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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is increased in osteoarthritis and regulates chondrocyte catabolic and anabolic activities

D.L. Long † [a], V. Ulici § [a], S. Chubinskaya ‡, R.F. Loeser † § *

† Section of Molecular Medicine, Wake Forest School of Medicine, Winston-Salem, NC, USA
‡ Department of Biochemistry, Rush University Medical Center, Chicago, IL, USA
§ Thurston Arthritis Research Center, Division of Rheumatology, Allergy and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA

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SUMMARY

Objective: We determined if the epidermal growth factor receptor ligand HB-EGF is produced in cartilage and if it regulates chondrocyte anabolic or catabolic activity.
Methods: *HB-EGF* expression was measured by quantitative PCR using RNA isolated from mouse knee joint tissues and from normal and osteoarthritis (OA) human chondrocytes. Immunohistochemistry was performed on normal and OA human cartilage and meniscus sections. Cultured chondrocytes were treated with fibronectin fragments (FN-f) as a catabolic stimulus and osteogenic protein 1 (OP-1) as an anabolic stimulus. Effects of HB-EGF on cell signaling were analyzed by immunoblotting of selected signaling proteins. MMP-13 was measured in conditioned media, proteoglycan synthesis was measured by sulfate incorporation, and matrix gene expression by quantitative PCR.
Results: *HB-EGF* expression was increased in 12-month old mice at 8 weeks after surgery to induce OA and increased amounts of HB-EGF were noted in human articular cartilage from OA knees. FN-f stimulated chondrocyte *HB-EGF* expression and HB-EGF stimulated chondrocyte MMP-13 production. However, HB-EGF was not required for FN-f stimulation of MMP-13 production. HB-EGF activated the ERK and p38 MAP kinases and stimulated phosphorylation of Smad1 at an inhibitory serine site which was associated with inhibition of OP-1 mediated proteoglycan synthesis and reduced aggrecan (*ACAN*) but not *COL2A1* expression.
Conclusion: HB-EGF is a new factor identified in OA cartilage that promotes chondrocyte catabolic activity while inhibiting anabolic activity suggesting it could contribute to the catabolic-anabolic imbalance seen in OA cartilage.

Introduction

The progressive degradation and loss of articular cartilage during the development of osteoarthritis (OA) is thought to be due to an imbalance in chondrocyte anabolic and catabolic activity[1]. An important goal of OA research has been to identify the factors that promote catabolic over anabolic activity with the hope that one or more of these mediators might serve as a therapeutic target. A host of soluble mediators capable of regulating chondrocyte activity have been found to be produced locally by the articular chondrocytes and act in an autocrine and paracrine fashion. These include various growth factors, cytokines, chemokines, Wnt family members, and toll-like receptor agonists, to name just a few. It is also well recognized that these and other factors produced by neighboring joint tissues, including the synovium, subchondral bone, and the menisci in the knee, can also regulate chondrocyte activity to promote catabolic over anabolic activity[2]. Despite the number of factors identified to date, it is not known which

* Address correspondence and reprint requests to: R.F. Loeser, Thurston Arthritis Research Center, Division of Rheumatology, Allergy and Immunology, University of North Carolina School of Medicine, Campus Box 7280, Chapel Hill, NC, 27599-7280, USA.
E-mail addresses: dllong@wakehealth.edu (D.L. Long), vulici@email.unc.edu (V. Ulici), Susanna_Chubinskaya@rush.edu (S. Chubinskaya), richard_loeser@med.unc.edu (R.F. Loeser).
[a] These authors contributed equally to this work.

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mediators would be the best targets for therapy and if other mediators not yet studied may also be involved.

In an attempt to discover novel biological mediators that could contribute to OA, we recently performed a computational analysis of gene expression microarray data generated using RNA isolated from knee joint tissues during a time course experiment in mice[3]. OA was induced using the destabilized medial meniscus (DMM) model and RNA was isolated from knee joint tissues at baseline, 2, 4, 8, and 16 weeks after surgery. We focused on signaling and metabolic pathways and found a subnetwork, significantly regulated during the development of OA, that included a potential protein–protein interaction link between matrix metalloproteinase (MMP)-2 and the epidermal growth factor receptor (EGFR) via heparin-binding epidermal growth factor-like growth factor (HB-EGF).

There is very little data on HB-EGF in joint tissues with one study showing a 2.76-fold difference in *HBEGF* gene expression in damaged relative to intact cartilage obtained at the time of joint replacement surgery for knee OA[4]. HB-EGF can serve as a ligand for the EGFR and activation of the chondrocyte EGFR by transforming growth-factorα (TGFα) has been shown to stimulate *MMP* expression and cartilage degradation as well as inhibit *Sox-9* expression and anabolic activity[5,6]. We postulated that HB-EGF could be another mediator that promotes catabolic over anabolic activity in cartilage. Therefore, the objective of the present study was to investigate HB-EGF expression and production in normal and OA cartilage and determine its effects on chondrocyte catabolic and anabolic activity.

Methods

Reagents

Phospho-ERK, phospho-p38, phospho-Smad1^{ser206}, phospho-Smad1$^{ser463/465}$/Smad5$^{ser463/465}$/Smad8$^{ser465/467}$, total Smad1, total p38, and total ERK antibodies were from Cell Signaling (Beverly, MA). MMP-13 antibody was from Abcam (Cambridge, MA). HB-EGF antibody, HB-EGF ELISA duoset, MMP-13 ELISA, EGF receptor inhibitor AG1478, ERK inhibitor U0126, and recombinant HB-EGF were from R&D Systems (Minneapolis, MN). P38 inhibitor SB203580 and MMP-2 antibody were from EMD Millipore (Billerica, MA). Control siRNA and smartpool siRNA against HB-EGF were from Dharmacon (Lafayette, CO). Amaxa nucleofection reagents for transfection were from Lonza (Walkersville, MD). Predesigned *MMP-13*, *HB-EGF*, *COL2A1*, *ID1*, *SMAD6*, *TGFα*, and α5 integrin (*ITGA5*) real-time PCR primers were from SuperArray Biosciences (Frederick, MD). Primers for *TBP*, *B2M*, and *ACAN* were from the Wake Forest School of Medicine DNA laboratory. Sequences for these are provided in Table S1. AMV Reverse Transcriptase and RT^2 SYBR® green ROX™ qPCR Mastermix were purchased from Promega and Qiagen, respectively. Recombinant fibronectin fragment containing the RGD cell binding domain was produced using an expression construct provided by Dr Harold Erickson (Duke University, Durham, NC). Vectastain Elite ABC kit and Nova Red substrate were from Vector Labs (Burlingame, CA). PicoGreen DNA assay was from Invitrogen (Carlsbad, CA). Mayer's Hematoxylin was from Sigma (St. Louis, MO).

Tissue acquisition and chondrocyte isolation

Normal human ankle articular cartilage was obtained from deceased tissue donors with no known history of arthritis from the Gift of Hope Organ and Tissue Donor Network (Itasca, IL) through the Department of Biochemistry at Rush University Medical Center (Chicago, IL). Tissue from a total of 35 individual donors with ages from 46 to 77 years (avg 64 years) was used for cell culture studies. Chondrocytes were isolated with sequential pronase and collagenase digestion and plated at high density monolayer as previously described[7]. All cells were used without passaging to ensure proper phenotype was retained.

Immunohistochemistry

Cartilage and medial meniscal sections used for immunohistochemistry were from young normal ($n = 4$, ages 36–48), old normal ($n = 4$, ages 68–76) and OA ($n = 4$, ages 64–90) donors and were a kind gift of Dr Martin Lotz (Scripps Research Institute, La Jolla, CA). Although the current study focused on HB-EGF in cartilage, we examined HB-EGF levels in the meniscus as a comparison to cartilage, in particular because the outer region of the meniscus contains blood vessels which would be expected to contain HB-EGF and could serve as a positive immunostaining control. The sections embedded in paraffin were deparaffinized followed by staining with Vectastain Elite Kit (Vector Labs) according to manufacturer's instructions. Tissue was incubated overnight at 4°C with a 10 μg/mL goat anti-HBEGF antibody. Tissue incubated with no primary antibody was used as a negative control. Staining was visualized with ImmPACT Nova Red substrate (Vector Labs). Nuclei were visualized by Mayer's Hematoxylin staining. Images were captured using the indicated objectives.

Quantitative real-time PCR

The RNA used for measuring HB-EGF expression in the DMM model of OA was from a previously published microarray study[8]. RNA from sham control and DMM knees joints was subjected to real-time PCR analysis using predesigned primers specific for mouse *HB-EGF*. *HB-EGF* levels were normalized to the expression of TATA box-binding protein measured in parallel samples. For studies using isolated human chondrocytes, RNA was isolated from confluent monolayers using TRIzol and primers specific for human *HB-EGF* were used. RNA isolation and real-time PCR were performed as previously described[9]. RNA was also isolated directly from frozen samples of articular cartilage obtained from normal tissue donors ($n = 7$, mean age 53 years) and OA ($n = 5$, mean age 57 years) patients who had undergone knee arthroplasty. For this, the tissue was first ground to a powder using the BioPulverizer system (BioSpec Products, Inc. #59012MS) which was pre-chilled in liquid nitrogen. 50–100 mg of cartilage powder from each sample was further homogenized in 2 ml of TRIzol using a 3 ml syringe with an 18G needle. RNA was isolated from the aqueous phase after Chloroform separation. Following isopropanol precipitation and 70% ethanol washes, the resulted RNA pellet was dissolved in 100 μl of dH_2O followed by a cleanup step using the RNeasy Mini Kit (Qiagen). RNA concentration was measured using the NanoDrop 2000 (Thermo Scientific) and quality was verified using the Agilent Technologies 2200 TapeStation. 600 ng total RNA per sample was then converted to cDNA using AMV Reverse Transcriptase (Promega). The resulted cDNA was diluted 4 times and analyzed by real-time PCR using the Bio-Rad CFX96 Real-Time System and CFX manager analysis software.

Immunoblotting

Conditioned media and cell lysates were collected and used for immunoblotting as previously described[7]. Primary antibodies were diluted to 1:1000 and immunoreactive bands were detected using chemiluminescence. All immunoblotting experiments were repeated at least 3 times with cells from different donors. For analysis of HB-EGF levels in conditioned media in response to