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Original Research

Vessel Ultrasound Sonographic Assessment of Soluble Receptor for Advanced Glycation End Products Efficacy in a Rat Balloon Injury Model

Hyun-Jin Tae, DVM, PhD[1,3,†], Natalia Petrashevskaya, PhD[1,†], Ismayil Ahmet, PhD[1,†], Sungha Park, MD, PhD[2], Mark I. Talan, MD, PhD[1], Edward G. Lakatta, MD[1], Li Lin, PhD[1,*]

[1] *Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health, Baltimore, Maryland*
[2] *Division of Cardiology, Cardiovascular Center, Yonsei University College of Medicine, Seoul, Korea*
[3] *Current affiliation: Department of Biomedical Science and Research Institute for Bioscience and Biotechnology, Hallym University, Chunchon, Korea*

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ABSTRACT

Objective: We aimed to assess the therapeutic efficacy of differentially modified soluble receptor for advanced glycation end products (sRAGE) in vivo using vessel ultrasound sonography and to compare the sonography data with those from postmortem histomorphologic analyses to have a practical reference for future clinical applications.
Methods: Vessel ultrasound sonography was performed in a sRAGE-treated rat carotid artery balloon injury model at different time points after the surgery, and therapeutic efficacy of different doses of sRAGE produced in Chinese hamster ovary cells and with different N-glycoform modifications were assessed.
Results: Vessel ultrasound sonography found that sRAGE produced in Chinese hamster ovary cells with complex N-glycoform modifications is highly effective, and is consistent with our recent findings in the same model assessed with histology. We also found that sonography is less sensitive than histology when a higher dose of sRAGE is administered.
Conclusions: Sonograph results are consistent with those obtained from histology; that is, sRAGE produced in Chinese hamster ovary cells has significantly higher efficacy than insect cell-originated sRAGE cells.

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Introduction

Inflammation is a natural protective mechanism that resolves infections and injuries of the target tissue.[1] Failure to effectively resolve the inflammation and return the affected tissue to homeostasis leads to maladaptation and precipitation of pathophysiologic consequences that often result in the development of chronic maladies, including cardiovascular diseases and diabetic complications.[2,3] It has been well established that injury-elicited inflammation in the vasculature often causes excessive proliferation of vascular smooth muscle cells within vessel walls and the subsequent expansion of the intima, leading to the eventual blockage of the vessel.[4–6] These remodeling processes are intensified especially in patients with diabetes. Although effective in combating neointima hyperplasia, current anti-inflammatory and antimitotic drugs often display significant side effects and toxicity that deem systemic applications unfeasible, and their local delivery is achieved via drug-eluting stents.[7,8] Therapeutic reagents that can be administered systemically stand the benefit of providing alternative avenues for treating acute vascular injuries as well as circumventing overall chronic inflammation in the vasculature.[9]

RAGE is a pattern recognition receptor that recognizes multiple endogenous ligands and triggers innate and adaptive immune responses.[10–14] Signaling via RAGE has been associated with vascular inflammation and implicated in the development of cardiovascular diseases.[15,16] Prior studies[17,18] have shown that administration of sRAGE can protect against injury-mediated vascular inflammation and neointimal expansion by functioning as a RAGE decoy. Such protection by sRAGE may also be extended to other inflammatory conditions, including diabetic complications and atherosclerosis.[19,20] Our recent study[21] demonstrated that N-glycoform modifications of sRAGE modulate its bioactivity: compared with sRAGE produced in insect Sf9 cells used in previous

* Address correspondence to: Li Lin, PhD, Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224
E-mail address: linli@mail.nih.gov (L. Lin).
† These authors contributed equally to this work.

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studies (ie, 5 μg/g body weight, daily injection for a week), a single, low dose of sRAGE produced in Chinese hamster ovary cells ($sRAGE^{CHO}$) (ie, 3 ng/g body weight) can substantially reduce neointima growth and inflammation in a rat carotid balloon injury model. These findings render $sRAGE^{CHO}$ an attractive therapeutic candidate with clinical potential.[13,22]

Although previous studies assessed how sRAGE treatment affected neointimal growth via histomorphologic analyses of postmortem vessel sections,[17,18,21] direct assessment of sRAGE action in vivo has not been performed. Vessel ultrasound sonography is a technique that can be used noninvasively in clinical practice to monitor arterial structure and function.[23–25] To further validate sRAGE efficacy to suppress neointimal growth, and to provide a basis for future clinical applications, we performed sonograph studies on carotid balloon denudation-injured rats before histology and compared these results with those observed in histomorphologic analyses. Such studies render an independent assessment of $sRAGE^{CHO}$ efficacy in vivo, and can validate its potential as a candidate therapeutic protein for treating vascular injury and inflammation. The present study was an extension of our previously published work.[21]

Materials and Methods

Subjects

Male Wistar rats (400–450 g) were purchased from Charles River Laboratories (Wilmington, Massachusetts) and maintained in a vivarium fed the National Institute on Aging on ad libitum food diet (NIH-07 mouse/rat diet; National Institutes of Health, Bethesda, MD) with access to filtered water. Each study group contained 6 to 15 rats.

Carotid artery balloon denudation injury procedure

The surgical procedure and postsurgery care have been described in detail,[21] and have been in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 3040-2, revised 1999), and with the institutional Animal Care and Use Committee approved protocol.

Production and administration of sRAGE

Immediately following the surgery, rats were administered the designated dose of sRAGE via intraperitoneal injection. Generation of $sRAGE^{CHO}$, $sRAGE^{Sf9}$, and $sRAGE^{CHO}$(N25T/N81T) expression vectors, as well as purification of sRAGE recombinant protein have been described in detail.[21]

Vessel ultrasound sonography

Vessel ultrasound sonography was conducted on the 0 Day (ie, surgery day, before surgery), and on the seventh and 14th day postsurgery. Rats were sedated with isoflorane (2% in oxygen) via facemasks, and put in the supine position. After shaving frontal neck skin hair, a 40 MHz probe was used to scan the carotid arteries. An M-mode tracing was recorded at 3 points in the long-axis view: 3 and 10 mm distal to the base, and 2 mm proximal to the bifurcation. Each M-mode tracing included the whole vessel wall thickness and lumen diameter. Vessel wall thickness was also recorded alone, using a zoom-in function. A B-mode scan was recorded at 2 mm proximal to the bifurcation. Each vessel was measured 5 times. The vessel wall thickness and lumen diameter at minimal and maximal points were measured using National Institutes of Health Image J software. The parameters from the nonoperated right carotid artery of the same rat were used as control.

Tissue collection and histomorphologic analysis

Isolation of carotid vessels and histomorphologic analyses have been described.[21] In all analyses, parameters from the nonoperated right side of the carotid artery of the same subjects were used as the normal control relative to the balloon-injured left side of the carotid artery.

Allocation concealment

Allocation concealment was applied to balloon denudation surgery, sonographic studies, and histomorphologic analyses. Investigators involved in the procedure were also blinded in respect to sRAGE types and dose administered.

Statistical analysis

Numerical data are expressed as means (SEM). Sonographic data were analyzed with multisample comparison ANOVA with post hoc Bonferonni corrections. A value of $P < 0.05$ was considered statistically significant.

Results

Beneficial effects of $sRAGE^{CHO}$ treatment measured by vessel ultrasound sonography 1 to 2 weeks after the injury

Our previous studies[21] had demonstrated that administration of sRAGE immediately after arterial injury is most therapeutically effective. To monitor $sRAGE^{CHO}$ effects in live rats with carotid arterial injury, we performed the ultrasound sonography procedure on rats before the surgery, and at 1 and 2 weeks postsurgery. Although at 1 week postsurgery the maximal vessel lumen diameter of injured vessels treated by $sRAGE^{CHO}$ is clearly distinguishable from that of placebo-treated vessels (Figure 1A), the measurement of average vessel wall thickness of these 2 groups was not clearly differentiated until 2 weeks postsurgery (Figure 1B), suggesting that sufficient time (ie, at least 1 week) is required to assess the benefits of $sRAGE^{CHO}$ treatment in a live animal model.

Correlation of ultrasound sonography and histomorphologic analyses

To test the correlation of sonographic data with that obtained from postmortem histologic measurement, we plotted the data from the 2 independent measurements, using the lowest effective dose (ie, 0.5 ng/g body weight). Despite the shrinkage of vessel cross-sections during the histologic process, reasonably high correlations between the data from the 2 measurements were apparent in the scatter plots (lumen diameter: $R = 0.72$; vessel wall thickness: $R = 0.76$) (Figure 2A and 2B), suggesting that the effect of $sRAGE^{CHO}$ treatment can be independently, and perhaps reliably monitored in vivo (Figure 2C).

Vessel sonographic assessment in rats treated with different sRAGE doses and sRAGE produced from different cells

On the basis of timing and correlation with histologic results shown in Figures 1 and 2, we also measured lumen diameter and vessel wall thickness at 2 weeks postsurgery in rats treated with $sRAGE^{CHO}$ at lower (0.5–1.5 ng/g body weight) (Figure 3A and 3B) and higher doses (1.5–6 ng/g body weight) (Figure 3C and 3D). When vessel wall thickness was measured, 1.5 ng/g and higher doses of $sRAGE^{CHO}$ treatment appeared to be statistically similar to those of nonoperated vessels (Figure 3A and 3C). However when lumen diameter was measured, dose-dependent improvement in

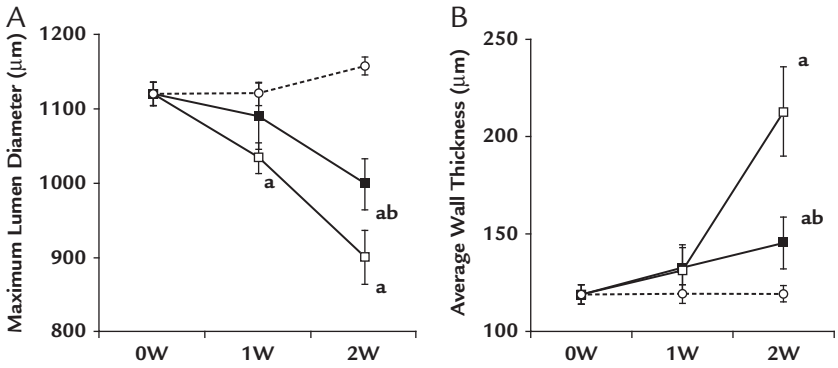

Figure 1. Observation of soluble receptor for advanced glycation end products (sRAGE) effects using vessel ultrasound sonography during 2 weeks of postarterial injury. (A) Maximal lumen diameter during the 2 weeks postinjury. (B) The average vessel wall thickness during the 2 weeks postinjury. Empty circle = nonoperated; filled square = balloon-injured and sRAGE-treated; empty square = balloon-injured and placebo-treated. Values are means (SEM); sRAGE administered = 0.5 ng/g body weight. a = $P <$ 0.05 sRAGE Chinese hamster ovary-treated samples versus nonoperated; b = $P <$ 0.05 sRAGE Chinese hamster ovary-treated samples versus placebo-treated samples.

groups treated with 0.5 to 3 ng/g $sRAGE^{CHO}$ was detected (Figure 3B and 3D), suggesting that the latter parameter may be more sensitive.

Our previous work[21] showed that specific N-glycoform modifications are a key determinant of sRAGE bioactivity, and that not only N-glycosylation, but also mammalian cell-specific,

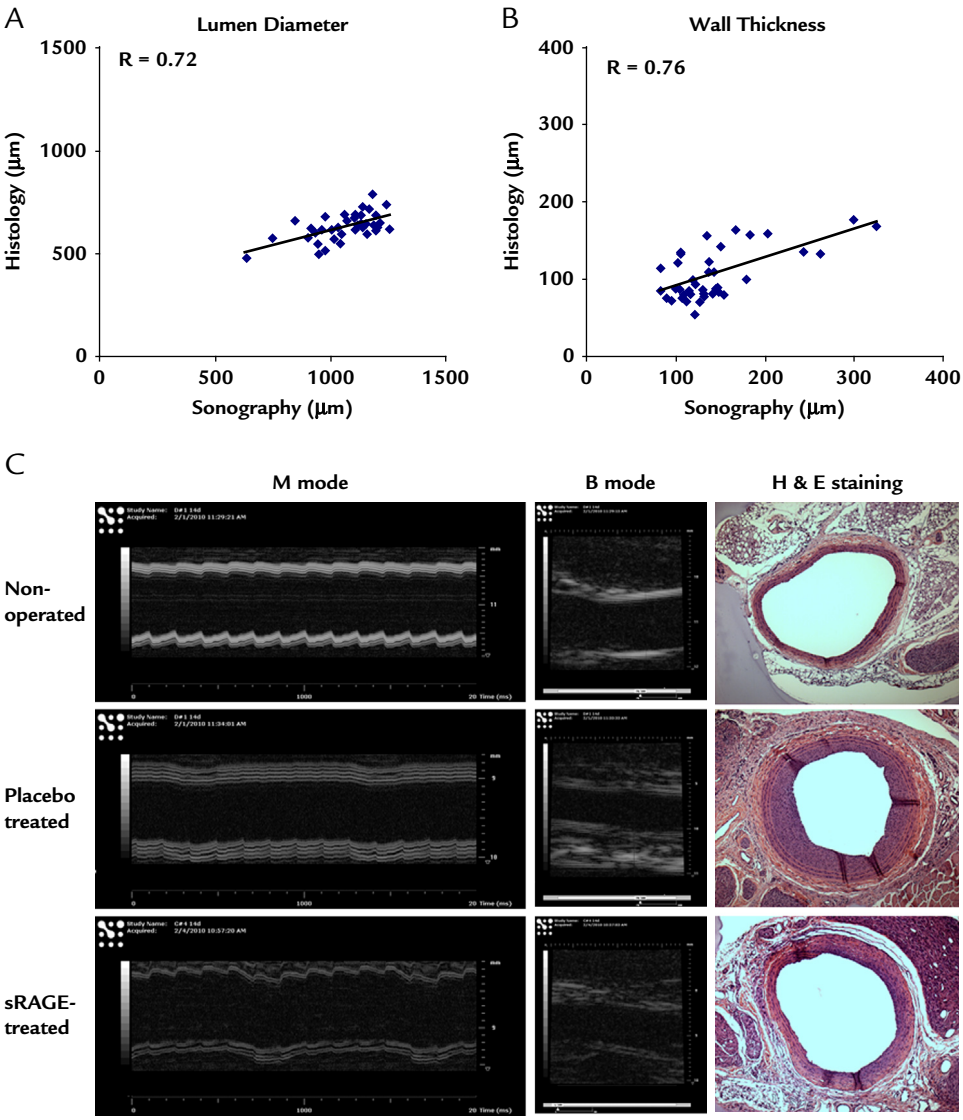

Figure 2. Correlation of ultrasound sonography and histology in soluble receptor for advanced glycation end products (sRAGE) (0.5 ng/g) treated carotid vessels shown in scatter plot of data from (A) lumen diameter and (B) vessel wall thickness at 2 weeks postinjury (nonoperated, injured with sRAGE, and placebo-treated, n = 12 of each group). (C) Representative sonographic and histologic (100 ×) images.

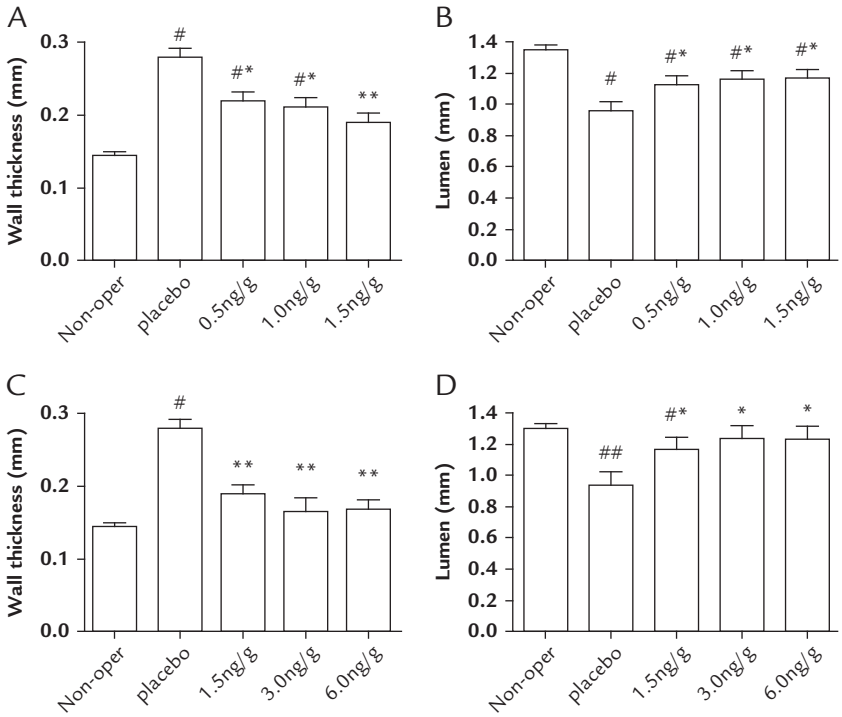

Figure 3. Soluble receptor for advanced glycation end products produced in Chinese hamster ovary ($sRAGE^{CHO}$) cells efficacy measured by vessel ultrasound sonography. (A and B) Low dose range (0.5 – 1.5 ng/g) of $sRAGE^{CHO}$ treatment measured by sonography. (C and D) high does range (1.5 – 6 ng/g) of $sRAGE^{CHO}$ treatment measured by sonography. (A and C) Average wall thickness. (B and D) Average lumen diameter. Nonoperated: n = 15; placebo-treated: n = 15; dose 0.5–1.5, 6 ng/g: n = 6; dose 3 ng/g: n = 7. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$, nonoperated versus placebo and each dose group; $^{*}P < 0.05$ and $^{**}P < 0.01$ for placebo versus each dose group.

complex-type N-glycosylation is critical for the observed high therapeutic efficacy of sRAGE. Independent assessment using sonography (Figure 4) with an established optimal dose of $sRAGE^{CHO}$ further confirmed a significantly higher efficacy of $sRAGE^{CHO}$ relative to that of nonglycosylated sRAGE (N25T/N81T) and sRAGE produced in insect Sf9 cells.

Discussion

The therapeutic value of sRAGE has been well recognized and tested in various animal models, including arterial injury models.[17,18,21,22] Administration of sRAGE, a decoy of RAGE, can reduce injury-associated, RAGE signal-mediated chronic inflammation and minimize maladaptation and remodeling in vasculature, thus decreasing the risk of cardiovascular diseases and other vessel-associated complications. Although previous studies discovered that sRAGE can be used to block neointimal growth and formation of atherosclerotic plaques in animal models, to achieve the blockage, a high dose and multiple administrations of sRAGE produced in insect Sf9 cells were employed.[17–19,26,27] Our recent study[21] demonstrated that specific N-glycoform modifications are the key determinant underlying sRAGE bioactivity and therapeutic efficacy: a low, single dose of sRAGE produced in CHO cells can significantly reduce injury-associated inflammation and neointimal growth. In addition to the observed low efficacy, glycoforms from insect cells are also immunogenic in mammals, and therapeutic glycoproteins must be produced from mammalian sources. Previous studies, including ours, had evaluated therapeutic effects of sRAGE mainly based on postmortem histomorphologic analyses of the vessel cross-sections and in vitro bioactivity assays. Although these assessments, especially histologic studies,

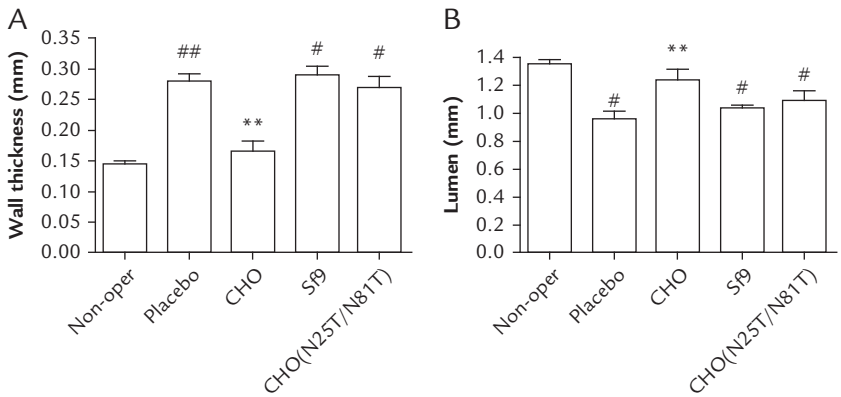

Figure 4. Modulation of soluble receptor for advanced glycation end products (sRAGE) efficacy via N-glycoform modification measured by vessel ultrasound sonography. (A) Average wall thickness. (B) Average lumen diameter. Dose used in the studies = 3 ng/g. Nonoperated: n = 15; placebo-treated: n = 15; sRAGE produced in Chinese hamster ovary cells (CHO): n = 7; $sRAGE^{Sf9}$ and $sRAGE^{CHO}$(N25T/N81T): n = 6. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, nonoperated versus placebo and each sRAGE group; $^{**}P < 0.01$, placebo versus each sRAGE group.

provided important and direct information of the vessel condition, including inflammation and formation of the neointima, these approaches cannot be used for clinical evaluation of treatment.

The present study demonstrates that injury of the vessel as well as the therapeutic effects of $sRAGE^{CHO}$ can be independently evaluated via vessel sonographic assessment. Similar to the results obtained from histology shown in our previous study,[21] current sonographic studies also showed a degree of dose-dependent attenuation of vessel lumen diameter, although this was less apparent in measured vessel wall thickness (Figure 3). Consistent with histology,[21] sonography also showed that paucimannose-glycan modified $sRAGE^{Sf9}$ and the non-glycosylated $sRAGE^{CHO}$(N25T/N81T) were ineffective when used in the same dose (3 ng/g) as $sRAGE^{CHO}$ (Figure 4). Previous studies[17,21] showed that $sRAGE^{CHO}$ administered immediately after arterial injury was most effective to restrict neointimal expansion. The manifested beneficial effects of $sRAGE^{CHO}$ are not readily detectable in vivo until at least 1 week after surgery and treatment, and the therapeutic outcome becomes apparent 2 weeks after surgery and treatment (Figure 1). These observations suggest that blocking RAGE alarmin ligands immediately after injury circumvents RAGE-mediated inflammatory signaling, and allows the injured vessel to undergo a repair process with less, or controlled, inflammation leading to a healing course with reduced remodeling. Compared with our previously published histology results,[21] vessel ultrasound sonography appears to be less sensitive to discern the difference between nonoperated vessels and vessels operated on with treatment when a higher dose of $sRAGE^{CHO}$ (3 ng/g) was used (Figure 4A), whereas such difference was discernable by histology. When a lower dose of $sRAGE^{CHO}$ was used (0.5 ng/g), the difference between treated and nonoperated vessels is clearly discernable and there was a better correlation between sonography and histology (Figure 2). This is likely due to the fact that 0.5 ng/g is not an optimal dose, and that remodeling of the vessel wall by balloon denudation was not suppressed as much as that in a situation when a higher dose was administered. These observations suggest that sonographic data should serve as a reference for application, and that the exaggerated benefits observed in higher sRAGE doses should be taken into consideration to determine a proper dose for the best treatment outcome.

Conclusions

Our research using vessel sonography to monitor sRAGE effects in live rats reaffirmed that specific N-glycoform-modified sRAGE, produced in CHO cells is highly effective in vivo to attenuate neointimal growth after arterial injury, compared with that produced in insect cells. This in vivo approach also provides a basis for monitoring sRAGE effects in future clinical applications.

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Conflicts of Interests

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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