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Involvement of H-Ras and reactive oxygen species in proinflammatory cytokine-induced matrix metalloproteinase-13 expression in human articular chondrocytes

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ABSTRACT

Proinflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) enhance degradation of cartilage-specific, type II collagen by matrix metalloproteinase-13 (MMP-13). We investigated the previously unknown role of H-Ras and reactive oxygen species (ROS) in the cytokine induction of MMP-13 gene expression in human articular chondrocytes by using pharmacological inhibitors, RNA interference (RNAi) and antioxidants. Manumycin A, an inhibitor of H-Ras farnesylation by farnesyltransferase, suppressed IL-1β- and TNF-α-induced MMP-13 mRNA and protein expression. Small interfering RNA (siRNA)-mediated H-Ras silencing down-regulated MMP-13 mRNA and protein induction by IL-1β and TNF-α. Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase/NOX) inhibitor, diphenyleneiodonium (DPI) suppressed cytokine-induced MMP-13 expression and superoxide production. Apocynin, another NOX inhibitor, also diminished MMP-13 induction. Deoxyglucose an antimetabolite of glucose metabolism reduced MMP-13 increase. Role of NOX-mediated ROS production was reaffirmed by the observation that the antioxidants, trolox, nordihydroguaiaretic acid (NDGA), quercetin and resveratrol downregulated cytokine-induced MMP-13 mRNA and protein expression. These results provide strong pharmacological and genetic evidence for the implication of H-Ras and NADPH oxidase-generated superoxide production in MMP-13 gene regulation by IL-1β and TNF-α. These proteins could be potentially targeted for therapeutic inhibition of MMP-13-driven cartilage erosion by using H-Ras and NOX inhibitors and antioxidants.

Introduction

Chondrocytes synthesize and maintain the principal cartilage extracellular matrix (ECM) components such as aggrecan and type II collagen for proper functioning of joints [1,2]. However, during the pathogenesis of common rheumatic diseases, rheumatoid arthritis (RA) and osteoarthritis (OA), aggrecan is destroyed first reversibly, followed by the irreversible digestion of collagen fibrils by proteases [3,4]. Proinflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) are the main stimuli that enhance cartilage-specific, type II collagen degradation by matrix metalloproteinase-13 (MMP-13 or collagenase-3) which preferentially cleaves type-II collagen [5–8]. MMP-13 also cleaves aggrecan in the interglobular domain [9]. Overexpression of MMP-13 in mouse joints mimics human OA-like joint destruction, while MMP-13-deficient mice are protected against cartilage erosion [10,11]. Therefore, MMP-13 is a major therapeutic target for reducing cartilage loss. IL-1 and TNF-α signals are transduced through their respective receptors by multiple pathways including mitogen-activated protein kinases (ERK, p38 and JNK MAPKs), activating protein-1 (AP-1) and nuclear factor kappa B (NF-κB) that are important targets for anti-arthritic drug development [12–14]. Indeed, components of synovial fluid-originated IL-1 signaling are activated in the upper zone of human OA cartilage [15].

Ras proteins such as Harvey-Ras (H-Ras) contain CAAX motif at their carboxy terminus that triggers farnesylation (attachment of farnesyl pyrophsphate) of the Cys by farnesyltransferase (FT). After farnesylation, AAX peptide is released and farnesylcysteine is methylated and the so processed Ras protein is targeted to cell membrane for its role in mitogenic signaling [16]. Inhibitors of Ras farnesylation (FTIs) cause its mislocalization and improper function including downstream signaling. Ras also contributes to Rac1-mediated nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase or NOX) assembly, reactive oxygen species (ROS) production and redox signaling [17,18]. NOX consist of

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catalytic subunits (Nox 1-5), p22phox, p47phox, p67phox and Rac1 [19]. Previously, we showed that adaptor proteins regulate IL-1-induced MMP-13 and a disintegrin and metalloproteinase with thrombospondin motif-4 or ADAMTS-4 expression in human chondrocytes [20,21]. However, role of H-Ras and ROS in the induction of MMP-13 gene expression has not been studied extensively in human chondrocytes and was the subject of this study. By using pharmacological inhibitors and RNA interference (RNAi)-mediated Ras knockdown and antioxidants, we demonstrate that H-Ras, NADPH oxidase and ROS mediate IL-1 and TNF-α-induced MMP-13 gene and protein expression in human chondrocytes.

Materials and methods

Chondrocyte cell culture and treatments

Normal human knee chondrocytes (purchased from Lonza, Walkersville, MD) were grown in Differentiation Bullekit medium as high-density short-term monolayer cultures where the chondrocytes maintained their differentiate phenotype. Chondrocytes were maintained in serum-deficient medium for 24 h for quiescence prior to treatments. Cells were either pretreated for 1 h with vehicles (DMSO, ethanol or water) or pharmacological inhibitors such as manumycin A (Cayman Chemical, Ann Arbor, MI), diphenyleneiodonium (DPI-in DMSO) (Sigma–Aldrich, Saint Louis MO), apocynin (in ethanol) or deoxyglucose (in water) (EMD Biosciences, Inc. La Jolla, CA) at final concentrations of 10–20 μM and then stimulated further with 0.1% BSA (control) or recombinant human IL-1β or TNF-α (R&D Systems, Minneapolis, MN) for 24 h. In separate experiments, quiescent human chondrocytes were pretreated with the antioxidants, trolox (100 μM, Calbiochem) and nordihydroguaiaretic acid (20 μM, Sigma), quercetin (100 μM, Calbiochem) and resveratrol (100 μM, Sigma) for 1 h and then incubated simultaneously with the antioxidant and IL-1β or TNF-α for further 24 h.

Transfection of small interfering RNA in chondrocytes

Cells were transfected with 200 nM of Ha-Ras-specific siRNA or negative control siRNA (Ras siRNA™ Assay Kit, Upstate, Charlottesville, VA, USA) by an improved calcium phosphate precipitation method [22] and then stimulated with IL-1β or TNF-α for 24 h and effect of gene knockdown on MMP-13 levels measured by RT-PCR and Western blot analysis.

MMP-13 RT-PCR analysis

Total RNA was extracted and analyzed by using MMP-13- and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)-specific primers [23] that yield 491 and 226 bp cDNA bands respectively as described earlier [20].

MMP-13 ELISA and Western blot

MMP-13 levels were measured in chondrocyte conditioned media by using a specific ELISA, SensoLyte MMP-13 ELISA kit (colorimetric) (AnaSpec, San Jose, CA, Catalog 72107). This 96-well sandwich Immunoassay quantitatively determines the pro and active forms of MMP-13 using a mouse anti-human MMP-13 monoclonal antibody, biotinylated goat anti-human MMP-13 antibody leading to yellow color development measured at 450 nm.

For MMP-13 Western blots, supernatants from chondrocyte cultures were precipitated with TCA (10% final), kept at −20 °C for 30 min and then centrifuged at 14,000 rpm for 20 min at 4 °C for concentrating the proteins. Supernatants were discarded and precipitated proteins were dissolved in 0.1 M NaOH. Sample loading buffer was added and boiled for 5 min and run on SDS–PAGE for proteins separation. After transfer by electrophoresis, the membranes were then blocked with 5% non-fat milk in PBS for 1 h, incubated with the 1:500 dilution of MMP-13 primary antibody (Sigma, Saint Louis, Missouri) diluted either in 5% milk in PBS or 5% BSA overnight at 4 °C. The blots were then washed four times and incubated for 2 h with HRP-conjugated secondary antibody. Immunoreactive bands were developed with Enhanced Chemiluminescence and visualized by fluorography.

Superoxide assay

After treatments with DPI for 20 min alone, and with cytokines for 1 h, chondrocytes were washed with phosphate-buffered saline and incubated with 500 μl of Tyrode's buffer containing 160 μM of cytochrome C (Sigma) and 120 nM of nitric oxide (NO) synthase

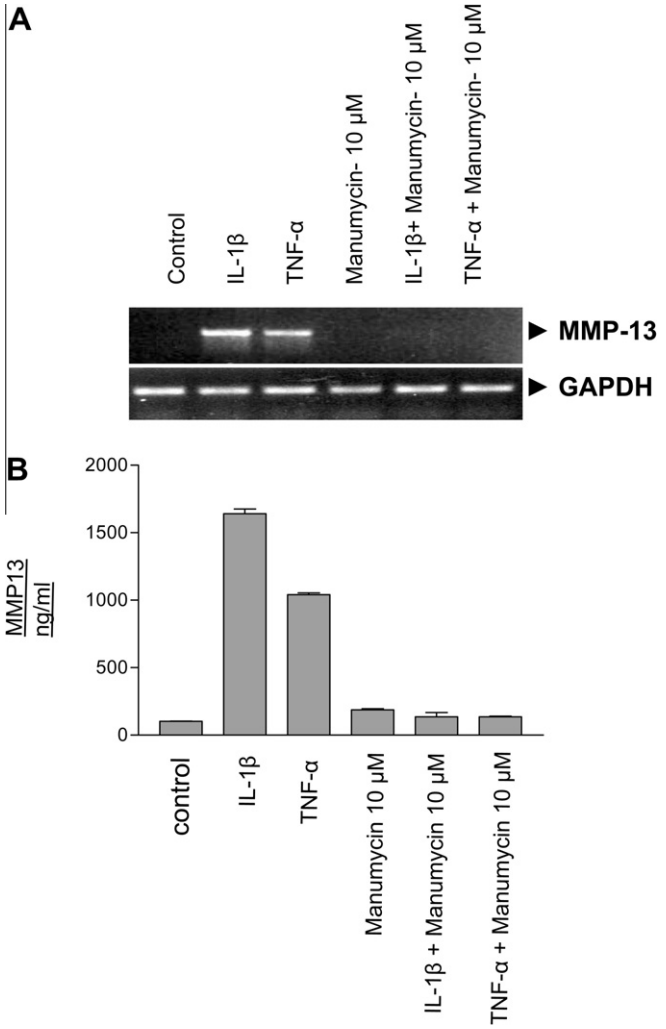

Fig. 1. Suppression of IL-1β- and TNF-α-induced MMP-13 mRNA and protein expression by manumycin A. Confluent human chondrocytes maintained in serum-deficient medium were pretreated with vehicle (Control; DMSO) or 10 μM of Manumycin A for 1 h and then stimulated with IL-1 β or TNF-α for 24 h. Total RNA from the cells was analyzed for MMP-13 and GAPDH mRNA levels by RT-PCR and the representative photograph of multiple (more than three times) experiments is depicted (A). The conditioned media were analyzed for MMP-13 levels by a specific ELISA. The absorbance values were read at 450 nM and a mean ± SD of three independent experiments is shown as bar graphs using the GraphPad Prism software (B).