Hyaluronan Fragments Stimulate Endothelial Recognition of Injury through TLR4*

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Prompt recognition and response to injury is critical for activation of epithelial innate defense mechanisms, recruitment of inflammatory cells, and initiation of the repair process. The response to injury may involve exposure to exogenous foreign molecules such as microbial coat components. Tissue damage in sterile compartments such as fetal skin wounds also triggers defense and repair mechanisms. Many exogenous triggers of injury or danger have been identified, such as LPS,1 peptidoglycan, and other microbial components (1). These molecules are recognized at least in part by pattern recognition receptors, an elegant and evolutionarily ancient system for recognition of exogenous danger (2). However, the mechanisms for recognition of injury to self are less well described. Intra-cellular components of necrotic cells not normally seen by immune cells, components of the ECM, and stress signals such as an increase in heat-shock proteins have all been implicated as endogenous indicators of injury (3–5). Among this group is hyaluronan (HA), an important structural component of the ECM that is also a common component of bacterial surfaces. This investigation sought to understand the role HA may play in the wound repair process.

HA typically exists as a high molecular weight polymer greater than 10^6 Da and composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid (6, 7). HA is synthesized at the cell surface and is a uniform glycosaminoglycan since its disaccharides are not sulfated or epimerized. Thus, unlike glycosaminoglycans such as heparan sulfate or chondroitin sulfates that encode specific activity by use of a diverse disaccharide sequence, the size, concentration, and location of HA are the only variables to consider when evaluating function. Recently, small molecular weight HA (sHA) has been implicated in several biological processes including angiogenesis, cell proliferation, maturation, migration, activation of protein tyrosine kinase cascades, and inflammatory gene expression (8–14). These sHA fragments may activate repair processes and signal the immune recognition apparatus that injury has occurred.

Microvascular endothelial cells (EC) exist in a crucial position. In the case of dermal microvascular EC, these cells traverse an interface between the surface epithelium and underlying connective tissue. They are able to sense danger and injury in the surrounding tissues and signal an immediate defense response (15). Their activation is essential in the recruitment, rolling, adhesion, and extravasation of leukocytes to the site of injury and inflammation, a process that is critical to protection against pathogens (16). In addition, ECs are able to respond to, and produce a variety of cytokines critical for signaling cells in both the proximal and distant environment that a repair or inflammatory process is underway.

Due to the role of the dermal microvascular endothelium in sensing injury and signaling repair, we sought to determine how ECs respond to HA released from the ECM following injury. Understanding how ECs respond to the presence of HA fragments and the mechanisms involved in this signaling pathway will lend perspective as to how tissue damage is recognized.
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and how immune modulators are recruited to the site of injury. Our findings suggest the pattern recognition receptor TLR4 participates in recognition of danger from both microbes and host injury.

EXPERIMENTAL PROCEDURES

Media and Reagents—Human microvascular endothelial cells isolated from neonatal dermis (cryopreserved at passage 3) were purchased from Cascade Biologies and grown in Endothelial Cell Growth Media consisting of Medium 131 (Cascade) supplemented with Microvascular Growth Supplement (Cascade) and 100 units/ml penicillin/100 μg/ml streptomycin (Invitrogen). Experiments were performed in 1%-serum media containing Endothelial Containing Media (Clonetics), 1% heat-inactivated fetal calf serum (HyClone), 1 μg/ml hydrocortisone acetate (Sigma), 5 × 10⁻⁵ M dbcAMP (Sigma), and 100 units/ml penicillin/100 μg/ml streptomycin (Invitrogen). sHA (4-, 6-, and 8-mer hyaluronan fragments) were a gift from J. Simon, Department of Dermatology, Freiburg University, Germany. High molecular weight hyaluronic acid from human umbilical cord and Proteus vulgaris chondroitinase ABC (EC 4.2.2.4) were from Sigma. Hyalgan (HG) was manufactured by Sanofi-Synthelabo and purchased from Besse Medical Supply. LPS was purchased from Sigma and endotoxin-removal columns were from Seikagaku (followed manufacturer's instructions for endotoxin removal). TLR4 antibody, HTA125, was a gift from T. Kikkawa (Tisch Children's Medical Center, San Diego, CA), and the IgG control antibody for cell culture experiments was purchased from Sigma. Polymyxin B was purchased from Calbiochem and Pep-1 was synthesized by SynPep (Dublin, CA) as described previously (17). The LAL assay was performed using the Pyrochrome kit from Seikagaku. Millipore Microcon centrifugal filter devices (MWCO 3000) were purchased from Fisher.

Wound Fluid Collection and Dot Blot—Wound fluid was isolated from sterile tubes inserted into the backs of mice for 48 h. Back fur was removed from mice with hair clippers and Nair. Mice were anesthetized with isofluorane, and betadine (povidine-iodine, 10%, Purdue Frederick) and an alcohol swab were used to clean the incision area. Incision was made by vertical tension of skin with forceps and single cut (± 1 cm long) with scissors. Blunt dissection of subcutaneous space was carefully performed with scissors and 6–8 sterile tubes (3.5-mm outside diameter of sterile nasal cannula tubing, Hudson Respiratory Care Inc.) were inserted into subcutaneous space. Wounds were closed with wound clips using wound clip applicator. After 48 h, mice were euthanized by over-anesthesia with halothane, back skin was washed with betadine, and wiped with an alcohol swab. The base of the wound clips were opened with scissors and wound fluid was collected in the wound space and in the tubing by micropipetting. Human wound fluid was collected from patients posturgery and PGs/GAGs were isolated from human and mouse wound fluid by anion exchange purification as previously described (18). For mouse samples only, wound fluid was pooled from several different mice before PG/GAG isolation.

Wound fluid and wound fluid GAG (WF/GAG) was dotted onto a nitrocellulose membrane using a dot blot apparatus. 1 μl of mouse wound fluid and 20 μl of human wound fluid was diluted in 200 μl of TBS, 200 μl of this was placed in one well of dot blot and the other 200 μl was serially diluted five times (2-fold dilutions each time). 1 μl of mouse WF/GAG (one pooled sample) was blotted onto the membrane, corresponding to ~0.05 μg of sulfated GAG. Approximately 0.5 μg of sulfated human WF/GAG from three individual samples was blotted onto the membrane. Western blotting solution (Roche Applied Science) was used to block the blot and for primary and secondary antibody incubations and 0.05% Tween 20 in 1× TBS (TBST) was used for all washes. After blocking, the blot was incubated with biotinylated-hyaluronic acid-binding protein (Seikagaku), diluted 1:1000, for 1 h at room temperature. Streptavidin-HRP, diluted 1:5000, was used as the secondary antibody, and the blot was developed with Western Lightning Chemiluminescence (PerkinElmer Life Sciences).

Mouse—Endothelial cells were incubated with 20 μg/ml sHA in 1%-serum media for 6 h in 100-mm tissue culture dish (Falcon, BD Systems), and RNA isolated by Qiagen RNeasy kit. RT-PCR was performed using Ambion Reverse Kit following the manufacturers instructions. 1 μg of RNA was used under the following PCR conditions: 80 °C for 3 min, 0 °C for 13 min, 42 °C for 1 h, 92 °C for 10 min, 4 °C hold. PCR was performed on the cDNA using IL-8 primers: 5′-GGAG-AAGTTTGGAGGAGGCTGAG-3′ (forward) and 5′-CAAGGCACAGTGGAACAGG-3′ (backward). IL-8 PCR conditions were as follows: 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min (23 cycles), 72 °C for 7 min, 4 °C hold.

In Vitro Cell Culture Experiments—All endothelial cells were grown in Endothelial Cell Growth medium and seeded in 96-well flat bottom plates (Costar, Corning Inc.) until confluent. 8–16 h before adding samples, the cells were washed once with 1% serum medium and then 100 μl of 1% serum medium added to cultures. Endothelial cells were between passages 5 and 8 when assayed. Samples were diluted in 1% serum medium (100-μl total volume) and added to cells for indicated time points. For experiments using TLR4 and control antibodies, the antibody was added first to the cells in 1% serum medium (50-μl volume) and then the sample (i.e. sHA) in 1% serum medium (50-μl volume) was added approximately 5 min after antibody addition. Following indicated incubation, cell medium was collected into sterile 96-well U-bottom plate (Costar, Corning Inc.) and spun down at 1000 rpm for 10 min. Cell medium was removed from any pelleted material, placed in a sterile, U-bottom 96-well plate, and a small sample was assayed for IL-8 by ELISA. The IL-8 ELISA kit was purchased from PharMingen and followed the manufacturer's protocol.

HA Digestion—HA and sHA was digested completely by incubating 15 μg with 50 million units of chondroitinase ABC in chondroitinase buffer (50 mM Tris base/60 mM sodium acetate, pH 8.0) at 37 °C overnight. Following digestion, samples were boiled for 3 min and then Microcon centrifugal filter devices were used to separate out the disaccharides generated from the enzyme that was retained in the filter. These samples were resuspended in 1% serum medium and added to endothelial cells for 24-hour incubation as described in in vitro cell culture experiments. Media samples were assayed for IL-8 production using IL-8 ELISA.

Mouse Experiments—Wild-type Balb/c mice and TLR4 mutant C3H/HeJ mice were purchased from Jackson Laboratories. Mice were injected intraperitoneally with 100 μl of total volume of PBS, 100 μg of sHA, 100 μg of HA, or 25 ng of LPS for indicated concentrations and times. After specified incubation time, mice were anesthetized with isofluorane and bled terminally by heart puncture. Blood was collected using a 20-gauge needle and 1-ml syringes (BD Systems). Serum was separated from whole blood collection by serum separator tubes (Ram Scientific) and placed at −20°C until assayed. MIP-2 and KC levels were measured in serum by MIP-2 and KC ELISA (R&D Systems), respectively, following manufacturers instructions. Animal procedures were approved by the Veterans Affairs San Diego Healthcare System Subcommittee on animal studies, protocol 02-037.

RESULTS

Soluble HA Is Released Following Injury—The biological activity and functional significance of HA depends on oligosaccharide length and location relevant to responding cell types. To determine if HA is released from the ECM following injury and to measure the amount available as a potential signal to cellular components of the wound environment, HA was measured in human and mouse wound fluid generated and collected under sterile conditions. Biotinylated HA-binding protein detected abundant amounts of large molecular weight HA present in soluble form in wound fluid from both species (human > 0.32 mg/ml, mouse > 9.6 mg/ml). This HA measurement technique did not detect small molecular weight forms of HA as determined by separate analysis of purified small molecular weight HA (sHA) (4–6 oligosaccharides). However, several prior investigations have shown that endogenous hyaluronidases or reactive oxygen species in the skin or inflammatory conditions will process large molecular weight HA into smaller molecular weight forms (6, 20, 21).

sHA Triggers an Increase in Endothelial Cell IL-8 Gene Expression—Release of HA and sHA into the wound environment will expose the microvasculature to polysaccharides normally confined to the ECM or present on bacterial surfaces. To de-
TABLE I

| Gene                                      | Mean fold increase |
|-------------------------------------------|--------------------|
| IL-8                                      | 56.13              |
| Melanoma growth stimulating factor        | 21.34              |
| Stromelysin 2/MMP-10                      | 10.15              |
| ICAM-1                                    | 5.03               |
| Metallothioneine from cadmium treated cells | 4.29            |
| p56 induced by IFN                        | 3.51               |
| Caveolin 1                                 | 3.19               |
| Syndecan 4                                 | 3.16               |
| Guanylate binding protein 2-interferon-inducible | 2.90         |
| Guanylate binding protein 1-interferon-inducible | 2.66         |
| Synuclein, α                               | 2.65               |
| IkB/MAD-3                                 | 2.59               |
| NFKB DNA binding subunit                  | 2.52               |
| Protein tyrosine phosphatase receptor type k | 2.38           |
| RGP3-G-protein signaling                  | 2.23               |
| Macrophage specific colony stimulating factor | 2.00          |
| Interferon-regulating factor 1             | 2.00               |

Controls

| Gene                                      | Mean fold increase |
|-------------------------------------------|--------------------|
| GAPDH                                     | 0.90               |
| β-actin                                   | 1.02               |

demonstrated at least 2-fold change in transcript abundance compared to control endothelial cell cultures treated with vehicle alone and with a variance between triplicates of less than 35%

To determine if sHA plays a role in sHA-induced IL-8 production by EC, the effects of a TLR4 blocking antibody, and responsiveness of mice lacking functional TLR4 were evaluated. TLR4-specific blocking antibody completely abolished IL-8 release by human EC after sHA exposure while control IgG had no effect (Fig. 2a). A decrease in IL-8 production was also seen after incubation with TLR4 antibody alone. The mouse model of IL-8 responsiveness to sHA further supported a requirement for sHA to associate with TLR4. Control mice congenic to TLR4-deficient mice were injected intraperitoneal with sHA for MIP-2 measurements at 0, 2, 6, and 24 h after injection. Significant MIP-2 induction was observed in control mice after 2 h (data not shown). Control and TLR4-deficient mice were then injected with either PBS or sHA and bled after 2 h. Control mice injected with sHA had a 6-fold increase in MIP-2 compared with injection with PBS. Mice deficient in TLR4 had little MIP-2 response to sHA injection (Fig. 2b). In separate experiments, injection of LPS in control mice increased MIP-2 to ~22 ng/ml. As expected, TLR4-deficient mice had diminished response to LPS, after injection of 25 μg of LPS MIP-2 levels slightly increased to 11 ng/ml (data not shown).

In addition to MIP-2, KC was also measured in serum sampled from control and TLR4-deficient mice injected with PBS or sHA. KC was elevated 7-fold in controls injected with sHA compared with those injected with PBS. TLR4-deficient mice were unable to respond to sHA injection and did not have elevated levels of KC (data not shown).

To confirm the TLR4-dependent response of EC to sHA was due to recognition of the oligosaccharide and not due to the presence of trace contamination, several complementary experimental controls were performed. LPS contamination was directly evaluated by LAL assay for the presence of endotoxin in sHA preparations. Maximum detectable endotoxin was at 0.314 EU/ml in a 50 μg/ml sHA sample. The minimum LPS concentration required to induce an IL-8 response from EC under the assay conditions used in the present study was 1EU/ml, three times greater than the maximal amount of LPS detectable in sHA (Fig. 3a). To further eliminate the possibility that trace LPS was responsible for activity, HA preparations containing both large and small molecular weight HA were treated by passage over LPS binding columns. This did not affect the ability of HA to stimulate IL-8 (Fig. 3b). As a control for the efficiency of the LPS removal column, LPS (1 EU/ml) was added to the pharmaceutical-grade high molecular weight HA preparation lacking sHA required for stimulation of EC, then similarly treated by LPS removal column. In these controls, column treatment removed IL-8 stimulation activity. Similarly, addition of polymyxin B to EC cultures inhibited the ability of LPS but not sHA to induce IL-8 (data not shown).

To directly demonstrate that sHA is responsible for stimulation of EC, a peptide inhibitor of HA binding (Pep-1) (17) and an enzyme specific for degradation of HA (chondroitinase ABC), were tested for ability to block IL-8 induction. Pep-1 completely blocked an increase in IL-8 by sHA (Fig. 3c). In separate experiments, Pep-1 did not inhibit LPS induction of IL-8 release from EC (data not shown). Chondroitinase ABC treatment that will degrade HA to inactive disaccharides abolished induction of IL-8 in both the HA preparation containing large and small molecular weight and sHA (Fig. 3d).

DISCUSSION

Following injury, inflammation accompanies the wound healing process and is essential for defense against opportunistic pathogens, tissue repair, and remodeling. ECM components,
such as glycosaminoglycans, have been implicated as innate signals of injury to the skin. Examples of glycosaminoglycans acting as inflammatory signals have included observations that small molecular weight HA induces dendritic cell maturation and that dermatan sulfate can induce expression of ICAM-1 on endothelial cells (13, 22). In the current study we demonstrate that HA becomes soluble after mechanical injury. HA fragments were found to stimulate human dermal microvascular endothelial cells and induced the release of IL-8 in tissue culture. This stimulatory activity could be reproduced in vivo and the recognition of sHA was mediated by TLR4. Thus, rapid recognition of injury can be mediated by release of previously insoluble glycosaminoglycans from the extracellular matrix. These observations suggest HA and TLR4 serve as a sensitive detection system for initiation of the wound defense and repair process.

HA normally exists in the epidermis and dermis as a high molecular weight polymer between 10^5 and 10^7 Da (6, 7). High molecular weight HA has been historically thought to promote tissue integrity and to provide scaffolding for cell migration, differentiation, and proliferation in the ECM (23). However, HA breakdown may serve as a signal that injury has occurred. HA fragments can diffuse between cells, and large molecular weight HA can transfer to lymph nodes by hydrodynamic movement (7). Under normal conditions prior to injury the endothelial cells lining the microvasculature of the skin will be physically separated from HA and the remainder of the ECM by their basement membrane. These cells are essential to the early inflammatory process as they lie at the interface between blood and tissues, are able to regulate the traffic of molecules and cells across the vessel wall (15) and participate in the modulation of inflammation in part through their response and production of cytokines (24). Following injury, release of sHA enables contact of these small polysaccharides with endothelial cells either by diffusion or as a consequence of the physical disruption of the blood vessel wall. Since the local environment of the dermis is rich in HA (25), and interwoven with the microvasculature, the interaction of these two compartments is an unavoidable consequence of any form of tissue damage. Analysis of wound fluid collected aseptically from human sur-

**FIG. 1.** IL-8, MIP-2, and KC increase in response to sHA exposure. a, dose-dependent increase in IL-8 release following addition of sHA to human dermal microvascular endothelial cells in vitro. Cells were treated with varying concentrations of sHA for 24 h. Inset shows IL-8 mRNA expression in endothelial cells incubated with 20 μg/ml sHA for 6 h. b, time dependence of IL-8 release following addition of 30 μg/ml sHA to endothelial cells in vitro. a and b, data are mean ± S.D. following subtraction of background IL-8 released by untreated cells (cell background for 2a is 751 pg/ml and for 2b is 807 pg/ml). c and d, MIP-2 and KC expression in mice injected with PBS or 100 μg of sHA. Mice injected with sHA had a 6-fold increase in MIP-2 expression over mice injected with PBS control (c) and a 7-fold increase in KC expression (d). c and d, data are mean ± S.D. from three mice per condition. Results are representative of experiments repeated at least three times.
gical wounds or under sterile conditions following subcutaneous injury in mice confirmed prior observations that HA is released in soluble form after mechanical injury (6, 26). Prior studies of skin and a variety of inflammatory conditions have shown that large molecular weight HA can be processed by hyaluronidases and reactive oxygen species to small molecular weight forms whose function in inflammation is unclear. Thus, evaluation of the consequences of an interaction between the newly generated small sHA fragments and dermal microvascular endothelial cells was a logical step toward further understanding the innate triggers of tissue repair.

Microarray analysis indicated that expression of a variety of genes changes in endothelial cells upon sHA stimulation. Interpretation of microarray data is limited by the scope of the observations and limited information on the biological relevance of some of this information (27). Quantitatively minor changes in steady-state abundance of some gene transcripts may have great physiological importance. However, IL-8 was chosen for further analysis because of the magnitude of its response (56-fold increase) and its role in neutrophil recruitment. Neutrophils are important cells in innate immunity and wound healing because they provide part of the first line of defense for the host. Neutrophils possess a variety of mechanisms for inactivating bacteria and debridement of injured tissues (28). IL-8 induces respiratory burst in neutrophils, generating both oxygen radicals and nitric oxide and releasing the lysosomal contents of the neutrophil, all of which participate in bacterial and tissue degradation (29, 30). In addition, IL-8 signals keratinocyte proliferation and migration, increases the adherence of monocytes, and increases lymphocyte chemotaxis, events important in all phases of wound repair (30, 31). Taken together, the activities of IL-8 and neutrophil recruitment represent a critical first step in preventing further bacterial infection and initiating clearing of the injured area.

**FIG. 2. IL-8 and MIP-2 induction by sHA is TLR4-dependent.**

*a*, ECs were treated with TLR4 antibody or control (IgG) followed by sHA addition. The TLR4 antibody HTA125 blocks sHA-induced production of IL-8 by endothelial cells *in vitro*. Data are mean ± S.D. of triplicate determinations. Results are representative of two experiments. *b*, wild-type and TLR4-deficient mice were injected with PBS or 100 μg of sHA. Wild-type mice respond to sHA injection by elevated serum MIP-2 while TLR4-deficient mice are unable to respond to sHA. Data are mean of triplicates ± S.D. and are representative of at least three experiments.

**FIG. 3. HA, not microbial contamination, is responsible for IL-8 induction in endothelial cells.**

*a*, minimum dose dependence of LPS-induced IL-8 production by ECs. Data are mean ± S.D. following subtraction of background IL-8 released by untreated cells (background of untreated cells is 1071 pg/ml). *b*, endotoxin removal does not block HA induction of IL-8. To confirm efficiency of endotoxin removal from crude HA mixture, LPS was added to high molecular weight HA (Hyalgan (HG)) and this solution assayed before and after column treatment. *c*, Pep-1, a peptide specific for binding HA, blocked sHA induced production of IL-8. *d*, chondroitinase ABC digestion of HA reduces IL-8 induction. Data are mean ± S.D. of triplicate determinations and are representative of two experiments.
In the mouse, KC and MIP-2 are most closely related to human IL-8 and were thus chosen for analysis to confirm the response observed to sHA in vitro (31–33). Following intraperitoneal injection, circulating MIP-2 increased 6-fold, consistent with the response observed in tissue culture. It is not clear from these data that the origin of MIP-2 is exclusively from endothelial cells in the mouse as this chemokine can be produced by several cell types (32, 34). However, the rapid response to sHA following intraperitoneal administration further confirmed the potential bioavailability of sHA to the vasculature, and established a useful animal model for identification of the mechanism responsible for recognition of sHA.

Inhibition by specific blocking antibodies, and use of a mouse line deficient in functional TLR4, suggests that the increase in human IL-8 or mouse MIP-2 and KC induced by sHA is dependent on TLR4. This evolutionarily ancient pattern recognition receptor is best known at present for its ability to recognize LPS on Gram-negative bacteria. Recently, several investigations have suggested that there are many different ligands for TLR4, including both exogenous and endogenous molecules. A major limitation in the interpretation of these results is conclusive evidence that the response observed was not due to undetected contamination by LPS. As LPS is a widely present and active at extremely low concentrations, rigorous demonstration of the identity of a TLR4 stimulatory molecule is required. In the present work, LAL assays confirmed that all reagents were free of endotoxin contamination or were well below the threshold endotoxin level required for TLR4 activation. In addition, polymyxin B, an LPS inhibitor, had no effect on sHA-treated ECs. Finally, experiments in which sHA was either inhibited by the HA blocking peptide Pep-1, or digested with chondroitinase ABC, resulted in subsequent loss of activation. The ability of these HA selective neutralizing approaches to inhibit IL-8 production by ECs further supports the conclusion that TLR4 activation is due to the presence of sHA and not contamination in the experimental setup.

In addition to sHA released from endogenous sources after injury, bacteria such as Group A Streptococcus are coated in HA and can secrete hyaluronidases. Therefore, HA fragments can originate from either host or bacteria, and would be indistinguishable by themselves. This suggests that the cellular activation triggered by this stimulus is not a self versus non-self recognition, but rather a specific phenomenon that signifies danger (35). The ability of bacterial hyaluronidases to degrade HA to inactive disaccharides as opposed to mammalian hyaluronidases that generate active hexa and octasaccharides may also reflect a system evolved by some bacteria to avoid detection.

As a signal of danger, it would be important to normal tissue homeostasis that the size and abundance of HA is regulated. Therapeutically, both high molecular weight HA and hyaluronidase (39) has been employed for modification of the host immune response (8, 40, 41). In the case of wound repair, the presence of HA fragments appear to be a conserved “danger” signal, communicating to the host the presence of tissue damage through a pathway shared with identification of foreign microbial challenge. Further understanding and manipulation of this innate response system may be a useful approach to modulate wound healing.

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