Published in final edited form as:

Wound Repair Regen. 2014 ; 22(4): 521–526. doi:10.1111/wrr.12192.

Hyaluronan enhances wound repair and increases collagen III in aged dermal wounds

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Abstract

Age-related changes in the extracellular matrix contribute to delayed wound repair in aging. Hyaluronan, a linear nonsulfated glycosaminoglycan, promotes synthesis and assembly of key extracellular matrix components, such as the interstitial collagens, during wound healing. The biological effects of hyaluronan are mediated, in part, by hyaluronan size. We have previously determined that dermal wounds in aged mice, relative to young mice, have deficits in the generation of lower molecular weight hyaluronan (defined as <300 kDa). Here, we tested the effect of exogenous hyaluronan of 2, 250, or 1,000 kDa sizes on full-thickness excisional wounds in aged mice. Only wounds treated with 250 kDa hyaluronan (HA250) were significantly improved over wounds that received carrier (water) alone. Treatment with HA250 was associated with increased expression of transcripts for the hyaluronan receptors CD44 and RHAMM, as well as collagens III and I. Analyses of dermal protein content by mass spectrometry and Western blotting confirmed significantly increased expression of collagen III in wounds treated with HA250 relative to control wounds. In summary, we find that HA250 improves wound repair and increases the synthesis of collagen III in aged dermal wounds.

Aging is associated with deficits in many stages of cutaneous wound repair including granulation tissue formation, fibroblast proliferation, and extracellular matrix (ECM) synthesis.¹ One important but understudied component of dermal ECM is hyaluronan (HA). HA is a linear disaccharide polymer that can range from 2 to 25,000 disaccharides with molecular masses up to 2×10^2 kDa. HA is the most abundant nonproteinaceous component of dermis and is necessary for the organization, assembly, and homeostasis of wound ECM, including dermal collagens.^{2–4} HA size (i.e., its molecular weight) determines its biological properties: higher molecular weight (HMW) forms of HA inhibit proliferation and migration of most cells, whereas lower molecular weight (LMW) forms (ranging from

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Conflict of Interest: The authors have no other conflicts of interest to declare.

oligosaccharides to polymers of 300 kDa) typically promote cell proliferation and angiogenesis.^{5–8} Although HA is associated with many cell types, resident fibroblasts are primarily responsible for HA secretion in the dermis. HA in wound dermis largely reflects the activity of HA synthases 2 and 3.^{9–11} Once secreted, HA size is regulated primarily by cleavage via hyaluronidases.¹²

Although it is generally accepted that HMW-HA can inhibit processes necessary for wound repair, and LMW-HA promotes inflammation, angiogenesis, and proliferation, the size of HA and its subsequent effects are tissue specific.^{13,14} Fetal skin contains high levels of HMW-HA, which is thought to induce healing without fibrosis and scar formation.¹⁵ It has been proposed that HMW-HA is critical to the scarless healing observed in fetal wounds by inhibiting myofibroblast differentiation and the subsequent expression of the fibrogenic transforming growth factor (TGF)- β 1.¹⁶ In contrast to the body of literature describing the involvement of HA in fetal wound repair, the influence of aging on HA content and MW in normal tissues remains incompletely characterized.^{7,17} There is evidence that in normal tissues, cleavage of HA into lower MW forms is altered by aging.^{7,17–20} We have found that in mice, age-related impairments in dermal wound repair are correlated with a deficit in the production of LMW-HA.²¹ In the nervous system, there is evidence that the presence of LMW-HA can block inflammatory signals that potentiate encephalitis. Moreover, HA oligomers are implicated in toll-like receptor signaling mechanisms that affect oligodendrocyte maturation and remyelination during inflammation of the central nervous system.^{22,23} Regardless of the tissue studied, it is generally accepted that cells can sense differences in HA size, which makes a compelling argument for testing the effects of different HA sizes in accessible locations such as the dermis.²⁴

We have recently found that aged wound dermis is less able to generate smaller forms of HA than young wound dermis.²¹ The present set of experiments was designed to determine the effect of HA of distinct sizes on dermal wound repair in aged mice.

MATERIALS AND METHODS

Animals

Male C57/BL6 mice of 24 months of age were obtained from the NIA Aged Rodent Colony (<http://www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/>). The Office of Animal Welfare at the University of Washington approved the care of mice and all procedures.

Dermal wound model

Aged (24 months old) mice received two dorsal, 6-mm full-thickness dermal wounds as previously described.^{21,25} Wounds were covered with Tegaderm (3M, St. Paul, MN) to delay wound contraction, a prominent feature of skin wound healing in rodents. Wounds were treated every 48 hours (beginning with day 0) with 100 μ L of endotoxin-free water containing 10 ng HA/ μ L (the optimal concentration based on studies by others and our pilot studies of dermal fibroblast responses in vitro).¹³ We evaluated HA of specific MWs of 2 kDa (HA2) ($n = 8$), 250 kDa (HA250) ($n = 11$), and 1,000 kDa (HA1000) ($n = 11$) (Hyalose,

Oklahoma City, OK). The HA solutions were delivered under the Tegaderm using a 30-gauge needle that did not touch the wound bed. Control mice ($n = 10$) were treated in an identical fashion every 48 hours with 100 μ L of carrier (endotoxin-free water) that lacked HA.

At time 0, 2 days, and 5 days after wounding, the wounds of the animals were digitally photographed from above, and the wound areas were measured from the images using the public domain program ImageJ (<http://rsbweb.nih.gov/ij/>). Results were recorded in square pixels and then converted to area (square mm). Mice were euthanized within 36 hours after the last wound measurement (which was also the last treatment with HA), and their wound tissue was harvested using a 12-mm biopsy punch. A 6-mm biopsy punch was then used to obtain wound bed samples (plugs) of the same size and from the same location within each parent wound.

Each wound plug was stripped of epidermis and divided into quarters: One quarter was processed for RNA extraction, two quarters were processed for protein, and one quarter was processed for analysis by mass spectrometry. In addition, a subset of wound plugs was processed for paraffin embedment and sectioning.

Measurement of transcripts by RT PCR

Total cellular RNA from mouse dermis was extracted using Trizol (Invitrogen, Grand Island, NY). RNA purity and integrity was assessed by spectrophotometric analysis. A total of 1 μ g of RNA was reverse-transcribed using an iScript kit (Bio-Rad Laboratories, Hercules, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using an ABI 7900 RT-PCR instrument with SYBR Green Master Mix (Bio-Rad) for mouse TGF- β 1, CD44, RHAMM, collagen III, and collagen I.

Primers were as follows:

TGF- β 1 F: GGACTCTCCACCTGCAAGAC

TGF- β 1 R: GACTGGCGAGCCTTAGTTTG

CD44 F: AGCGGCAGGTTACATTCAA

CD44 R: CAAGTTTTGGTGGCACACAG

RHAMM F: CCTTTGAAGCCGAGAAACAG

RHAMM R: GGTGACCAAGTAGCTGTGC

Collagen III F: ACCAAAAGGTGATGCTGGAC

Collagen III R: GACCTCGTGCTCCAGTTAGC

Collagen I F: GAGCGGAGAGTACTGGATCG

Collagen I R: GTTCGGCTGATGTACCAGT

All experiments were performed at least twice, and each sample was analyzed in at least duplicate. Samples were normalized to GAPDH mRNA.²⁶ Fluorescent signals were analyzed during each of 40 cycles consisting of denaturation (95 °C, 15 seconds) and

annealing (54 °C, 15 seconds). Relative quantitation was calculated using the comparative threshold cycle method.

Mass spectrometric protein assay

Wound beds were retrieved in an identical fashion as described above. Equivalent portions were placed in 200 µL of NP40 lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1% Nonidet P40, 0.02% NaN₃) (Invitrogen) and homogenized using a Bullet blender (Next Advance, New York, NY). The wound homogenates were then analyzed by selected reaction monitoring (SRM) mass spectrometry. Concentration of total protein was determined by the bicinchoninic acid assay (BCA Protein Assay Kit, Pierce, Rockford, IL). Approximately 200 µg of total protein (25–39 µL of homogenate) was placed in a 10-kDa cutoff Amicon Ultra filtration device (Millipore, Billerica, MA) in order to remove the detergent and protease inhibitors prior to tryptic digestion.²⁷ Briefly, homogenate was combined with 200 µL of 8 M urea/50 mM ammonium bicarbonate prior to centrifugation. The retentate was solubilized in 400 µL of the urea buffer prior to centrifugation, and this was repeated. The retentate disulfide bonds were reduced in 200 µL of the urea buffer plus 2 µL of 500 mM tris (2-carboxyethyl) phosphine for 40 minutes at 37 °C, and the resulting cysteine thiols were alkylated by the addition of 2.5 µL of 500 mM iodoacetamide. Excess reactants were removed by centrifugation, and the retentate was washed with 400 µL of the urea buffer followed by two washes with 400 µL of 50 mM ammonium bicarbonate without urea. Tryptic digestion proceeded overnight in 100 µL of 0.04 µg/µL trypsin in 50 mM ammonium bicarbonate at 37 °C with rotary shaking. Tryptic peptides were collected in the flow-through after centrifugation, and the Amicon filtration device was washed with 200 µL of 50 mM ammonium bicarbonate. The wash and flow-through were combined and acidified to a final concentration of 0.5% trifluoroacetic acid. A 75 µm × 30 cm capillary column self-packed with 3 µm C18 (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was operated at a flow rate of 250 nL/min with a 2-hour gradient of 0–35% acetonitrile in 0.1% formic acid. The column effluent was analyzed using a Thermo TSQ Vantage triple quadrupole mass spectrometer with electrospray ionization (Thermo Scientific, San Jose, CA). The SRM transitions were selected with the aid of the computer program Skyline,²⁸ and each pooled wound tryptic digest was randomly analyzed in triplicate. Summed transition peak areas from HA-treated wounds were compared with peak areas from the same peptides from control wounds. These ratios were normalized to the average of all analyzed peptides, and protein level ratios and confidence limits were calculated from the normalized peptide ratios.

Western blotting

The wound homogenates used for SRM mass spectrometry were also analyzed by Western blotting. Dermal wound extracts of equivalent total protein content (BCA) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20 µg of protein per lane) under nonreducing or reducing conditions, transferred to nitrocellulose, and probed with 2–5 µg/mL of the following antibodies: mouse monoclonal antibody against CD44 (Cell Signaling, Danvers, MA); rabbit polyclonal antibody against collagen I (Millipore); and rabbit polyclonal antibody against collagen III (Abcam, Cambridge, MA). Blots were probed with the appropriate horseradish peroxidase-conjugated secondary antibodies

(Jackson ImmunoResearch, West Grove, PA) (1 µg/mL) and visualized by enhanced chemiluminescence (GE Healthcare LifeSciences, Piscataway, NJ). Bands were scanned, and digital images were quantified using ImageJ.

Immunohistochemistry

A subset of wound plugs was dehydrated, embedded in paraffin, and sectioned at 5 µm. Sections from the middle of the wound were mounted on slides, deparaffinized, stained with a rabbit polyclonal antibody specific for collagen III (Abcam) (2–5 µg/mL), and incubated with a biotinylated polyclonal goat antibody to rabbit IgG (Amersham, Pittsburgh, PA) (5 µg/mL). Collagen III immunostaining was visualized with a Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories, Burlingame, CA) in conjunction with 3,3'-diaminobenzidine.

Statistics

Tests for significance between control and treatment groups were performed using a two-tailed Student's *t* test with unequal variance. Significance was defined by $p < 0.05$.

RESULTS

HA improves wound repair in aged mice

We began our studies to determine if the addition of topical HA of specific sizes enhanced the closure of full-thickness wounds in aged mice. We found that over a 5-day period, aged mice showed improvement in wound closure when they received HA2, HA250, or HA1000 relative to control mice that received carrier (water) only. However, improvement in wound closure achieved statistical significance only in mice treated with HA250 ($p = 0.024$, Figure 1). Wounds of control mice (injected with water only) healed in a manner nearly identical to wounds in aged mice that received no fluid injections (as performed in our recent study),²¹ thereby indicating that the carrier for HA (water) did not influence wound healing in the control mice of the present study.

HA increases expression of transcripts for HA receptors and collagens during wound repair

We then examined the expression of key mediators of wound repair in the presence of HA2, HA250, and HA1000 relative to control wounds. As shown in Figure 2, RNA was extracted from dermal wounds, and RT-PCR was performed for CD44 and RHAMM, the primary receptors for HA.²⁹ The expression of TGF-β1 and collagens III and I, which are integral to improved dermal wound repair, was also measured.^{30,31} HA2 significantly increased expression of TGF-β1 ($p < 0.05$), CD44 ($p < 0.005$), and collagen I ($p < 0.05$). HA1000 was associated with significantly higher levels of CD44 ($p < 0.005$), RHAMM ($p < 0.005$), collagen III ($p < 0.05$), and collagen I ($p < 0.05$). Treatment with HA250 also significantly increased the expression of transcripts for the HA receptors CD44 ($p < 0.005$) and RHAMM ($p < 0.005$) but reached a more stringent value for statistical significance with respect to both collagen III ($p < 0.0005$) and collagen I ($p < 0.005$). The effect of HA250, relative to control, on expression of TGF-β1 had a p -value = 0.05.

HA increases deposition of collagen III during wound repair

Dermal wounds have multiple components that are increasing simultaneously as healing progresses, regardless of the presence of interventions such as HA. To enhance the detection of differences among the wounds, measurements of changes in protein expression in wound dermis exposed to HA2, HA250, and HA1000, relative to controls, were made using SRM mass spectrometry. Two of the proteins, TGF- β 1 and RHAMM, were below the levels of detection, so no quantitative changes in these proteins could be established. Expression of CD44, collagen I, and the housekeeping proteins (GAPDH and actin) exhibited no significant changes by mass spectrometry. The only significant change that was greater than 25% and had a narrow confidence interval was an increase in collagen III in wounds treated with HA250 compared with control wounds (Figure 3A, asterisk).

To confirm the observation by mass spectrometry that HA250 significantly increases collagen III expression in dermal wounds, Western blots of wound extracts were immunolabeled with an antibody specific for collagen III and the staining quantified by densitometry. Results showed a significant increase in collagen III in wound dermis treated with HA250 ($p < 0.05$) relative to control wound dermis exposed to carrier only (Figure 3B). Western blots for CD44 and collagen I did not detect differences between control and any of the HA-treated wounds (data not shown). Immunohistochemical staining of a subset of wounds for collagen III yielded similar results: an increase in expression of collagen III in dermis of HA250-treated wounds relative to control wounds (Figure 3C).

DISCUSSION

There are many interventions to improve wound repair, but more are needed.³² HA is a logical therapeutic candidate due to its high expression, close interaction with collagens, hydrophilic nature, and multifaceted effects on cell behaviors. These features make a compelling argument for the use of HA in accessible locations such as the dermis.^{14,24} Indeed, HA is already safely and widely used in humans—in the dermis as a space-filling agent and as a therapeutic intervention in skeletal joints.^{33,34} The effect of HA on tissues is determined by its size, and there is increasing evidence that cells and tissues sense and respond to HA of specific MWs.^{14,21,24,35}

In the present study, we found that HA250 was the most beneficial, relative to HA2 and HA1000, in promoting the closure of wounds in aged dermis. Wound closure is a readout that represents the combination of a variety of interacting dermal processes necessary for effective healing.^{1,21} Although all forms of HA seemed to improve healing, we acknowledge that the statistical analysis supported only the use of HA250. There is biological plausibility as to why HA250 would have the greatest benefit on wound repair. Namely, HA in the 250-kDa size class neither triggers pathways inhibitory to wound repair as higher MW (>300 kDa) forms of HA do, nor does it provide the inflammatory signals in the manner of very low MW (<20 kDa) forms of HA.^{13,14,24} Of course, it is recognized that the maintenance of any specific size (MW) class of HA is transient after the HA is introduced into humans or animals due to rapid degradation of the molecule by hyaluronidases. However, it has been shown that periodic readministration of HA during the course of treatment seems to

counteract HA degradation and maintain the effects generated by the form of undegraded HA that was initially used.^{14,21,24,35}

There are several potential mechanisms by which HA250 induces beneficial responses. In this study, we found the expected induction of transcripts for the primary HA receptors in the dermis, CD44 and RHAMM. The subsequent pathways activated by these receptors promote cellular migration and proliferation.²⁹ HA250 also increased the expression of TGF- β 1 with a *p*-value that approached significance (*p* = 0.05). The effect on TGF- β 1 is not unexpected as activated CD44 interacts with TGF- β 1 to promote myofibroblast differentiation and a fibrogenic phenotype, which promotes subsequent deposition of interstitial collagens. The latter include collagen III, a provisional collagen found in the early phases of wound repair, and collagen I, which is typically produced during the later phases of repair involving ECM remodeling.³⁶ The finding of significant induction of transcripts does not necessarily predict corresponding quantitative increases in protein, but measuring mRNA expression is particularly useful in analysis of wound beds, where there is a very high degree of turnover of newly synthesized and preexisting ECM components. Moreover, normal, unwounded dermis has very high levels of extant collagens, which make the detection of changes in postwound collagen levels challenging.

The use of mass spectrometry for quantitative and targeted analysis of specific proteins has many analytical advantages over antibody-based measurements. For example, mass spectrometry does not have the problems of cross reactivity and sensitivity to fixation that are characteristic of antibody probes, and mass spectrometry can analyze multiple molecular targets in tissues more rapidly and, often, at lower cost than antibody-based methods. However, normalizing mass spectrometric data generated from wounds is challenging. Normalizing to cellular proteins is not possible, given that there is a difference in cellularity of wounds vs. normal dermis. Normalizing to extracellular proteins is problematic in that it is difficult to identify specific proteins that do not change as wound repair proceeds. To address these problems, we normalized the ratio of proteins in HA-treated and control wounds from the average of all measured proteins. Using this accepted technique, we found that the increase of CD44 in HA250-treated wounds was relatively minor in comparison with changes in mRNA transcripts. This result might be attributable to an imperfect correlation between levels of mRNA transcription and translation of transcripts into protein.³⁷ To evaluate the influence of HA on the structure of healing wounds, we focused on collagens III and I, as these molecules are the primary ECM components that confer mechanical stability during dermal repair.^{1,32,36} Although collagen III is often referred to as the provisional collagen that is deposited early in dermal repair, and collagen I is described as the more permanent, structural collagen that is deposited later, in reality, these collagens often colocalize in vitro and in vivo.^{38–40} Consequently, their expression is often studied in concurrent, rather than sequential, fashion. Changes in total amounts of collagen I after HA treatment were difficult to discern, which is not surprising given the high levels of this collagen type in both normal and wounded dermis. However, mass spectrometry was able to detect statistically significant increases in collagen III in HA250-treated wounds relative to other proteins that were measured. Notably, the mass spectrometric data were supported by RT-PCR, which showed significant increases in levels of collagen III mRNA, and by Western blots, which also showed significant increases in collagen III protein. Others have

shown that HA in a range of sizes can induce the synthesis of collagens in a tissue-specific manner. Effects of HA on the synthesis of collagen III are mediated by fibrogenic growth factors via mechanisms similar to those that mediate the influence of HA on deposition of collagen I.^{13,34,38–40}

In summary, we found that exposure of wounds to HA improves dermal healing in aged mice, with the most significant benefit resulting from treatment with HA of the 250-kDa size class. The improvement in wound repair was associated with increased expression of transcripts for HA receptors that mediate wound repair processes and of transcripts for collagens III and I, which provide structural support in the healing wound. Significant HA250-induced increases in levels of collagen III protein were shown by both mass spectrometric and Western blot assays. We propose that HA, and specifically HA250, has potential clinical utility for improving cutaneous wound healing in aging.

Acknowledgments

The authors wish to thank Matthew N.R. Johnson for his technical assistance and Virginia M. Green, PhD, for editorial review of the manuscript.

Source of funding: This work was supported by R21AG33391 (M.J.R.) and the Nathan Shock Center of Excellence in Basic Biology of Aging at the University of Washington (M.J.M.). The MacCoss Laboratory receives instrumentation and financial support for research from Thermo Scientific.

Glossary

ECM	Extracellular matrix
HA	Hyaluronan
HMW	Higher molecular weight
LMW	Lower molecular weight

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Figure 1.

Hyaluronan (HA) of 250 kDa significantly improves wound repair in aged mice. Closure of full-thickness wounds in aged mice was significantly improved in wounds that were treated with HA250 (change in wound area = $13.2 \pm 2.1 \text{ mm}^2$) ($n = 11$), relative to control wounds treated with water carrier only (change in wound area = $7.2 \pm 1.1 \text{ mm}^2$) ($n = 10$). Benefit of HA250 was maximal at day 5 postwounding ($p = 0.024$). Wound closure with HA2 (change in wound area = $11.8 \pm 2.4 \text{ mm}^2$) ($n = 8$) and HA1000 (change in wound area = $12.2 \pm 2.1 \text{ mm}^2$) ($n = 11$) had p -values of 0.115 and 0.052, respectively, at day 5 postwounding. * $p < 0.05$, error bars are SEM.

Figure 2.

Hyaluronan (HA) induces mediators of wound healing. Wounds from aged mice treated with HA2 ($n = 8$), HA250 ($n = 11$), HA1000 ($n = 11$), or water carrier only (controls = C) ($n = 10$) were retrieved within 36 hours after receiving the final injection of HA or carrier at day 5 postwounding. Epidermis was removed, and wound dermis was processed for RNA isolation. Reverse transcription-polymerase chain reaction (RT-PCR) was performed for key mediators of dermal wound repair. Relative to control wounds, wound dermis treated with HA of any molecular weight (MW) showed increases in several of the mediators, but only wounds treated with HA250 showed statistically significant increases in CD44, RHAMM, collagen III, and collagen I with p values < 0.005 . The effect of HA250 on transforming growth factor (TGF)- β 1 had a p -value = 0.05. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

Figure 3.

Hyaluronan of 250 kDa (HA250) increases the synthesis and deposition of collagen III during wound repair in aged mice. Wounds from aged mice treated with HA2 ($n = 8$), HA250 ($n = 11$), HA1000 ($n = 11$), or controls (C) (water carrier only, $n = 10$) were retrieved after receiving the final injection at day 5 postwounding. Epidermis was removed, and wound dermis was extracted for total protein. (A) Mass spectrometric analyses showed a significant increase in collagen III in the wounds treated with HA250 over those exposed to control (* $p < 0.05$). Expression of CD44, collagen I, and the housekeeping protein, GAPDH, exhibited no biologically significant changes by mass spectrometry. (B) Quantification of Western blots of dermal wound extracts using an antibody specific for collagen III confirmed the expected increase in collagen III in dermis treated with HA250 relative to

control wounds ($*p < 0.05$). (C) Immunohistochemical staining for collagen III in a representative wound treated with HA250 relative to a control wound not treated with HA. In the HA250-treated wound, expression of collagen III (arrows) was increased in the wound bed as well as the adjacent dermis, relative to the control wound. Both images are at the same magnification. Scale bar = 50 μm .

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