Published in final edited form as:

Arthritis Rheumatol. 2014 May ; 66(5): 1266–1271. doi:10.1002/art.38391.

Stress inducible gene nuclear protein-1 (NUPR1/p8) regulates MMP-13 expression in human articular chondrocytes

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Abstract

Objective—Nuclear protein-1 (nupr1) is a stress-inducible protein involved in gene transcription. The objectives of this study were to determine (a) if chondrocytes express nupr1 and (b) whether nupr1 regulates MMP-13 expression.

Methods—Paraffin-embedded cartilage sections from normal human and osteoarthritic cartilage were immunostained using anti-nupr1 antibody. Total RNA to measure nupr1 expression was isolated from joint tissue obtained 8-weeks after surgery from young (12-week-old) and older (12-month-old) mice that underwent destabilization of medial meniscus (DMM) to induce osteoarthritis. Human chondrocytes were stimulated with 1-10ng/ml of IL-1 β , 25- μ M *tert*-butylhydroperoxide, or 2- μ M of thapsigargin and nupr1 expression was analyzed by quantitative-PCR. In addition, chondrocytes were transfected with siRNA to knockdown nupr1 expression and then stimulated with IL-1 β overnight. After incubation, the conditioned media was collected and analyzed for MMPs.

Results—Increased nupr1 immunostaining was noted in human osteoarthritic cartilage relative to normal cartilage. Expression was increased in mouse joint tissue from 12-month-old mice that underwent DMM surgery relative to sham controls. Stimulation of chondrocytes with IL-1 β induced a 2-fold increase in nupr1 mRNA within 1hr and peaked to 4-fold at 6hrs. Treatment of chondrocytes with *tert*-butylhydroperoxide to induce oxidative stress, but not with thapsigargin to induce endoplasmic reticulum stress, increased nupr1 mRNA expression by over 2-fold. Knocking down nupr1 inhibited IL-1 β -mediated induction of MMP-13.

Conclusions—Nupr1 is expressed in cartilage, and its levels are increased in OA. Nupr1 expression is required for IL-1 β -mediated expression of MMP-13. Thus, our study suggests a novel pathway for regulation of IL-1 β -mediated production of MMPs in chondrocytes.

Keywords

Nuclear protein-1; MMP-13; OA; human articular chondrocytes; oxidative stress and endoplasmic reticulum stress

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Authors have no conflict of interest with regard to work.

Progressive loss of articular cartilage is a hallmark of osteoarthritis (OA). During OA, augmented production of matrix-degrading enzymes, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin-1 motifs (ADAMTSs), is due to increased catabolic activity of chondrocytes. Among the MMPs, MMP-13 (collagenase 3) plays an important role in cartilage degradation and has been previously shown to be overexpressed in OA (1). Several cytokines and chemokines, including IL-1 β , IL-6, and TNF α , promote catabolic activity in chondrocytes (2). IL-1 β is a major proinflammatory mediator that induces increased production of MMP-3 and MMP-13 by chondrocytes (2). However, the mechanisms involved in the regulation of MMP expression in OA have not been fully elucidated.

Nuclear protein 1 (nupr1/p8) is a stress-inducible nuclear protein whose expression is induced by multiple stress stimuli, including lipopolysaccharide, carbon tetrachloride, TNF- α , and TGF- β (3). Nupr1 is highly conserved across the phylogenies and belongs to the family of high mobility group (HMG) transcription regulators involved in cellular stress responses (3). Nupr1 plays an important role in controlling cell cycle, apoptosis, and autophagy (3 and references therein). Recently, Gorrupi et al has shown that nupr1 also plays an important role in MMP-9 and -13 expressions in cardiac fibroblasts (4).

In the present study, we investigated whether nupr1 is present in OA cartilage and determined if it is involved in regulation of MMP expression by chondrocytes. In human OA cartilage, nupr1 was present and its expression was increased in the DMM model of OA in mice. Inhibition of nupr1 expression reduced IL-1 β -mediated expression of MMP-3 and -13.

MATERIALS AND METHODS

Materials

Collagenase-P was purchased from Roche Applied Science. Pronase was purchased from Calbiochem. Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1), antibiotics, and fetal bovine serum were obtained from Invitrogen. Nupr1/p8 antibody was obtained from Santa Cruz. Nupr1 and TBP primers and SybrGreen PCR Master Mix were purchased from Qiagen. Nitrocellulose membranes and ECL chemiluminescence detection kits were purchased from Amersham Biosciences. Human nupr1-specific siRNA was obtained from Thermo Fisher Scientific. MMP13 and MMP3 antibodies were obtained from Abcam. MMP2 antibody was from Millipore. IL-1 β and human pro-MMP-13 ELISA kit was purchased from R&D Systems. RNAlater was purchased from Life Technologies. tert-butylhydroperoxide (tBHP) and thapsigargin were purchased from Sigma-Aldrich. Full length nupr1 plasmid construct was provided by Dr. Juan L. Iovanna (Centre de Recherche INSERM, Marseille, France).

Methods

Chondrocyte isolation and culture conditions—Normal human ankle cartilage was obtained from tissue donors (ages 55-84 years old) with no prior history of arthritis from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) through the Rush University Medical Center (Chicago, IL) or from the National Disease Research Interchange

(Philadelphia, PA) with Institutional Review Board approval and appropriate consent within 24 hours of the donor's death. Each cartilage specimen was graded for degenerative status based on the five-point Collins scale, as modified by Muehleman *et al.* (5); cartilage samples with a score of less than 2 were used as normal samples. The OA cartilage was discarded tissue obtained after knee replacement surgery performed at the Wake Forest Baptist Hospital (Winston-Salem, NC). Normal ankle cartilage shavings were digested with 0.2% Pronase for 1 h and then with 0.025% Collagenase-P (Roche Applied Science) overnight. High-density monolayer cultures were established by plating cells in 6-well plates at 1×10^6 cells/ml (2 ml/well) in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum. Plates were maintained for 3–5 days until they reached confluence prior to experimental use.

Immunohistochemistry—Immunostaining was performed with a nupr1-specific rabbit polyclonal antibody (antibody dilution 1:200) on paraformaldehyde-fixed, paraffin-embedded human knee cartilage sections from 3 normal tissue donors (68, 70 and 76 years old, respectively) and 3 OA patients (64, 84 and 88 years old, respectively). The cartilage samples were scored macroscopically and were kindly provided by Dr. Martin Lotz (Scripps Research Institute, La Jolla, CA.).

Cartilage sections were deparaffinized with xylene, rehydrated through a series of decreasing concentrations of ethanol, and washed with Tris-buffered saline (TBS). Antigen retrieval was achieved with proteinase K treatment. Sections were washed with TBS, and blocked with blocking buffer for 30 minutes at RT and incubated with primary antibody (1:200) in blocking buffer overnight at 4°C. Sections were then washed three times with TBS, and then incubated with biotinylated secondary antibody for 1 hr at room temperature. Sections were washed with TBS and incubated with ImmPACT™ NovaRED detection reagent (Vector Laboratories) for 30 min following manufacturer's instructions, counterstained with hematoxylin (Sigma), dehydrated, and mounted. For the negative controls, rabbit antiserum was substituted for the nupr1 antibody.

Quantitative Real-Time PCR—Total RNA was isolated from cultured human chondrocytes by the standard TRIzol method and from mouse joint tissue according to previously published techniques (6). The mouse tissue RNA was obtained from samples collected in a previously published gene array study that examined differences in gene expression between young adult (12 week-old) and older adult (12 month-old) mice, where OA was induced by destabilization of the medial meniscus (DMM) (6).

2 µg of total RNA was used to synthesize cDNA, with oligo(dT)15 as a reverse primer. Equivalent amounts of cDNA were used for real-time PCR in a 25 µl reaction mixture with 12.5 µl of 2X SYBR Green PCR Master Mix and 1 µl of specific primer pairs. Reactions were run in triplicate with 40 cycles of amplification on an ABI Prism 7000 real-time PCR machine (Applied Biosystems). The sequences of primers used were as follows: TATA box-binding protein (TBP), sense (5'—TGACAGGAGCCAAGAGTGAA-3') and antisense (5'—CACATCACAGCTCCCCACCA-3'); for nupr1, commercially available primers were used. The expression level of nupr1 was normalized relative to the expression of TBP measured in parallel samples.

Chondrocyte stimulation and immunoblotting—Confluent primary chondrocyte monolayers were made serum-free overnight and then stimulated with 1-10 ng/ml of IL-1 β , 25- μ M *tert*-butylhydroperoxide (tBHP) (a substrate of glutathione peroxidase that causes oxidative stress), or 2 μ M of thapsigargin (to induce endoplasmic reticulum stress). RNA was isolated at the indicated time points and nupr1 expression was analyzed by qPCR or by analysis of protein using cell lysates and immunoblotting. In some experiments, chondrocytes were transfected by the nucleofection method, using a human chondrocyte nucleofection kit (Lonza) with 1 μ M siRNA specific to nupr1 (to knock down expression) or cells were transfected with 2 μ g of a plasmid vector containing full-length nupr1 construct (overexpression) and then stimulated with IL-1 β overnight. After incubation, the conditioned media was collected and immunoblotted for nupr1, MMP-13, MMP-3, and MMP-2. In some experiments, MMP-13 production was also analyzed by human pro-MMP-13 ELISA (R&D systems). Immunoblots were quantified by using Image J software.

RESULTS

Nupr1 is increased in OA

Nupr1 protein levels were first examined in normal human and OA knee cartilage by immunohistochemistry (Fig. 1A). In normal knee articular cartilage, very little nupr1 was detected. However, in OA cartilage, nupr1 staining (red stain) was very strong and compact (Fig 1A & B, arrows), suggesting that immunostaining was primarily cellular. Thus, our data show that nupr1 is expressed in chondrocytes and suggest that its levels are increased in OA cartilage.

Additionally, we examined the expression of nupr1 in RNA isolated from knee joint tissue obtained in a previous study where gene expression was measured in young and older adult mice that had undergone DMM surgery or sham surgery as a control (6). Expression of nupr1 was increased in joint tissue samples from older DMM mice compared to age-matched sham-operated controls and also compared to young DMM mice (Fig. 1C). The difference between young DMM and sham controls was not significant. Interestingly, nupr1 expression was elevated in old sham mice compared to young mice; however, the increase was not statistically significant. Although the results are for total mouse joint tissue, not just cartilage, these findings provide evidence that nupr1 expression is upregulated in OA joints from older animals and may be important in development of OA.

Oxidative stress and IL-1 β induce nupr1 expression in chondrocytes

Since nupr1 is a stress-inducible protein, we wanted to examine if cellular stresses, including oxidative stress and endoplasmic reticulum (ER) stress, upregulate its expression. Stimulation of human chondrocytes with tBHP, a compound that causes oxidative stress by increasing the levels of oxidized glutathione (7), increased nupr1 mRNA expression by over two fold (Fig. 2A). This effect was not seen with thapsigargin, a known ER stress inducer, which activates UPR by inhibiting the ER Ca²⁺ ATPase. Thus, our data suggests that oxidative stress but not ER stress stimulates expression of chondrocyte nupr1.

Since IL-1 β is a major cytokine that plays an important role in OA pathology, we stimulated human chondrocytes with IL-1 β . Treatment of chondrocytes with IL-1 β induced a 2-fold increase in nupr1 mRNA expression as early as 1 hr (Fig. 2B), which peaked to 4-fold at 6 hrs and returned to baseline after 24 hrs of treatment. These data suggest that IL-1 β induces rapid, strong, and transient expression of nupr1 in chondrocytes.

Since our data showed that oxidative stress increased nupr1 mRNA expression and elevated levels of nupr1 induce MMP-13 expression, we examined whether induction of oxidative stress would increase production of MMP-13. Stimulation of human chondrocytes with tBHP alone did not induce MMP-13 (Fig. 2C). However, when tBHP was added with a low dose of IL-1 β (1 ng/ml) MMP-13 production was increased but it was not statistically significant compared to IL-1 β alone (Fig. 2C). These data suggest that oxidative stress alone is not sufficient to induce nupr1-mediated MMP production and that additional signals generated by IL-1 β are needed to induce MMP-13 production.

Nupr1 is required for IL-1 β -stimulated MMP production

Since nupr1 expression was regulated by IL-1 β , we examined nupr1's role in IL-1 β -mediated production of MMPs. Knocking down nupr1 expression in chondrocytes by siRNA (Fig. 3A) inhibited IL-1 β -mediated induction of MMP-13 and to a lesser extent MMP-3. Levels of MMP-2, used as a constitutively expressed control, did not change (Fig. 3B). Overexpression of nupr1 in chondrocytes increased expression of MMP-13 significantly and this was similar to the level seen with IL-1 β (Fig. 3C). Addition of IL-1 β to cells overexpressing nupr1 did not significantly increase the level of MMP-13 over cells expressing nupr1 alone or control cells (transfected with empty vector) treated with IL-1 β . Taken together, these data demonstrate a role for nupr1 in regulating MMP-13 in chondrocytes.

DISCUSSION

Chondrocytes are thought to undergo increased stress as a result of excessive mechanical loading of the joint and from age-related oxidative stress (8). This cellular stress can alter normal chondrocyte function by promoting increased catabolic activity that results in production of matrix-degrading enzymes, including MMPs and ADAMTSs. The result is loss of cartilage matrix and development of OA. MMP-13 is one of the major collagenases that degrade type II collagen, and it is upregulated in OA. However, the factors that cause upregulation of MMP-13 expression during OA have not been fully elucidated.

IL-1 β is a major catabolic cytokine that regulates expression of matrix metalloproteinase, including MMP-13 (3). IL-1 β stimulates increased production of reactive oxygen species (ROS), including hydrogen peroxide, and induces oxidative stress in the cell (9). Expression of nupr1 is upregulated by cellular stress, including oxidative stress and ER stress in various cell types (10, 11). Here we demonstrate that induction of oxidative stress (but not ER stress) in isolated human chondrocytes induced nupr1 mRNA expression by over 2-fold. Nupr1 expression is regulated by activating transcription factor (ATF4), a transcriptional activator induced by various cellular stresses, including, oxidative stress (12). The upstream signaling pathways leading to activation of ATF4 dictate its downstream gene targets.

During oxidative stress, ATF4 is activated by MAP kinases (13). We have previously shown that tBHP induces activation of the MAP kinase pathway in chondrocytes (14). Thus, we hypothesize that in chondrocytes, the oxidative stress-MAP kinase-AFT4 pathway may be responsible for inducing nupr1 expression. In support of our hypothesis, a recent study has shown that the extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) MAP kinases were required for expression of nupr1 (15).

Increased expression of nupr1 has been implicated in several diseases, including cancer and cardiac hypertrophy (4), and is thought to play a role in the transcriptional regulation of several genes, including MMPs. Stimulation of chondrocytes with IL-1 β upregulated nupr1 expression, and knocking down its expression inhibited IL-1 β -mediated production of MMP-13. Our observation is consistent with a previous study in cardiac fibroblasts (6), where nupr1 expression was required for TNF α -mediated induction of MMP-9 and MMP-13. Chromatin immunoprecipitation data presented in the study (6) showed that nupr1 was bound to the promoter regions of MMP-9 and MMP-13 in association with the cJun component of AP1; however, no direct DNA binding capabilities of nupr1 were demonstrated. These data suggest that nupr1 acts as a cofactor in regulating MMP gene expression in conjunction with other transcription factors. Further mechanistic studies are required to completely understand how nupr1 regulates the expression of MMPs in chondrocytes.

In conclusion, to the best of our knowledge, this is the first study to show that chondrocytes express nupr1 and that its expression is increased in OA cartilage. In addition, our study also demonstrates that nupr1 expression is required for IL-1 β -mediated expression of MMPs (MMP-3 and -13). Thus, our study provides a novel pathway for IL-1 β -mediated production of MMP-13 in chondrocytes.

Acknowledgments

Normal and OA cartilage sections used in this study were provided by the Dr. Martin Lotz laboratory (supported by NIH grant AG007996).

This work was supported by the National Institute of Arthritis, Musculoskeletal and Skin Diseases AR61050-01 (to RRY), AR49003-11 (to RFL) and the National Institute on Aging AG044034-01 (to RFL).

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Figure 1.

Nupr1 levels are increased in OA. A) Nupr1 was detected in human knee articular cartilage by immunohistochemistry. Normal cartilage shows faint staining for nupr1. Strongly positive immunostaining for nupr1 (red stain) was noted in chondrocytes from human OA tissue (red arrows). Images are representative of three different normal donors (ages; 68, 70 & 76) and three different OA subjects (ages; 64, 84 & 88) (ages of donors shown: normal 68 yrs and OA 64 yrs). B) Visualization of Nupr1 at 40x magnification. C) Nupr1 mRNA expression in the joint tissues of young (12-week-old) and older (12-month-old) adult mice that underwent sham or DMM surgery, analyzed by quantitative real-time PCR using mouse nupr1-specific primers. The data were normalized using the TATA box-binding protein (TBP) as a control, and are presented as a ratio. RNA isolated from joint tissues from 9 animals for each condition were pooled in groups of 3, resulting in 3 independent pools used for RT-PCR.

Figure 2.

Effects of oxidative stress and IL-1 β on nupr1 and MMP expression. A) Normal human chondrocytes (n=3, ages of donors: 58, 61 & 85 yrs) were treated with 25 μ M tertbutylhydroperoxide (tBHP) or with 2 μ M thapsigargin (TG) overnight. Expression of mRNA for nupr1 was examined by qPCR using human nupr1-specific primers. B) Normal human chondrocytes (n=3, ages of donors; 66, 72 & 75 yrs) were treated with IL-1 β for the indicated times; nupr1 expression was analyzed by quantitative real-time PCR. Data were normalized with TBP as a control and are presented as nupr1/TBP ratio. Values presented are means $\pm$ standard deviation. C) Normal chondrocytes (n=3; ages of donors 61, 67, and 72 yrs) were treated with 25 μ M tBHP or IL-1 β (1 ng/ml) or in combination overnight and the conditioned media was analyzed for MMP-13 production by ELISA.

Figure 3.

Nupr1 expression is required for IL-1 β -induced MMP production. A) Normal human chondrocytes (n=3, ages of donors 70, 75, & 82 yrs) were transfected with control siRNA or with siRNA specific for nupr1. Total RNA was isolated, and quantitative real-time PCR was performed to determine mRNA expression of nupr1, using the TATA box-binding protein (TBP) as a control. mRNA expression is presented as the nupr1/TBP ratio. Data presented as mean $\pm$ standard deviation. B) Cultures shown in A were treated with or without IL-1 β overnight. Conditioned media was collected and immunoblotted to measure MMP-13 and MMP-3 levels. Levels of MMP2 were used as a loading control. C) Chondrocytes (n=3, ages of donors 67, 72 & 82 yrs) were transfected with control plasmid or plasmid vector containing a full-length nupr1 construct and treated with or without IL-1 β overnight. After transfection, cell lysates were made and nupr1 expression was analyzed by immunoblotting with nupr1 antibody. After IL-1 β treatment, conditioned media was analyzed for MMP-13 and MMP-2 by immunoblotting. D. Densitometric analysis was performed on blots obtained in 3 independent experiments similar to the one shown in C lower panel.