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Genomic Analysis and Differential Expression of HMG and S100A Family in Human Arthritis: Upregulated Expression of Chemokines, IL-8 and Nitric Oxide by HMGB1

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We applied global gene expression arrays, quantitative real-time PCR, immunostaining, and functional assays to untangle the role of High Mobility Groups proteins (HMGs) in human osteoarthritis (OA)-affected cartilage. Bioinformatics analysis showed increased mRNA expression of Damage-Associated Molecular Patterns (DAMPs): HMGA, HMGB, HMGN, SRY, LEF1, HMGB1, MMPs, and HMG/RAGE-interacting molecules (spondins and S100A4, S100A10, and S100A11) in human OA-affected cartilage as compared with normal cartilage. HMGB2 was down-regulated in human OA-affected cartilage. Immunohistological staining identified HMGB1 in chondrocytes in the superficial cartilage. Cells of the deep cartilage and subchondral bone showed increased expression of HMGB1 in OA-affected cartilage. HMGB1 was expressed in the nucleus, cytosol, and extracellular milieu of chondrocytes in cartilage. Furthermore, HMGB1 was spontaneously released from human OA-affected cartilage in *ex vivo* conditions. The effects of recombinant HMGB1 was tested on human cartilage and chondrocytes *in vitro*. HMGB1 stimulated mRNA of 2 NFκB gene enhancers (NFκB1 and NFκB2), 16 CC and CXC chemokines (IL-8, CCL2, CCL20, CCL3, CCL3L1, CCL3L3, CCL4, CCL4L1, CCL4L2, CCL5, CCL8, CXCL1, CXCL10, CXCL2, CXCL3, and CXCL6) by ≥10-fold. Furthermore, HMGB1 and IL-1β and/or tumor necrosis factor α (but not HMGI/Y) also significantly induced inducible nitric oxide synthase, NO, and interleukin (IL)-8 production in human cartilage and chondrocytes. The recombinant HMGB1 utilized in this study shows properties that are similar to disulfide-HMGB1. The differential, stage and/or tissue-specific expression of HMGB1, HMGB2, and S100A in cartilage was associated with regions of pathology and/or cartilage homeostasis in human OA-affected cartilage. Noteworthy similarities in the expression of mouse and human HMGB1 and HMGB2 were conserved in normal and arthritis-affected cartilage. The multifunctional forms of HMGB1 and S100A could perpetuate damage-induced cartilage inflammation in late-stage OA-affected joints similar to sterile inflammation. The paracrine effects of HMGB1 can induce chemokines and NO that are perceived to change cartilage homeostasis in human OA-affected cartilage.

Introduction

The pathogenesis in osteoarthritis (OA) is observed in the joints, cartilage, and subchondral bone (Haseeb and Haqqi, 2013). Human OA-affected cartilage as compared with normal cartilage is an active site for chondrocyte hypertrophy, angiogenesis and chondrogenesis, inflammation, inflammation resolution, and extracellular matrix repair and remodeling (Pelletier *et al.*, 2001; Attur *et al.*, 2002a; Haseeb and Haqqi, 2013). The chondrocytes in different zones (I to IV) and subcondral regions are phenotypically and functionally distinct (Haseeb and Haqqi, 2013). Increased amounts of NFκB activity and catabolic activity by interleukin (IL)-1β, tumor necrosis factor α (TNFα), nitric oxide (NO), matrix metalloproteinase (MMPs), and eicosanoids in OA are associated with dysfunctional cartilage homeostasis (Pelletier *et al.*, 2001; Attur *et al.*, 2002a; Haseeb and Haqqi, 2013). Unlike the overt inflammation exhibited in

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rheumatoid arthritis (RA), OA displays ''covert inflammation'' (Attur *et al.*, 2002a). We and others have previously shown that human OA-affected cartilage (as compared with normal cartilage) exhibits an inflammatory response devoid of the cardinal signs of inflammation (redness and swelling with heat and pain—*rubor et tumor cum calore et dolor*) due to unique architecture (avascular, aneural, and alymphatic) of the cartilage (Pelletier *et al.*, 2001; Attur *et al.*, 2002a). In spite of the lack of cardinal signs of inflammation in human OA-affected cartilage, a substantially increased level of mRNA and proteins representing several inflammatory mediators have been reported (Amin *et al.*, 1996, 1999; Attur *et al.*, 2001, 2002a; Pelletier *et al.*, 2001; Haseeb and Haqqi, 2013). Human OA- and RA-affected PBMCs can be segregated based on an inflammatory response by using gene expression arrays (Amin *et al.*, 2007).

High Mobility Groups proteins (HMGs) are chromosomal proteins that can be subdivided into three super families, namely HMGA, HMGB, and HMGN, which contain an AT-hook domain, an HMG–box domain, and nucleosomal binding domain, respectively (Sparvero *et al.*, 2009; Chen and Nuñez, 2010; Harris *et al.*, 2012). It should be noted that the HMGN protein family of HMGs reduces the compactness of the chromatin fiber in the nucleosomes, thereby augmenting transcription. HMG proteins are involved in functions associated with transcription, replication, recombination, DNA repair, inflammation, cell differentiation, development, autophagy, and hypertrophy (Adjaye *et al.*, 2005; Sparvero *et al.*, 2009; Chen and Nuñez, 2010; Harris *et al.*, 2012). Recently, a new class of molecules, Damage-Associated Molecular Patterns (DAMPs), which includes HMGBs, SRY, LEF1, S100, AGE, and extracellular matrix proteins (e.g. heparin sulfate), has been identified (Sparvero *et al.*, 2009; Chen and Nuñez, 2010). These DAMPs have unique properties to initiate and perpetuate inflammatory responses via their receptors in the absence of infections (Sparvero *et al.*, 2009; Chen and Nuñez, 2010). This is termed sterile inflammation (Sparvero *et al.*, 2009; Chen and Nuñez, 2010). Many DAMPs such as HMGB1 (high-mobility group box 1) and heat-shock proteins are cytosolic and or nuclear proteins, which can also be released into the extracellular milieu after tissue injury (Sparvero *et al.*, 2009; Chen and Nuñez, 2010; Harris *et al.*, 2012). HMGB1, which can bind and bend DNA during transcription, can also function as a leaderless secretory protein. The predominant nuclear HMGB1 can translocate to the cytosol, where it undergoes post-translational acetylation. The cytosolic HMGB1 is subsequently secreted into the extracellular milieu. The post-translational process also includes modification of redox states of HMGB1, which partially dictates the bioactivity of extracellular HMGB1 in biological processes by interacting with DNA, several co-ligands, and receptors (Yang *et al.*, 2005, 2010; Tang *et al.*, 2012; Janko *et al.*, 2014). For example, all-thiol HMGB1 generated *in vitro* is engaged in chemotaxis and leukocyte recruitment. Disulfide-HMGB1 produced *in vitro* induces cytokines and inflammation, whereas the oxidized HMGB1 exhibits no immune responses and may be involved in inflammation resolution (Tang *et al.*, 2012; Yang *et al.*, 2012). Furthermore, HMGB1 released by apoptotic cells or necrotic or dead cells are terminally oxidized and unable to induce cytokines and chemokines (Urbonaviciute *et al.*, 2009; Venereau *et al.*, 2012; Yang *et al.*, 2012). The all-thiol HMGB1 and disulfide HMGB1 may be readily interchangeable (by reactive oxygen species [ROS] or reactive nitrogen species [RNS]) depending on the presence of an electron donor or acceptable in the environment (Venereau *et al.*, 2012; Janko *et al.*, 2014). For example, LPS-stimulated cells induced both all-thiol and disulfide HMGB1 (Venereau *et al.*, 2012; Tang *et al.*, 2012). All-thiol HMGB1 is prevalent immediately after (*in vitro*) muscle injury, but it switches to disulfide HMGB1 after a few hours (Venereau *et al.*, 2012). Finally, the sustained ROS/RNS production eventually generates the terminal oxidation of HMGB1 ''oxidized HMGB1'' (Janko *et al.*, 2014). This extracellular HMGB1 can trigger and prolong inflammatory responses via its cell-surface receptors (Toll-Like Receptors [TLR2, TLR4], Mucin domain 3 [TIM3], and Receptor for Advanced Glycation End products [RAGE]) and intracellular proteins/receptors such as spondins and S100 calcium binding proteins (Sparvero *et al.*, 2009; Chen and Nuñez, 2010; Harris *et al.*, 2012).

HMGB1 serum levels significantly correlated with disease activity scores (DAS-28) in patients with arthritis (Goldstein *et al.*, 2007). One of the key characteristics of OA-affected cartilage was chondrocyte hypertrophy and increased synthesis of type X collagen, which could be driven *in vitro* by RAGE receptor of HMGB1 (Cecil *et al.*, 2005). Animal models of OA (STR/ort mice) and collagen-induced arthritis showed increased levels of HMGB1 in the synovial membrane, cartilage, meniscus, and ligaments (Palmblad *et al.*, 2007; Kyostio-Moore *et al.*, 2011). The multifunctional activity of HMGs and its functions in tissue injury in other systems prompted us to analyze their expression in one of the target tissues in human OA. The present study shows differentially expressed HMGs and S100As in normal and human OA-affected cartilage using a genomic approach and the functional effect of HMGB1 in chondrocytes and cartilage. The discussion highlights the similarities in HMG expression of cartilage in man and mouse and the ability of HMGB1 to position itself to change cartilage homeostasis.

Materials and Methods

Reagents

All cell culture media, FCS, cytokines, and PCR primers were obtained from Gibco BRL, Pepto. Tech. Inc., and Yamanuchi Pharmaceuticals. IL-8 ELISA kits were purchased from R and D Systems. Rabbit anti-HMGB1 specific antibodies were a gift from Dr. Kevin Tracy (The Feinstein Institute for Medical Research-LIJ School of Medicine, NY).

Generation of native HMGB1

The native recombinant HMGB1 was cloned, expressed, and purified as previously reported (Wang *et al.*, 1999; Li *et al.*, 2004). Briefly, a full-length cDNA was cloned and expressed in *Escherichia coli*, and the native recombinant HMGB1 was generated. The protein was affinity purified and tested for endotoxin contamination by chromogenic *Limulus amebocyte* lysate assay and other methods as previously reported (Li *et al.*, 2004; Rao *et al.*, 2011; Yang *et al.*, 2012). The HMGB1 in every batch was also tested for its ability to induce inflammatory mediators (such as Nitric Oxide or PGE_2) in cells before it was used in experiments to

ascertain its proinflammatory activity. It should be noted that the medium used in this study was devoid of any reducing or oxidizing agents. The purified native recombinant HMGB1 was provided by Dr. Kevin Tracy's Laboratory.

Procurement of human cartilage and demographics

Normal and OA-affected cartilage from human knees was procured as previously reported (Amin *et al.*, 1997; Attur *et al.*, 2000; Dave and Amin, 2013). The clinical samples represent a broad ethnic and age group, where OA is significantly susceptible (Haseeb and Haqqi, 2013). Briefly, knee OA-affected cartilage was obtained from donors who were undergoing knee replacement surgery under the guidelines of the Institution Review Board for use of human tissues in translational research. Nonarthritic knee cartilage was mostly obtained from patients with fractures or from accident victims after knee amputation or from the National Development and Research Institute (NDRI). The cartilage samples were scored based on the histology in the slides, pathology, and clinical records. The demographics of donors (including males, females, age, source, and ethnic backgrounds) are described in Supplementary Table S1(Supplementary Data are available online at www.liebertpub.com/dna) and (Dave and Amin, 2013). The classification of cartilage was based on OARSI scores (Pritzker *et al.*, 2006; Dave and Amin, 2013).

Human OA-cartilage explant assay for soluble HMGB1

The OA-cartilage *ex vivo* assay was performed as previously reported in our laboratory (Amin *et al.*, 1996; Attur *et al.*, 2002b). The assay for the detection of extracellular HMGB1 from human cartilage was similar to that reported for mouse chondrocytes (Liu-Bryan and Terkeltaub, 2010). Briefly, knee articular cartilage from OA-affected joints of donors was cut into discs and placed in a 24-well plate. Articular cartilage from different parts of the joint was pooled for the experiment. The cartilage was incubated in Ham's F-12 medium (2 mL), and 1% endotoxin free human albumin (and devoid of any oxidizing or reducing agent) in the presence and absence of different modulators. The medium from each well was aspirated and analyzed for HMGB1 or NO as described. Soluble extracellular-cartilage HMGB1 was evaluated by loading 50 μL of supernatants onto a thin 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in the presence of a positive control (1 μg of recombinant human HMGB1), and Western blotted to follow the specific immuno-reactivity to 29 kDa HMGB1 and the corresponding molecular weight. Bound antibody was detected by a secondary antibody conjugated with horseradish peroxidase, and developed using the enhanced chemiluminescence system (Amersham Corp.) on Kodak Xomatic X-ray film. The presence of a 29 kDa immune-reactive protein was identified in the cartilage supernatant that co-migrated with the internal positive control. Nitric Oxide was estimated as their stable end product, nitrite, from the cartilage or cell supernatant using a Griess assay (Amin *et al.*, 1996).

Isolation and cultivation of human chondrocytes

Human chondrocytes were isolated and cultivated as previously reported (Dave and Amin, 2013). Chondrocytes were released from cartilage, and equal amounts of cells (1×10^5 cells per well) were cultivated in 24-well plates as formerly described (Amin *et al.*, 1996; Attur *et al.*, 2002b). They were subject to various experimental conditions in the presence and absence of different modulators.

Western blotting of inducible nitric oxide synthase

Primary human chondrocytes were released from cartilage, and equal amounts of cells were cultivated (Dave and Amin, 2013). The medium was changed, and the cells were challenged with different modulators for an additional 24 h. Cells were harvested and washed with PBS. Total protein was extracted from the cells and estimated by BCA reagent (Pierce). Cell extracts were loaded onto the gel with molecular-weight markers and subjected to Western blot analysis (Amin *et al.*, 1996). The blots were probed with specific anti-inducible nitric oxide synthase (iNOS) monoclonal antibodies as described by BD Transduction Laboratories and developed using ECL Western blot system (Amersham Corp.), and Kodak Xomatic X-ray film.

Quantitative real-time PCR analysis

Primer and probe were designed using the Primer Express Algorithm (PE Applied Biosystems). The quantitation was performed by quantitative real-time PCR (qPCR) with a flourogenic probe. qPCR was performed for specific transcripts (gene) using RNA from a minimum of three normals (N) and six to seven OA-affected cartilage donor samples (OA). The corresponding primer probes were utilized for each gene analyzed. Amplitaq Gold DNA polymerase was used for amplification in a Prism7700 System (PE Applied Biosystems). To exclude PCR amplification of contaminating genomics DNA, we performed PCR (RT minus control) with RNA, which was not reverse transcribed into DNA. In these experiments, RT minus controls were negative. The values for HMGB1 were presented as units of relative expression after normalizing the expression with GAPDH in each cartilage sample. The rest of the qPCR data was presented as units of relative expression after normalization with the expression of GAPDH and after conversion of the values into percent normal before they were calculated for mean ± SD and *p*-values (Attur *et al.*, 2002c; Rao *et al.*, 2011).

Histological analysis of cartilage

Histological evaluation was performed on sagittal sections of normal and OA-affected cartilage (Attur *et al.*, 2009). After dissection of cartilage samples, specimens were fixed in buffered formalin and embedded in paraffin blocks. Sections were stained with specific anti-HMGB1-antibodies. The HMGB1 was stained in brown, and the nucleus was stained in blue.

Gene expression arrays and bioinformatics

RNA extraction from cartilage, sample pooling, and gene expression arrays were performed as previously reported in our laboratory (Attur *et al.*, 2001, 2002b, 2009). Briefly, RNA was extracted from normal (OARSI score: 0–1) and OA-affected cartilage (OARSI score: 3–5). Equal amounts of total RNA were pooled into six different

cohorts (N1 to N6) of normal and (OA1 to OA6) of OA, respectively. Each cohort of normal or OA-affected cartilage groups consisted of RNA that represented a minimum of 5 and a maximum of 12 donors. Probes were prepared from the pooled cartilage samples, hybridized to U133A gene chips as described (www.affymetrix.com), and previously reported for normal and OA-affected cartilage (Attur *et al.*, 2002c). In the 2nd set of gene expression arrays, the primary human chondrocytes were cultivated for 24 h, after which the medium was aspirated, and fresh medium was added to the cells with (5 μg/mL of HMGB1) and without HMGB1 (Wt) for an additional 24 h. The chondrocytes were harvested, and the RNA was isolated as described earlier. Probes were prepared from Wt, and HMGB1-treated cells, and hybridized to U95 gene chips as described (www.affymetrix.com) and previously reported (Attur *et al.*, 2002c). Gene expression microarray raw data (CEL files) was background corrected and normalized using the Bioconductor package ''affy'' with ''RMA'' method; and differential expression and statistical analysis was performed using Linear Models (Smyth, 2004). Probes to EnsEMBL genes (version 70) were annotated using Biomart database. When more than one probe was annotated to the same gene, average expression was taken. Significances of differentially expressed genes were considered if the multiple-tests corrected (FDR) p-value was ≤ 0.05. Heatmap was produced on median-centered expression value (difference of expression of each gene from the median expression value of all samples of that gene) using Gitools (Perez-Llamas and Lopez-Bigas, 2011). Stable housekeeping genes used in these analyses were selected from evidence-based selection and a meta-analysis of 13, 629 human gene arrays (De Jonge *et al.*, 2007).

Statistical analysis and data presentation

The Student's t-test was employed for statistical analysis as previously reported (Rao *et al.*, 2011; Dave and Amin, 2013). The data (from a minimum of triplicates) are presented as mean ± standard deviation. The p-value ≤ 0.05 was considered significant.

Results

Gene expression arrays and qPCR of normal and OA-affected cartilage

A systems biology approach to human OA using genomics, proteomic, and bioinformatics approaches was initiated as described (Attur *et al.*, 2002c). Normal and OA-affected cartilage was harvested from a broad spectrum (age and ethnic background) of donors as shown in Supplementary Table S1. Total RNA was isolated from cartilage and stored for further use. Five to twelve RNA samples were pooled in equal amounts and generated six different cohorts of normal and six cohorts of OA-affected cartilage. Gene expression

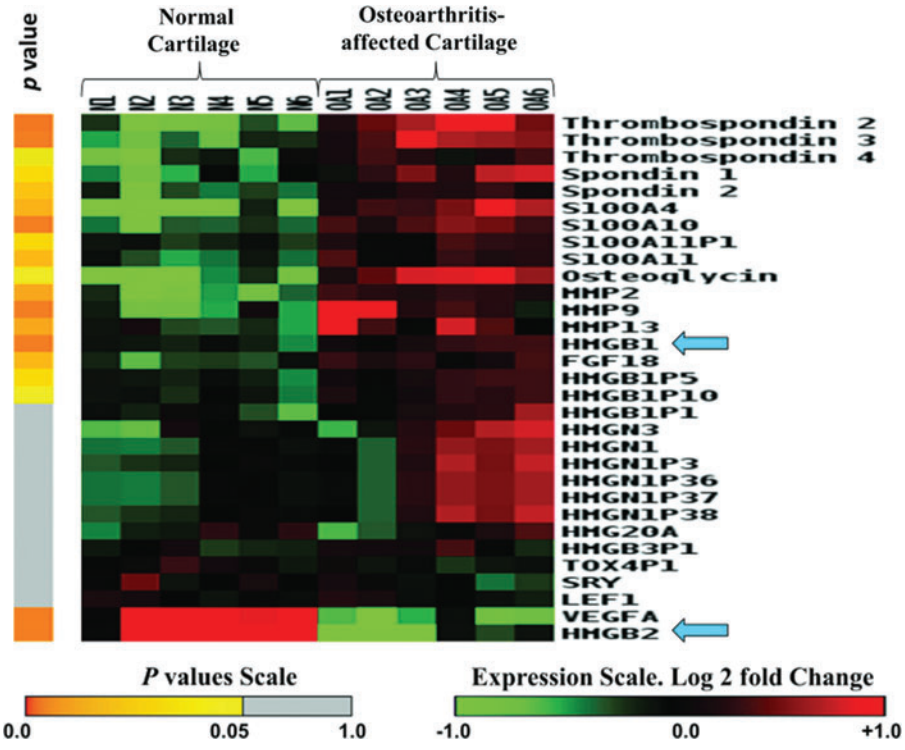

FIG. 1. Gene expression array of Damage-Associated Molecular Patterns (DAMPs) and High Mobility Groups proteins (HMGs) in normal and OA-affected cartilage. Heatmap shows up- and down-regulated transcripts from normal (N) and OA-affected (OA) cartilage. Gene expression arrays were performed from cohorts of six pools of normal and six pools of OA. Gene expression profiles are shown in rows. Color toward red indicates higher expression, and color toward green indicates lower expression as compared with median expression as shown on the scale. The statistical analysis is described on the left-hand side of the figure in the form of a heat map in yellow and gray, where $n = 6$. The significant p-values ($p \leq 0.05$) are represented in yellow. The detailed characteristics of clinical samples are described in Supplementary Table S1. Details about the fold changes and statistical values for each gene are described in Supplementary Table S2. Color images available online at www.liebertpub.com/dna

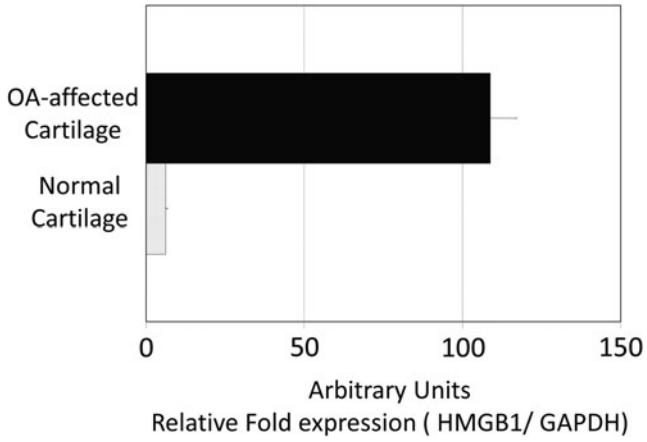

FIG. 2. qPCR of mRNA of HMGB1 in normal and OA-affected cartilage. Total RNA was isolated and quantitated from three normals and three OA-affected cartilage samples from different donors. The data are presented normalizing the values with GAPDH mRNA (housekeeping gene) and SD.

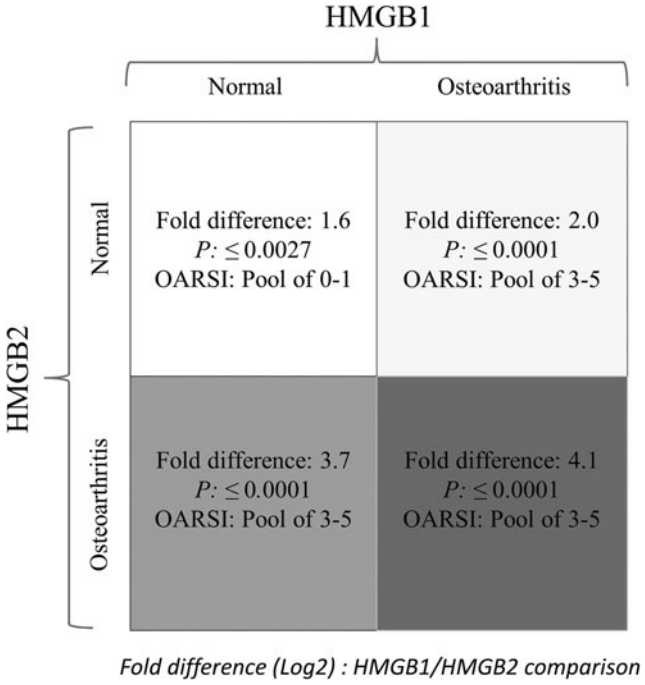

FIG. 3. Bioinformatics and statistical analysis of HMGB1 and HMGB2 in normal and OA-affected cartilage. HMGB1 and HMGB2 share 80% homology at the amino-acid level (Sparvero *et al.*, 2009). The gene expression of HMGB1 and HMGB2 was compared in both normal and OA-affected cartilage to examine their expression during the disease process. The figure describes the ORASI score of the cartilage in normal and OA-affected cartilage and the corresponding expression of HMGB1 and HMGB2 with their *p*-values.

arrays, normalization, statistical, and bioinformatics analysis were performed and each cohort represented data generated from a separate gene chip ($n=6$) as shown in Figure 1. The heatmap showed a significant difference in expression between HMGB1 and HMGB2. HMGB1 increased and HMGB2 decreased in human OA-affected cartilage as compared with normal cartilage (Fig. 1). The differential expression of HMGB1 and two of its pseudogenes (HMGBP5 and HMGBP10) were statistically significant. The expression of nucleosomal HMGN1 and HMGN3 was also increased in four of the six cohorts of human OA-affected cartilage as compared with (none) in normal cartilage (Fig. 1). Similar to HMGB1, S100 proteins interacted with RAGE, promoting cellular recruitment to tissues (Sims *et al.*, 2010). Gene expression arrays showed a statistically significant increase in mRNA expression of S100A4, S100A10, and S100A11 in human OA-affected cartilage as compared with normal cartilage. A detailed list of genes, fold changes, and *p*-values for each transcript is described in Supplementary Table S2 in the addendum. Several proteins (such as MMP-2, -9, and -13, osteoglycin and spondins) that are known to be up-regulated in human OA-affected cartilage as compared with normal cartilage (Pelletier *et al.*, 2001; Attur *et al.*, 2002a, 2009; Haseeb and Haqqi, 2013) also showed an increase in the expression of their respective mRNA in these gene arrays (Fig. 1). The changes in the gene expression arrays were further validated by qPCR analysis of selected transcripts. qPCR analysis of HMGB1 from a separate set of three normal and three OA-affected cartilage samples is shown in Figure 2. Other transcripts were validated using qPCR and another set of clinical samples (three to seven donors) of normal cartilage (N) and OA-affected cartilage (OA) as described in the ''Materials and Methods'' section. For example, Thrombospondin 2 (TSP2), (N: 207 ± 129, vs. OA: 803 ± 274), Fibroblasts Growth Factor-18 (FGF-18) (N: 220 ± 104, vs. OA: 475 ± 175), Osteoglycin (OGN), (N: 678 ± 653, vs. OA: 2146 ± 537), and vascular endothelial growth factor (VEGF) (N: 91 ± 18, vs. OA: 30 ± 4.0) were also observed to be differentially expressed and statistically significant ($p \leq 0.05$) similar to those observed in the heatmaps in Figure 1. Human HMGB2, as compared with HMGB1, showed significant and differential expression of transcripts in both normal and OA-affected cartilage (Fig. 3).

Immunohistological staining of HMGB1 in normal and OA cartilage and release of HMGB1 from cartilage

Going forward, we focused our attention on an archetypical alarmin (HMGB1) involved in noninfectious inflammation whose mRNA expression was up-regulated in human OA-affected cartilage. The tissue-specific and subcellular localization of HMGB1 is well documented (Sparvero *et al.*, 2009; Chen and Nuñez, 2010). We, therefore, examined the expression of HMGB1 protein in normal and OA-affected cartilage. Immunohistological staining of normal and OA-affected cartilage showed HMGB1-positive cells in both normal and OA-affected cartilage (Fig. 4). Kim *et al.* (2000) reported that although there were apoptotic cells in OA-affected cartilage, apoptotic cells were rarely seen in normal cartilage. The staining (with variable intensity) was more pronounced near the surface and deep region of the normal and OA-affected cartilage as compared with the mid-region. The deep region of the OA-affected cartilage showed an increased number of HMGB1-positive cells as compared with the normal cartilage. As expected, Western blotting of cartilage extracts from normal and OA-affected cartilage showed the presence of HMGB1 (unpublished data) similar to those observed in normal and arthritis-affected chondrocytes from monkeys (Loeser *et al.*, 2005). The

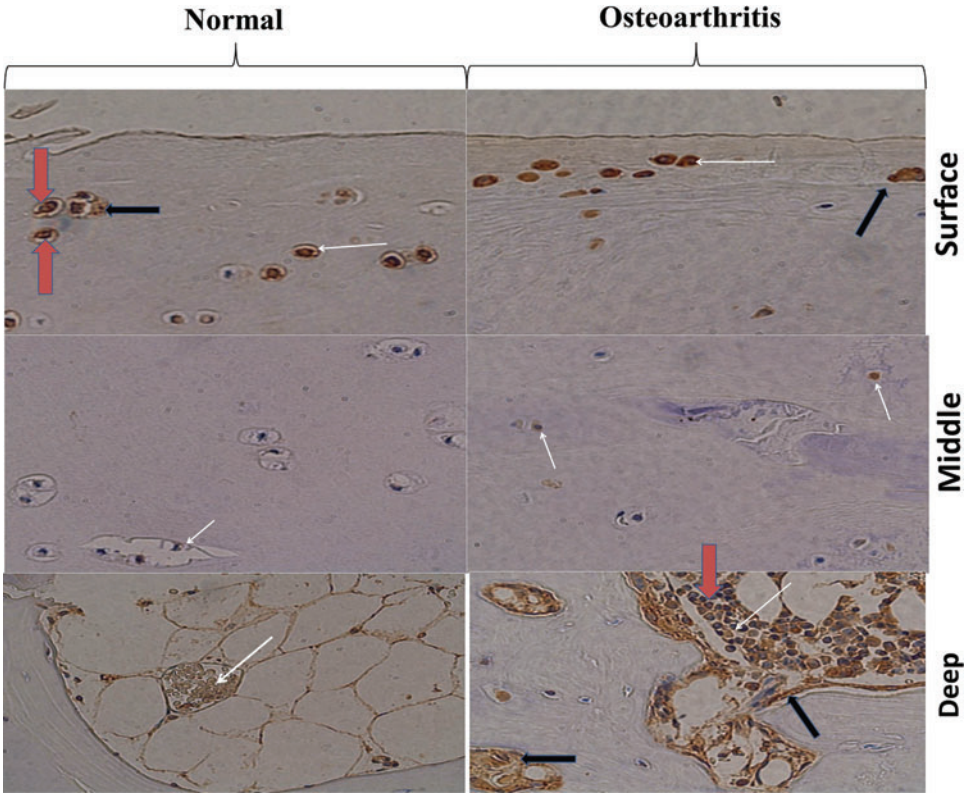

FIG. 4. Immunohistological staining of HMGB1 in normal and OA-affected cartilage. The figure shows the surface, middle, and deep region of the normal and OA-affected cartilage. Cartilage samples were stained (brown) with anti-HMGB1 antibodies + chromphore. The nuclear staining is represented in blue. Some cells show both blue and brown staining of nuclear HMGB1 as indicated by the red arrow. The white arrow shows the HMGB1-positive cells with cytoplasmic staining The black arrow shows HMGB1 staining in the extracellular milieu. Color images available online at www.liebertpub.com/dna

cartilage exhibited tissue-specific expression of HMGB1 (within the cartilage) with a variable intensity of HMGB1-positive cells in two different regions. In summary, immunostaining showed differential expression of HMGB1 with distinct functions in different zones of the cartilage.

All nucleated cells harbor HMGB1 in the nucleus. There are typically $\sim 10^6$ HMGB1 molecules per cell. Some chondrocytes showed increased staining in the nucleus as indicated by the red arrow. The presence of HMGB1 in human chondrocytes was also observed in both the cytosol and extracellular region (lacunae) of the cells as marked in Figure 4. The HMGB1-positive cells in normal and OA-affected cartilage appeared with a large nucleus, suggesting the presence of healthy cells. Extracellular HMGB1 is known to serve as an alarmin and/or a proinflammatory cytokine, by damaged/necrotic cells and/or activate/inflammatory cells (Yang and Tracey, 2005; Yang *et al.*, 2005; Sparvero *et al.*, 2009; Chen and Nuñez, 2010). In view of the immunostaining of cartilage described earlier, damaged, necrotic, and hypertrophic chondrocytes in OA-affected cartilage, we applied our human cartilage *ex vivo* assay to examine the spontaneous release of HMGB1 outside the cartilage (Amin *et al.*, 1996, 1997). The OA-affected cartilage was washed with sterile saline, diced, and incubated in a 24-well plate in triplicate in *ex vivo* conditions to evaluate the spontaneous accumulation of HMGB1 in the serum-free medium containing 1% endotoxin-free human albumin. The assay for the HMGB1 was performed by taking an equal volume of the supernatants from the medium and loading them onto SDS-PAGE gel after 24 and 48 h. The recombinant HMGB1 was used as the positive control for Western blotting to examine the release of HMGB1 from cartilage into the supernatant. Our analysis showed a 29 kDa immune-reactive protein present at 24 and 48 h (similar to recombinant 29 kDa HMGB1) that was spontaneously released by OA-affected human cartilage in *ex vivo* conditions in the cartilage milieu (Fig. 5). IL-1β is known to augment inflammatory mediators in OA-affected cartilage, and it increases apoptotic cells (Zhou *et al.*, 2008). Preliminary

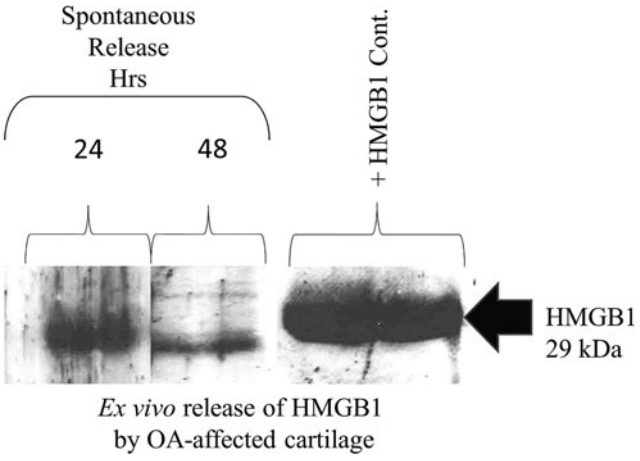

FIG. 5. Spontaneous release of HMGB1 by OA-affected cartilage. Human OA-affected cartilage was incubated in *ex vivo* conditions. The spontaneous release of HMGB1 was determined at 24 and 48 h. An equal amount of supernatant was loaded onto the gel. One lane of the recombinant HMGB1 that was loaded onto the gel is shown on the right-hand side of the figure. Western blot of HMGB1 was performed as described in the ''Materials and Methods'' section. A 29 kDa signal of HMGB1 corresponding to the positive control in the Western blot analysis was observed as shown by an arrow.

experiments were performed by incubating the OA-affected cartilage in the presence and absence of IL-1β. There was about 50% decrease in the accumulation of HMGB1 in the medium as evaluated by western blotting by IL-1β-treated cells (data not presented). These observations and those described earlier rule out the possibility of apoptotic cells in normal cartilage as a major source of extracellular/cartilage HMGB1.

Genomic analysis of the effect of HMGB1 on chemokines, IL-8, and iNOS in human chondrocytes

Sterile injury (in the absence of an infection) has been reported to induce and promote the autocrine and paracrine effects of HMGB1 (Klune *et al.*, 2008). Previous studies have shown that *E. coli*-derived native recombinant HMGB1 can induce inflammatory mediators (Wang *et al.*, 1999; Li *et al.*, 2004; Liu-Bryan and Terkeltaub, 2010; Venereau *et al.*, 2012) and also promote chemotaxis (Taniguchi *et al.*, 2007, 2011; Kew *et al.*, 2012). This suggests that the recombinant HMGB1 harbors at least both: all-thiol HMGB1 and disulfide HMGB1 in unknown proportions which may be interchanged (Venereau *et al.*, 2012; Yang *et al.*, 2012). The recombinant HMGB1 may provoke an inflammatory response and/or migration of stem cells toward the inflamed region to promote repair and regeneration (Klune *et al.*, 2008). Since the functional role of HMGB1 in human chondrocytes remains indefinable in human OA-affected cartilage, we performed gene expression arrays to identify the role of soluble HMGB1 in human chondrocytes, which are the governing cells in the cartilage. Primary human chondrocytes from OA-affected cartilage were incubated with 5 μg/mL of HMGB1 and assayed by gene expression arrays to mimic the paracrine effects in human chondrocytes. In the present communication, we examined the expression of chemokines, IL-8, and iNOS. The expression of each transcript was represented as a ratio of HMGB1-treated cells with uninduced cells (Wt). The changes (ratio) in the basal expression of four housekeeping transcripts in HMGB1/Wt were approximately 1.0. These housekeeping genes did not show any appreciable difference in the presence of HMGB1. Table 1 shows 17 gene transcripts that were identified based on (1) increased mRNA expression (by >10-fold) in HMGB1-treated cells as compared with untreated cells; (2) their potential role in inflammation, chemotaxis, and/or cartilage homeostasis. These included 16 different chemokines (of CC and CXC family) whose average expression was increased by 57-fold (Table 1). This included the CC (CCL2, CCL20, CCL3, CCL3L1, CCL3L3, CCL4, CCL4L1, CCL4L2, CCL5, and CCL8) chemokines. These transcripts also represented gene products representing pro and/or anti-inflammatory activity, apoptosis and anti-apoptosis functions, cell growth/differentiation, cell–cell, and cell–matrix adhesion as described in Table 1.

Table 1. Increased Expression of Chemokine, iNOS, and NFκB Enhancer mRNA by HMGB1-Treated Human Chondrocytes

No.	*Gene symbol*	*Gene description (and related function)*	*Fold change ratio HMGB1/Wt*
1	*NOS2*	Nitric oxide synthase 2, inducible (inflammation and oxidative stress)	104
2	*IL8*	Interleukin 8 (chemotaxis and proinflammatory)	188
3	*CCL2*	Chemokine (C-C_motif)_ligand_2 (chemotaxis, osteoclast differentiation)	53
4	*CCL20*	Chemokine (C-C_motif)_ligand_20 (MIP3A, pro-inflammatory)	173
5	*CCL3*	Chemokine (C-C_motif)_ligand_3 (MIP-1α, macrophage & inflammation)	30
6	*CCL3L1*	Chemokine (C-C_motif)_ligand_3-like_1 (pro-inflammatory activity)	30
7	*CCL3L3*	Chemokine (C-C_motif)_ligand_3-like_3 (pro-inflammatory activity)	30
8	*CCL4*	Chemokine (C-C_motif)_ligand_4 (MIP-1β, macrophage & inflammation)	13
9	*CCL4L1*	Chemokine (C-C_motif)_ligand_4-like_1 (chemotaxis, pro-inflammatory)	13
10	*CCL4L2*	Chemokine (C-C_motif)_ligand_4-like_2 (pro-inflammatory activity)	13
11	*CCL5*	Chemokine (C-C_motif)_ligand_5 (RANTES, pro-inflammatory activity)	228
12	*CCL8*	Chemokine (C-C_motif)_ligand_8 (MCP-2, chemotaxis, inflammation)	57
13	*CXCL1*	chemokine_(C-X-C_motif)_ligand_1_(GRO1 growth and oncogene)	38
14	*CXCL10*	Chemokine (C-X-C_motif)_ligand_10 (chemotaxis, colony formation)	11
15	*CXCL2*	Chemokine (C-X-C_motif)_ligand_2 (MIP-α2, macrophage & inflammation)	40
16	*CXCL3*	Chemokine (C-X-C_motif)_ligand_3 (GRO 3 oncogene, adhesion, migration)	28
17	*CXCL6*	Chemokine (C-X-C_motif)_ligand_6 (chemotaxis)	23
18	*NFκB1*	Nuclear factor NF-kappa-B p105 subunit (inflammation, UV, free radicles)	16
19	*NFκB2*	Nuclear factor NF-kappa-B p100 subunit (inflammation, UV, phagocytosis)	4
20	*RPL24*	Ribosomal protein-L24 (housekeeping gene)	0.6
21	*RPS26*	Ribosomal protein-S26 (housekeeping gene)	1.0
22	*RRP1B*	Ribosomal RNA processing 1 homolog B (housekeeping gene)	1.0
23	*LDHA*	Lactate dehydrogenase A (housekeeping gene)	0.9

Human chondrocytes were stimulated with 5 μg/mL of HMGB1 for 24 h, and the cells were harvested as described in the ''Materials and Methods'' section. The total RNA from control (Wt) cells and HMGB1-treated cells were subjected to gene expression array. The signal was normalized as described in the ''Materials and Methods'' section, and the data were represented as a percent increase as compared with control cells. The table shows selected transcripts (Chemokines and iNOS) up-regulated by ≥1000% as compared with normal unstimulated cells. The variations in NFκB are presented as changes in Log2. The terms in the parentheses represent the general function of the gene related to the pathophysiology of OA as described in Gene Expression Atlas or Wikigene or Genecard or Wikipedia based on the maximum information available on each site.

iNOS, inducible nitric oxide synthase.

Although most of these highly induced transcripts show pleiotropic functions of chemokines that bind to more than one receptor (Allen *et al.*, 2007), some of the general functions of these transcripts in inflammation and cell homeostasis are described via a hyperlink to Gene Expression Atlas or Wikigene or Genecard or Wikipedia based on the maximum information available from each site. Commercially available recombinant HMGB1 (Zhang *et al.*, 2013) of native recombinant HMGB1 used in this study (Wang *et al.*, 1999) or chemically modified HMGB1: disulfide HMGB1 (but not all-thiol HMGB1) induced activation of the NF-kB pathway, and increased levels of cytokines and chemokines (Kew *et al.*, 2012; Venereau *et al.*, 2012). Our gene expression arrays using the native HMGB1 showed activation of two members of NFκB family members (Hayden and Ghosh, 2012) via increased expression of mRNA for NFκB light chain enhancers (NFκB1 by 16.3-fold and NFκB2 by 3.6-fold) as shown in Table 1. Both NFκB1 and NFκB2 promote the NFκB pathways during inflammation, UV radiation, and generation of free radicals (Hayden and Ghosh, 2012).

Regulation of CXC chemokine and IL-8 by HMGB1

The human OA-affected chondrocytes showed >10-fold increased expression of CXC (CXCL1, CXCL10, CXCL2, CXCL3, and CXCL6) chemokines. Injury to cartilage is a recognized sequela of neutrophil activation and chemotaxis by IL-8 in arthritic joints (Lotz *et al.*, 1992). Recently, *in vitro* studies also showed the ability of IL-8 to promote chondrocyte hypertrophy (Yammani, 2012). We have previously shown that the spontaneous release of IL-8 and nitric oxide was from human OA-affected cartilage in *ex vivo* condition, but unlike nitric oxide, IL-8 was not regulated by endogenous IL-1β (Attur *et al.*, 1998), but IL-8 could be induced *in vitro* by IL-1β in human chondrocytes (Amin and Abramson, 1998; Haseeb and Haqqi, 2013). The mRNA expression of IL-8 was induced much higher (188-fold increase) as compared with the average expression of other chemokines shown in Table 1. We, therefore, compared the modulation of IL-8 in human chondrocytes in the presence of IL-1β and two different concentrations of HMGB1 as shown in Figure 6. Although we have previously reported significantly increased levels of IL-8 in OA-affected cartilage as compared with normal cartilage (Attur *et al.*, 1998), there was a much higher increase in the level of IL-8 by IL-1β. However, a tenfold increase in levels of IL-8 was observed in the presence of 5 μg/mL of HMGB1 when compared with IL-1β. These observations show the ability of HMGB1 to super-induce IL-8 and other related chemokines in human chondrocytes as shown in Table 1 and Figure 6.

Regulation of iNOS by HMGB1

Human OA-affected cartilage has been reported to spontaneous release NO (Abramson *et al.*, 2001a, 2001b). Hypoxia and or oxidative stress (similar to that observed in OA-affected cartilage) is known to induce HMGB1 in nonimmune cells (Klune *et al.*, 2008). The instinctively released HMGB1 by cells under stress can act as danger molecules by signaling neighboring cells of ongoing damage and might also trigger an inflammatory signaling cascade (Yang and Tracey, 2005; Harris *et al.*, 2012). We, therefore, examined whether human chondrocytes were

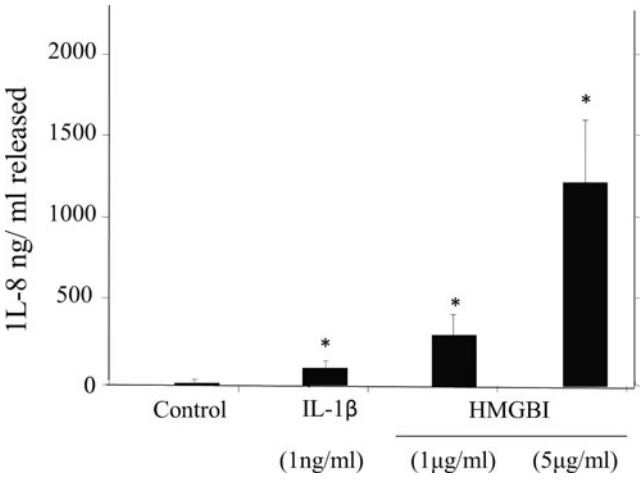

FIG. 6. Regulation of IL-8 by HMGB1 and IL-1β in human chondrocytes. Human OA-affected chondrocytes were seeded in triplicates for 24 h as described in the ''Materials and Methods'' section. One μg/mL of IL-1β or 1–5 μg/mL of HMGB1 was added to the cells. The production of IL-8 was estimated at the end of 72 h. Data are expressed as mean ± SD ($n = 3$). The p-values for IL-8 are compared with the nonstimulated cells, where $*p \leq 0.05$.

sensitive to soluble recombinant HMGB1, using nitric oxide as another model multifunctional inflammatory mediator that is also induced under stressful conditions. Nitric oxide is also known to be modulated by IL-1β and TNFα (Attur *et al.*, 1998; Patel *et al.*, 1998). The up-regulation of iNOS mRNA was about 104-fold in HMGB1-treated cells as compared with basal levels in untreated cells (Table 1). Primary chondrocytes from OA-affected cartilage were stimulated with IL-1β. TNFα, HMGB1, and HMGI/Y (negative control) and the levels of nitric oxide were examined at 48 h. IL-1β, TNFα, and HMGB1 (but not HMGI/Y) showed significantly increased levels of NO production as compared with nontreated or HMGI/Y-treated cells. Western blot analysis of human chondrocytes stimulated with IL-1β, TNFα, HMGB1, and HMGI/Y showed induction of 130 kDa iNOS in IL-1β, TNFα, and HMG1-stimulated cells but not in HMGI/Y or unstimulated cells. The increase in NO was consistent with the induction of iNOS mRNA and protein in human chondrocytes (Fig. 7).

Paracrine effects of HMGB1 in human OA-affected cartilage

The earlier experiments demonstrated that HMGB1 generated within the cartilage cells could seep out of the cartilage in *ex vivo* conditions. The paracrine effects of inflammatory mediators in synovial fluids have been reported to influence chondrocyte functions (Haseeb and Haqqi, 2013). Human arthritis-affected synovial fluid harbors 1–10 μg/mL of HMGB1 (Kokkola *et al.*, 2002). We next examined whether the paracrine effects of HMGB1 in organ cultures of OA-affected cartilage could influence the levels of nitric oxide within the cartilage. Human OA-affected cartilage was incubated with different concentrations of IL-1β and HMGB1 in two different experiments. There was a significant increase in the accumulation of NO in IL-1β- and HMGBI-treated cartilage as compared with nontreated cartilage (Fig. 8). Similar to

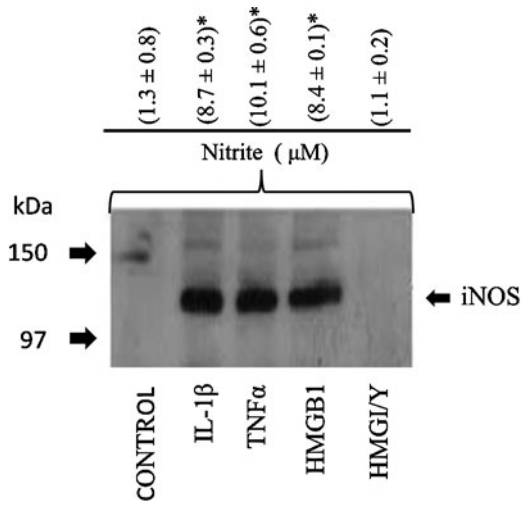

FIG. 7. Effect of HMGB1, IL-1β, TNFα, and HMGI/Y on NO in human chondrocytes. Human OA-affected chondrocytes were grown for 24 h before they were stimulated with IL-1β (10 ng/mL), TNFα (1000 U/mL), HMGB1, or HMGI/Y (2.5 μg/mL) for an additional 24 h. Cell extracts were prepared, and an equal amount of total protein was analyzed for expression of iNOS by western blotting as previously reported. The 130 kDa iNOS is shown by an arrow. In a similar experiment, NO levels were estimated at 48 h in triplicate as shown in the upper panel. Significant levels of NO were calculated using Student's *t*-test. ($*p \leq 0.01$) where $n = 3$, and the values were compared with the control cells.

IL-1β, these experiments show that 1–5 μg/mL of HMGB1 can exert paracrine effects in human OA-affected cartilage.

Discussion

Differential expression of several members of the HMG group of transcripts was observed in normal and diseased cartilage with sterile injury. Furthermore, it unveiled the role of HMGB1 in ''covert or silent'' inflammation in chondrocytes and cartilage during the pathogenesis of human OA. The mRNA expression showed a distinct pattern of transcripts that correlated with the OARSI histological (grade 3–5) of the OA-affected cartilage and grade 0–1 of normal cartilage. The experimental protocol of pooling mRNA from similar clinically defined samples revealed consistency in gene expression among different cohorts. Furthermore, the log2 (two- to four-fold) changes in mRNA expression (between normal and OA-affected cartilage) could be independently validated using an alternate technology such as qPCR. Indeed, some of these differentially expressed transcripts observed in our arrays were previously reported throughout the literature (Amin *et al.*, 1999; Pelletier *et al.*, 2001; Attur *et al.*, 2002a, 2002c, 2009; Haseeb and Haqqi, 2013).

The expression of HMGB1 and HMGB2 (which also binds to DNA) was tightly coordinated toward common functions in cartilage (Muller *et al.*, 2001; Sparvero *et al.*, 2009), but they exhibit distinct expression in normal and diseased cartilage. Both the HMGs exhibit pronounced expression in the superficial and deep zone of normal and OA-affected cartilage. The decreased expression of HMGB2 was associated with cartilage erosion and aging in mice (Taniguchi *et al.*, 2007, 2009). Further studies in mice showed that HMGB2 expression was inversely correlated with differentiation of mesenchymal stem cells (MSC), and overexpression by transfection of HMGB2 gene into MSCs suppressed chondrogenesis (Taniguchi *et al.*, 2009, 2011). Collectively, the age-related loss of HMGB2 or HMGB1's gain in articular cartilage may represent a trend for the decline in adult cartilage stem cell populations and cartilage erosion (Taniguchi *et al.*, 2007, 2011, 2009; Kyostio-Moore *et al.*, 2011). HMGN1 HMGN3, and HMGB1 were reported to reduce the compactness of the chromatin fiber and augment transcription (Bustin, 2010). In addition, HMGN1 is

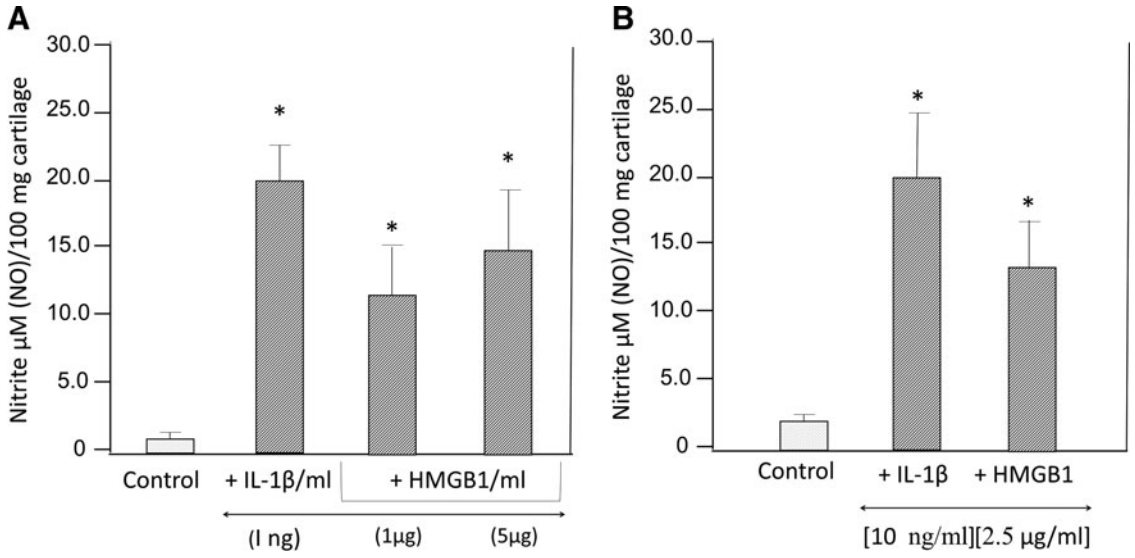

FIG. 8. Paracrine effect of HMGB1 on NO in human cartilage *in vitro.* Human OA-affected cartilage was incubated in triplicate in *ex vivo* conditions in two separate experiments **(A, B)** in the presence of IL-1β and HMGB1 for 48 hrs. The levels of NO were estimated in the form of nitrites, where $n = 3$. The *p* (*)-values between control-unstimulated cells and IL-1β or HMGB1-treated cartilage was ≤ 0.01.

preferentially localized to DNAase I hypersensitive sites, promoters, functional enhancers, and transcription factor binding sites (Cuddapah *et al.*, 2011).

The present study shows the simultaneous increase of TSP2, OGN, FGF-18, and S100 and decrease in VEGF, which are relevant to the pathophysiology of OA-affected cartilage and repair (Palmblad *et al.*, 2007; Sparvero *et al.*, 2009; Li *et al.*, 2013). For example, TSP2 regulated the ratio of cartilage to bone during fracture healing (Taylor *et al.*, 2009), and *TSP2*$^{-/-}$ mice showed abnormalities in collagen fibrils (Kyriakides *et al.*, 1999). The OGN protein has been detected in cartilage (De Ceuninck *et al.*, 2005), and it is involved in the regulation of collagen fibrillogenesis and linkages to the muscles (Ge *et al.*, 2004; Tanaka *et al.*, 2012). Indeed, *OGN*$^{-/-}$ mice showed an increased diameter of collagen fibrils (Tasheva *et al.*, 2002). FGF-18, which signals through FGF receptor 3, promotes chondrogenesis (Ellman *et al.*, 2013). A recombinant version of FGF-18 (sprifermin) may enter clinical trials as a potential treatment for OA (Ellman *et al.*, 2013). VEGF couples hypertrophic cartilage remodeling, ossification, and angiogenesis during endochondral bone formation, proliferation, differentiation, and/or survival of osteoclasts, osteoblasts, and chondrocytes (Hans-Peter *et al.*, 1999; Carlevaro *et al.*, 2000; Murata *et al.*, 2008). The limiting availability of VEGF may impact the healing and remodeling of human cartilage in OA.

Western blot analysis of human cartilage extracts confirmed the presence of HMGB1 in normal and OA-affected cartilage (unpublished data), similar to those observed in monkeys (Loeser *et al.*, 2005). In view of the expression and secretion of HMGB1 in normal and OA-affected cartilage by relatively healthy cells, lack of apoptotic cells in normal cartilage, presence of HMGB1 in normal cartilage, and the effects of IL-1β on HMGB1 accumulation in OA-affected cartilage support the assertion that the major amount of HMGB1 was most likely released by active secretion and not apoptotic or dead cells. The increased level of HMGB1-positive cells in the deep zone of human OA-affected cartilage was similar to up-regulated HMGB1 and S100 (at week 20) in cartilage of animal models of OA (Kyostio-Moore *et al.*, 2011). In mice, HMGB1-positive cells (hypertrophic chondrocytes) in the deep zone also secreted HMGB1 in the cartilage milieu in mice (Taniguchi *et al.*, 2007). Likewise, distinct expression of HMGB1 has been reported in other pathophysiological conditions with sterile injury such as colitis, ischemia, sepsis, endotoxemia, and systemic lupus erythematous (Sparvero *et al.*, 2009; Kyostio-Moore *et al.*, 2011; Harris *et al.*, 2012; Kang *et al.*, 2013).

There was a similarity in HMGB1 expression in human (this study), mice, and bovine cartilage (Heinola *et al.*, 2010): (1) Nucleosomal staining was predominant in chondrocytes from normal cartilage as compared with OA-affected cartilage; (2) OA-affected cartilage showed increased intensity of extra-/pericellular deposition of HMGB1; (3) There was also an increased staining of HMGB1 in the subchondral bone plate and osteophyte formation region of bovine cartilage similar to human cartilage (Heinola *et al.*, 2010); (4) Similar to OA-affected bovine and human cartilage, arthritis models in mice showed increased expression of HMGB1 in the cartilage; (5) cytosolic HMGB1 was observed in hypertrophic chondrocytes, growth plate region in mice (Taniguchi *et al.*, 2007), and human OA-affected cartilage; (6) activated murine (Johnson *et al.*, 2003; Liu-Bryan and Terkeltaub, 2010) and human chondrocytes released a 29 kDa HMGB1; and (7) HMGB1 from murine chondrocyte (Kyostio-Moore *et al.*, 2011) have been reported to function as a chemoattractant for osteophytes, osteoblasts, and endochondral ossification (Taniguchi *et al.*, 2007). HMGB1 in the extracellular milieu in the deep region of human OA-affected cartilage may promote chemotaxis by forming a complex with CXCL12 and signaling via CXCR4, similar to chemokine-like function of HMGB1 in an inflammatory bone marrow microenvironment of patients with chronic idiopathic neutropenia (Velegraki *et al.*, 2012). (8) HMGB1 induces MMP-3 and -13 in murine (Liu-Bryan and Terkeltaub, 2010) and human chondrocytes (Unpublished data); (9) HMGB1 or HMGB2 gene deletion (or decreased expression) showed decreased chondrogenesis, reduced superficial zone cellularity, and early onset of OA similar to human OA-affected cartilage (Taniguchi *et al.*, 2009, 2011). (10) Activated human and mice synovial cells show increased levels of HMGB1 (García-Arnandis *et al.*, 2001, Palmblad *et al.*, 2007).

The mechanism of action of HMGB1 was compared with murine models of arthritis and bovine cartilage. Briefly, a single injection (5 μg) of intra-articular HMGB1 in the knee joint of rodents induced HMGB1, which preceded the clinical onset of the diseases, resulted in moderate synovitis that lingered for about 28 days, with increased TNFα and IL-1β at this stage of the diseases (Pullerits *et al.*, 2003; Andersson and Harris, 2004). Furthermore, an injection of HMGB1 in joints of *TNF*α$^{-/-}$ mice induced arthritis similar to *TNF*α$^{+/+}$ mice (Pullerits *et al.*, 2008). It should be noted that the lack of HMGB1 expression in mice had detrimental effects on homeostasis of bone and cartilage (Taniguchi *et al.*, 2007). Seol *et al.* (2012) showed that injury to bovine cartilage and chondrocyte death stimulated the emergence of chondrogenic progenitor cells via HMGB1 release and RAGE-mediated chemotaxis. This process could be inhibited by Glycrrhizin, a chelator of HMGB1 and anti-HMGB1 antibodies. Thus, inflammatory cell recruitment (by all thio-HMGB1) and activation of inflammatory mediators (by disulfide HMGB1) may depend on mutually exclusive (and interchangeable) redox forms of HMGB1: These different redox forms of HMGB1 may function via different mechanisms, time lines of secretions by the same cell, binding to different receptors, and interact with different matrix proteins (such as DNA, lipids, IL-1β, CXC chemokines, and stromal cell-derived factors) in the presence of other inflammatory mediators and redox state of the tissue (Tang *et al.*, 2012, Janko *et al.*, 2014). It is quite possible that the release of dynamic redox-regulated HMGB1 may contribute to an orderly orchestrated response to early inflammatory response in cartilage, recruitment of progenitors of chondrocytes, and subsequent resolution of inflammation (by the oxidized HMGB1). The potential role of different redox form(s) of HMGB1 operating in the complex interplay of chondrogenesis in normal and OA-affected cartilage remains elusive. However, the recombinant HMGB1 previously reported to induce inflammatory mediators in macrophages (Wang *et al.*, 1999) also induced NFκB, chemokines, and nitric oxide in cartilage and chondrocytes in both *in vitro* and *ex vivo* conditions.

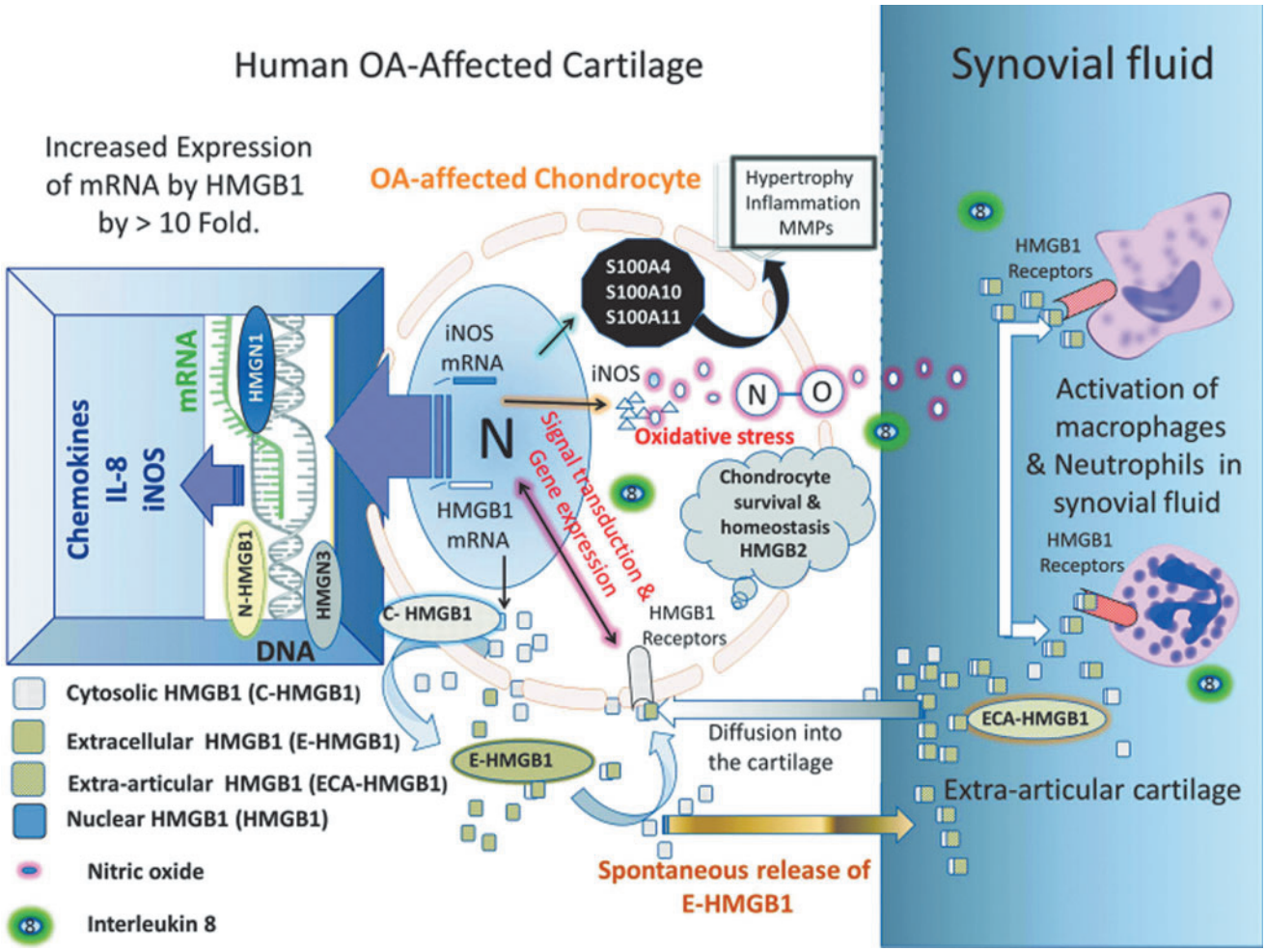

FIG. 9. The doppelganger life of HMGB1 in OA-affected cartilage: architectural factor, extracellular signaling, sterile inflammation, and cartilage homeostasis. The up-regulation of mRNA for HMGB1, HMGN1, and HMGN3 and a decrease in expression of HMGB2 in human OA-affected cartilage exhibited the interaction among HMG. HMGB1 is observed within the nucleus (N-HMGB1) of all nucleated cells. It was also observed in chondrocytes. There are typically $\sim 10^6$ HMGB1 molecules per cell. The HMGB1 protein was observed in the cytosol (C-HMGB1) of chondrocytes and extracellular milieu (E-HMGB1), where it can adopt its role as a cytokine (Yang and Tracey, 2005; Yang *et al.*, 2005). The extracellular HMGB1 (1) may bind to its receptor (red) and induce signal transduction (Wahamaa *et al.*, 2007; Sparvero *et al.*, 2009). HMGB1 exhibited superinduction of the CC and CXC family of chemokines, IL-8 and iNOS gene expression (by $\geq 1000\%$), as shown throughout this study. (2) may interact with matrix and or chemokines (CXCL12) and exhibit other functions (Diener *et al.*, 2013); (3) be released outside the cartilage (ECA-HMGB1), where it can enter the synovial fluid in the joints (Kokkola *et al.*, 2002). ECA-HMGB1 may (1) activate other inflammatory cells and/ or (2) might also penetrate back into the cartilage and activate the HMGB1 receptors on chondrocytes (Klune *et al.*, 2008). Increased levels of HMGN1 and HMGN3 facilitate gene transcription (Bustin, 2010) and superinduction of chemokines, iNOS and IL-8, in OA-affected cartilage. NO and IL-8 can enter the synovial fluid. IL-8 can attract inflammatory cells. Most of the HMGB1-induced genes are involved in pro- or anti-inflammatory activity, chemotaxis and cell/matrix adhesion, matrix metabolism, and cartilage homeostasis as referenced in Table 1. Increased levels of nitric acid and oxidative stress have long been recognized to alter catabolism in OA-affected cartilage, degradation, and cartilage repair (Amin and Abramson, 1998; Abramson *et al.*, 2001a). This oxidative environment in OA-affected cartilage may create an environment for post-translational modification of HMGB1 depending on its oxidation state within the cartilage (Janko *et al.*, 2014) as described in some *in vitro* experimental settings. Oxidation of di-sulfides of HMGB1 has been reported to favor an inflammatory response, and terminal oxidation of HMGB1 may promote inflammation resolution (Yang *et al.*, 2012; Diener *et al.*, 2013; Janko *et al.*, 2014). The decreasing level of HMGB2 is well documented in arthritis-affected cartilage and is shown throughout this study. HMGB2 plays a critical role in chondrocyte survival and cartilage homeostasis (Taniguchi *et al.*, 2007; Taniguchi *et al.*, 2009). The human OA-affected cartilage also showed increased expression of other DAMPs such as S100A4, S100A10, and S100A11. They have been reported to have catabolic and proinflammatory activity in chondrocytes (Yammani, 2012). Color images available online at www.liebertpub.com/dna

DAMPs (including S100A4, S100A10, S100A11, LEF1, and SRY) signal via the RAGE receptors and/or Wnt pathway. S100A4 is up-regulated in synovial tissue and fluids of OA and RA with increased secretion of MMP-13 (Yammani, 2012). In mice, *S100A4*$^{-/-}$ animals showed reduced joint inflammation, impaired bone resorption and regulation of osteoclast, increased cartilage and bone destruction, and decreased expression of MMPs (Bian, 2011; Erlandsson *et al.*, 2013). S100A10 expression decreased the production of TNFα, IL-1β, and IL-10 in chondrocytes (O'Connell *et al.*, 2010; Song *et al.*, 2012). The S100A11 induces chondrocyte hypertrophy and matrix catabolism (Yammani, 2012). Thus, similar to the HMGs, different species of S100 proteins have a critical role in cartilage inflammation and resolution.

Our observations suggest that there are relatively more similarities in expression and possibly common functions shared by DAMPs in man and mouse cartilage and joint tissues. For example, (1) increased levels of the S100A family of proteins stimulate chondrocyte hypertrophy and catabolic activity (Cecil and Terkeltaub, 2008; Van Lent *et al.*, 2008; Bian, 2011; Yammani, 2012); (2) Age-related loss of HMGB2 is associated with reduced cellularity and onset of OA. HMGB2 and LEF-1 enhance Wnt signaling and promote superficial zone chondrocyte survival in mice. Loss of the HMGB2-Wnt signaling interaction is one of the mechanisms in aging-related cartilage (Taniguchi *et al.*, 2007, 2009, 2011); (3) SRY-related high-mobility-group (HMG) box transcription factors (SOXs) exhibit a distinct role in different stages of chondrogenic differentiation and survival (Ikeda *et al.*, 2005); (4) Similar to HMGB1, Spondin 1 was increased in OA-affected cartilage. It regulates the differentiation of chondrocytes and endochondral bone formation in mice (Palmer *et al.*, 2010).

Several chemokines that were up-regulated (>by 1000%) by HMGB1 in human chondrocytes not only bind to more than receptor, but also influence cartilage homeostasis (Borzi *et al.*, 2004; Haringman *et al.*, 2004). Briefly, (1) CCL20 is required for osteoclast proliferation, differentiation, osteolytic bone lesion, and subchondral bone formation of arthritis-affected cartilage (Lisignoli *et al.*, 2007, 2009); (2) Chondrocyte hypertrophy can be induced by GRO1 (Pulsatelli *et al.*, 1999; Borzi *et al.*, 2002; Cristino *et al.*, 2008); (3) CXCL10 is noted to promote osteoclast formation (Grassi *et al.*, 2003) and recruits subchondral bone mesenchymal progenitor cells (Endres *et al.*, 2010); (4) CXCL1 is known to induce hypertrophic differentiation, apoptosis, and calcification in chondrocytes (Merz *et al.*, 2003); (5) CCL5 up-regulated NO, IL-6, MMP-1, −3, and -13 production in cartilage (Alaaeddine *et al.*, 2001; Borzi *et al.*, 2004); and (6) CXCL12 can form a complex with HMGB1 and signal via the CXCR4 receptor to recruit cells to damaged tissue (Schiraldi *et al.*, 2012). IL-8 can induce S100A11, which, in turn, can induce chondrocyte hypertrophy (Yammani, 2012). These observations suggest that some of the homeostatic functions in cartilage and subchondral bone by HMGB1 may be mediated via a complex interaction of chemokines.

The paracrine effects of HMGB1 may further activate macrophages and neutrophils in arthritis-affected joints (Yang and Tracey, 2005; Yang *et al.*, 2005). Although low and variable levels of nitric oxide are essential during endochondral ossification, catabolic and homeostatic activity in human cartilage (Amin *et al.*, 1999; Wang *et al.*, 2011), high levels of NO have detrimental effects in cartilage (Amin and Abramson, 1998). Granted that HMGB1 can bind to a multitude of known receptors in chondrocytes, the functional receptor for HMGB1 in human OA-affected cartilage remains elusive.

In summary, our observations bolster the complex role of these alarmins, DAMPs, and HMGs as innate and endogenous regulators of injury-induced sterile inflammation in the cartilage and joints, their collaborative role in chondrogenesis, hypertrophy, and maintenance of the homeostasis in mice and human cartilage as shown in Figure 9. There is significant focus on the therapeutic intervention of HMGB1 (Pulsatelli *et al.*, 2013). A drug such as Atrovastatin (Lipitor) reduces increased levels of hyperlipidemia and HMGB1 in the serum (Jin *et al.*, 2012). Therapeutic benefits through HMGB1-directed mechanisms involve HMGB1 inhibitory ligands such as Toll-like receptor antagonists, RAGE antagonists, and alpha7 nicotinic acetylcholine receptor agonists (Lamore *et al.*, 2010). Antibodies that neutralize HMGB1 are known to confer protection against tissue damage and injury in joints (Yang and Tracey, 2005; Sparvero *et al.*, 2009). Anti-HMGB1 therapy was comparable to TNF-blocking regiments in animal studies of arthritis (Andersson and Harris, 2004) and, thus, shows significant promise.

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Disclosure Statement

No competing financial interests exist.

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