucose increases AGE levels and that the biologically active enantiomer, D-glucose, was required for AGE accumulation. We then explored whether hyperglycemic culture impaired the expression of CXCR-4 but found that HL-60 cells incubated in normal glucose versus high glucose had virtually

Figure 3. Hyperglycemic culture increases AGE content but does not affect the expression of CXCR-4, RAGE, or mDia-1 in HL-60 cells. HL-60 cells were preincubated in regular IMDM (normal glucose), IMDM +25 mM D-glucose (high glucose), or IMDM +25 mM L-glucose (glucose decoy) for 24 h. Cell aliquots were either fixed with 4% paraformaldehyde and stained with anti-AGE or anti-CXCR-4 antibodies or processed for protein extraction and separation by SDS-PAGE. **(A)** Representative images of HL-60 cells stained for AGEs (green) with nuclear counterstaining (blue). **(B)** MFI by image analysis of AGE staining normalized to the isotype control. $N=4$. * $p<0.01$ versus normal glucose; one-way ANOVA, Fisher's LSD posttest. **(C)** Flow cytometric analysis of CXCR-4 expression showing cell number versus CXCR-4-related fluorescence intensity. Fluorescence gated at 5%. Representative data from $N=6$ experiments. **(D)** Representative of $N=2$ western blots for RAGE and mDia-1 content in incubated HL-60 cells. Also included is a group of HL-60 cells incubated in high glucose +500 ng/mL sRAGE. Housekeeping GAPDH is shown as internal control. AGE, advanced glycation end product; MFI, mean fluorescence intensity; RAGE, receptor for AGEs; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

identical distributions in CXCR-4 expression across the cell population (Fig. 3C). Finally, we investigated whether any of the culture conditions had an impact on the expression of RAGE, or its intracellular signaling partner mDia-1, but found no differences as seen by western blot analysis (Fig. 3D).

Hyperglycemia impairs SDF-1-mediated signaling, which is restored by sRAGE

Since hyperglycemic culture impaired SDF-1-directed HL-60 cell migration while there was no change in CXCR-4 expression, we focused on in-

tracellular SDF-1-mediated signaling events that might be adversely impacted by hyperglycemia. SDF-1 triggers a burst in ROS, which is critical to propagate the signal downstream to eventually cause cell migration.^{24,25} We therefore investigated the ability of SDF-1 to increase ROS levels in HL-60 cells after preincubation in normoglycemic versus hyperglycemic conditions. For this purpose, after 24 h of preincubation in normal glucose, high-glucose, glucose decoy, or high-glucose + sRAGE conditions, HL-60 cells were stained with the superoxide-sensitive dye DHE. HL-60 cells in the

high-glucose group exhibited an approximately twofold increase in baseline ROS levels, compared to those in the normal glucose group (Fig. 4A). This increase was completely abolished in the high-glucose + sRAGE group and significantly (although more partially) decreased in the glucose decoy group. There was a significant impact of these preincubation conditions on the SDF-1-mediated change in ROS levels. In the normal glucose group, SDF-1 triggered an ~25% increase in ROS levels, and a similar magnitude change was seen in the glucose decoy group (Fig. 4B). In contrast, a slight decrease in ROS levels before and after SDF-1 stimulation was measured in the high-glucose group. The slow decrease appeared to continue at the same rate as before SDF-1 addition, suggesting that SDF-1 had no effect on ROS levels in this group. sRAGE, in contrast, prevented, and even to

some extent enhanced, the SDF-1-triggered change in ROS levels. Taken together, these results support the notion that hyperglycemic conditions increase baseline ROS levels through a RAGE-mediated mechanism, and that elevated ROS mask, and/or prevent any ROS generation by the SDF-1 signaling pathway.

sRAGE increases efficacy of exogenous SDF-1 in a diabetic murine excisional wound model

Next, we hypothesized that sRAGE would potentiate the effect of SDF-1 applied *in vivo* in *Lepr^{db}* diabetic mice. A liposomal formulation for SDF-1 was used to increase SDF-1 stability *in vivo*.¹¹ The wounds were covered with a porous collagen-glycosaminoglycan matrix (IntegraTM) used as carrier, onto which vehicle (PBS), or 0.88 μ g of liposomal SDF-1, was applied. Some animals

Figure 4. Hyperglycemic culture elevates baseline ROS levels, abrogating SDF-1-mediated signaling, which is then restored by sRAGE. HL-60 cells were preincubated in regular IMDM (normal glucose), IMDM +25 mM D-glucose (high glucose), IMDM +25 mM L-glucose (glucose decoy) for 24 h, or 25 mM D-glucose +500 ng/mL sRAGE (high glucose + sRAGE). HL-60 cells were then stained with the ROS-sensitive dye dihydroethidium and plated on fibronectin-coated wells. Baseline fluorescence images were taken; cells were washed with PBS buffer, and new images were taken after 1 and 3.5 min. The cells were then switched to PBS containing 100 ng/mL SDF-1, and new images were taken after 1 and 3.5 min. **(A)** MFI per well during entire experimental timeline. **(B)** MFI increases because of addition of SDF-1 (MFI at 3.5 min post-SDF-1 addition minus MFI immediately prior SDF-1 addition). $N=5$. $**p<0.01$, $*p<0.05$ versus high glucose; one-way ANOVA, Fisher's LSD posttest. PBS, phosphate-buffered saline; ROS, reactive oxygen species.

receiving liposomal SDF-1 were also administered 20 μ g of sRAGE at 0, 2, 4, and 6 days postwounding (Fig. 5). SDF-1 liposome treatment alone resulted in a significant improvement in wound closure percentage at 35 days postwounding compared to vehicle control. The combination sRAGE + liposomal SDF-1 further increased wound closure rate, with a significantly more closed wound at 28 days postwounding compared to the vehicle control as well as the SDF-1 liposome groups.

DISCUSSION

The data presented herein show that hyperglycemic conditions inhibit chemotactic responses to SDF-1 in HL-60 and mouse PBMCs. Furthermore, sRAGE, which has been used to block proinflammatory signals emanating from AGE binding to RAGE,^{14,26} restores SDF-1-directed migration *in vitro*. Hyperglycemic culture also caused the accumulation of AGEs without changing the expression of the relevant receptor RAGE and its signaling partner mDia-1. Furthermore, there was no impact of hyperglycemic culture on surface levels of the SDF-1 receptor CXCR-4. However, hyperglycemic culture increased baseline ROS levels in HL-60 cells and prevented the ROS burst that normally occurs upon SDF-1 stimulation. sRAGE returned the ROS baseline to normal and restored the SDF-1-induced ROS burst. Taken together, these data suggest that SDF-1-directed

migration is impaired by hyperglycemic conditions because of increased ROS levels from RAGE signaling by accumulated AGEs.

The relationships between SDF-1 and ROS, as well as RAGE and ROS, have been previously and independently studied in various cell types. Upon SDF-1 binding to the CXCR4 receptor, there is a signaling pathway that involves transient production of ROS and the activation of phosphoinositide 3-kinase (PI3K) to eventually promote cell migration and survival.^{24,25} Cells in culture increase intracellular ROS within seconds when stimulated with SDF-1.²⁷ The activation of RAGE increases levels of ROS in the cell²⁸ and signals mDia1, an intracellular signaling partner to RAGE, which has been implicated in smooth muscle cell migration and expansion.²⁹ It is clear that ROS is important for migration; when nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the enzyme that generates superoxide, is blocked in neutrophils, they experience decreased migration.³⁰ However, excess levels of ROS are also detrimental as van Aalst *et al.* showed that incubation of endothelial cells with oxidized low-density lipoprotein both increased ROS levels and decreased cell migration compared to cells cultured in a normal medium.³¹ Prolonged exposure to ROS does not necessarily decrease CXCR4 expression as prior studies have shown that only very high levels of AGEs (500 μ g/mL) decreased receptor expression.¹²

Thus, ROS is a common intracellular messenger in the signaling pathways activated by CXCR4 and RAGE. On the one hand, the ROS spike is integral to the SDF-1 signaling pathway,^{24,25} and on the other hand, AGE binding to RAGE increases ROS levels.²⁸ Since the site of ROS generation in both cases is in the cytosol,^{24,28} it is plausible that the two pathways intersect in a negative way. For example, by increasing baseline ROS generation, AGEs may deplete substrates available to NADPH oxidase (Nox),³² thus impairing the ability to generate the ROS burst in response to SDF-1. Alternatively, ROS generation may still occur but it may be washed out in the high-ROS background, thus preventing the signal from being carried further downstream. In summary (Fig. 6), we hypothesize that under normal glucose conditions, there is a baseline level of superoxide, which spikes upon SDF-1 stimulation, thus resulting in cell migration. High glucose increases AGEs and superoxide but prevents the superoxide burst when SDF-1 is added, thus impairing migration. The addition of sRAGE restores superoxide levels, the SDF-1-induced superoxide spike, and thus cell migration.

Figure 5. sRAGE improved wound closure percentage at days 28 and 35. Treatments of HEPES-sucrose buffer (vehicle control), SDF-1 liposomes, and an sRAGE-SDF-1 liposome combination were applied to a 1 × 1 cm excisional wound in genetically diabetic *Lepr^{db}* mice. *N* = 5. *Value is significantly higher than vehicle at days 28 and 35 (*p* < 0.05; one-way ANOVA, Fisher's LSD posttest). HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Figure 6. Postulated pathway for decreased SDF-1 migration in high glucose. **(A)** Under normoglycemia, there is a baseline amount of glucose, AGEs, and ROS in the cellular environment. When SDF-1 is added, there is a spike of superoxide, which is integral to the signal transduction pathway leading to cellular migration. **(B)** In hyperglycemic conditions, there is increased glucose, AGEs, and RAGE, leading to high baseline ROS in the cell. When SDF-1 is added, no superoxide burst occurs, leading to impaired downstream signaling and migration. **(C)** In hyperglycemic conditions, but in the presence of sRAGE, AGEs and ROS levels return to their normal baseline, allowing once again for the SDF-1 to cause a spike in superoxide, thus restoring signaling and migratory response.

A limitation of the *in vitro* system used herein is that hyperglycemic conditioning is carried out over a few hours, while diabetic complications progress over prolonged periods (typically weeks in mice and years in humans). We also use HL-60 cells as a model system because of their stable expression of CXCR4. It is likely that more widespread changes, perhaps affecting the cell migration apparatus, such as F-actin,³³ occur during chronic diabetic conditions that cannot be fully addressed by short-term treatment with sRAGE. Nevertheless, we found that sRAGE also improved SDF-1-mediated

migration of PBMCs isolated from *Lepr^{db}* diabetic mice, suggesting that acutely administered sRAGE reverses, at least to some extent, the migration impairment that results from prior chronic exposure of the cells to the diabetic environment. This was also confirmed by our observation that sRAGE potentiated the ability of liposomal SDF-1 to accelerate the closure of excisional wounds in diabetic mice. It is noteworthy that a liposomal form of SDF-1 was used for this purpose because it has more potent and long-lasting effects than free SDF-1.¹¹

Prior reports have indicated that sRAGE alone modulates the inflammatory response and also improves skin wound healing. For example, in an *in vivo* diabetic mouse wound model, sRAGE decreased inflammation and improved collagen deposition as well as growth factor secretion.¹⁴ *In vitro* studies with AGE-conditioned epithelial cells showed decreased secretion of interleukin-6, a proinflammatory cytokine, after sRAGE treatment.¹³ Thus, while sRAGE may improve endogenous wound healing processes, this report also shows that sRAGE potentiates exogenously applied therapeutic agents, such as SDF-1, to improve wound healing *in vivo*.

INNOVATION

There is currently one FDA-approved growth factor treatment for use even though many have shown promise in preclinical animal models. Given the depletion of factors in diabetic wounds that stall healing, we set out to identify ways to improve exogenous growth factor treatments. The use of sRAGE to block RAGE binding has previously been shown to have immunomodulatory effects; in this report, we show the potential for sRAGE to also improve the cellular response to exogenous growth factors in hyperglycemic environments.

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AUTHOR DISCLOSURE AND GHOSTWRITING

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KEY FINDINGS

- Hyperglycemic *in vitro* culture conditions decrease SDF-1–directed migration of HL-60 and PBMCs, increase intracellular AGEs, and dysregulate ROS signaling.
- sRAGE restores ROS signaling and, in addition, reverses the migration impairment.
- In genetically diabetic mouse excisional wounds, the use of sRAGE improves percent wound closure of SDF-1 liposome-treated animals.

Tissue Engineering and Organ Fabrication in the Department of Surgery at the Massachusetts General Hospital, Harvard Medical School, Boston, MA. **Henry C. Hsia** was the Director of the Wound Center-Plastic Surgery program at the Robert Wood Johnson Medical School, Rutgers University. He recently he moved to the Yale University School of Medicine to become the Founding Director of the Yale Regenerative Wound Healing Center, New Haven, CT. **Ann Marie Schmidt** is the Dr. Iven Young Professor of Endocrinology, Department of Medicine, also holds appointments in the Department of Biochemistry and Molecular Pharmacology, as well as the Department of Pathology, and directs the Diabetes Research Program at the New York University Langone Medical Center, New York, NY. **François Berthiaume** is an Associate Professor of Biomedical Engineering at the Rutgers University, New Brunswick, NJ. Before joining Rutgers, he was a postdoctoral fellow and junior faculty at the Massachusetts General Hospital, Harvard Medical School, and the Shriners Hospitals for Children. He originally received a PhD in Chemical Engineering from the Pennsylvania State University.

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Abbreviations and Acronyms

AGEs = advanced glycation end products
 APC = allophycocyanin
 CXCR4 = C-X-C chemokine receptor type 4
 DAPI = 4',6-diamino-2-phenylindole dihydrochloride
 DHE = dihydroethidium
 DSPA = distearoylphosphatidic acid
 DSPC = distearoylphosphatidylcholine
 EDTA = ethylenediaminetetraacetic acid
 HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 HL-60 = human leukemia-60
 IMDM = Iscove's modified Dulbecco's medium
 NADPH = nicotinamide adenine dinucleotide phosphate
 PBMC = peripheral blood mononuclear cells
 PBS = phosphate-buffered saline
 PET = polyethylene terephthalate
 PI3K = phosphoinositide 3-kinase
 RAGE = receptor for AGEs
 RIPA = radioimmunoprecipitation
 ROS = reactive oxygen species
 RPMI = Roswell Park Memorial Institute
 SDF-1 = stromal cell-derived factor-1
 SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 SEM = standard error of the mean
 sRAGE = soluble receptor for AGEs