FEBS Letters 589 (2015) 4088–4096

journal homepage: www.FEBSLetters.org

Extracellular heat shock protein 70 promotes osteogenesis of human mesenchymal stem cells through activation of the ERK signaling pathway

Erman Chen [1], Deting Xue [1], Wei Zhang, Feng Lin, Zhijun Pan *

Department of Orthopedics, Second Affiliated Hospital, School of Medicine, Zhejiang University, 310000 Hangzhou, People's Republic of China

ARTICLE INFO

Article history:
Received 14 August 2015
Revised 22 October 2015
Accepted 13 November 2015
Available online 19 November 2015

Edited by Zhijie Chang

Keywords:
Extracellular HSP70
Human mesenchymal stem cell
Osteogenesis
ERK signaling pathway
Bone nonunion

ABSTRACT

Heat shock proteins have protective effects when cells are exposed to stress. However, the relationship between extracellular heat shock protein 70 (eHSP70) and osteogenesis of hMSCs has not been reported. The results of this study showed that HSP70 (200 ng/ml) increases alkaline phosphatase activity and promotes hMSC mineralization. Under osteogenic induction conditions, HSP70 significantly upregulated the expression of osteo-specific genes, such as the runt family transcription factor Runx2 and osterix (OSX). Comparative expression profiling by microarray and pathway analyses revealed that HSP70 promotes osteogenesis of hMSCs through activation of the ERK signaling pathway. HSP70 may be a potential therapeutic agent for the treatment of bone nonunion.

1. Introduction

Management of fracture nonunion or bone defects is one of the most challenging clinical problems in orthopedic trauma. The incidence of nonunion or bone defects following fractures is increasing. Fracture nonunion or bone defects can be caused by infection, tumor resection, and skeletal abnormalities [1]. Despite advanced and optimized surgical procedures, approximately 5–10% of fractures sustained annually in the United States fail to complete the bony union process [2]. From a physiological viewpoint, growth factors, osteoprogenitor cells, and the extracellular matrix play a crucial role in creating the foundation for successful bone healing. It was reported that human mesenchymal stem cells (hMSCs) are recruited to the fracture sites after the occurrence of a fracture [3]. A recent study showed that injecting bone marrow into the bone fracture site contributes to healing of the fracture [4]. Because MSCs are involved in fracture healing, enhancing hMSC osteogenesis may be a useful therapeutic strategy for promoting fracture union.

Human mesenchymal stem cells (hMSCs) have the potential to differentiate into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes [5]. hMSC differentiation into mature functional osteoblasts is a complex process involving many transcriptional factors and signaling pathways. Several studies have reported that growth factors [6], cytokines [7], or mechanical loading [8] influence MSC differentiation into osteoblasts. Several studies have reported that fractures, especially multiple injuries, subject the internal body environment to stress [9–11]. In this situation, the hMSCs would be acting under stressors in the local microenvironment of the fracture site. To date, there is no study examining the effects of the stress microenvironment on the osteogenesis of MSCs.

Heat shock proteins (HSPs) are a highly conserved intracellular protein family found in all eukaryotic and prokaryotic cells [12]. Although some HSPs are constitutively expressed, upregulation of expression can be induced by exposure to a variety of cellular stressors, including heat shock, inflammation, fractures, and infection [13,14]. Hsp70 is a highly inducible member of the 70kDa family of heat shock proteins and mainly involved in the folding,

Author contribution: Study design: Zhijun Pan and Erman Chen. Study conduct: Zhijun Pan, Erman Chen, and Deting Xue. Data interpretation: Erman Chen, Deting Xue, Wei Zhang, and Feng Lin. Data analysis: Erman Chen, Deting Xue, Wei Zhang, and Feng Lin. Drafting manuscript: Zhijun Pan and Erman Chen. Revising manuscript content: Zhijun Pan and Erman Chen. Final manuscript approval: Zhijun Pan and Erman Chen. Zhijun Pan takes responsibility for the integrity of the data analysis.

* Corresponding author at: No. 88, Jiefang Road, Hangzhou, People's Republic of China.

E-mail address: chenerman_zju@163.com (Z. Pan).

[1] First author: These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.febslet.2015.11.021
0014-5793/

assembly, degradation, and repair processes of intracellular proteins. It regulates protein activity, mediating protein transport, and maintaining the protein self-stabilization system [15–17]. Recently, some studies have shown that inflammatory cytokines released by necrotic cells play an important role in the process of fracture healing [18,19]. Whether eHSP70 affecting the fracture healing process is still unclear.

In the current study, we investigated the effects of eHSP70 on osteogenic differentiation of hMSCs. The results show that eHSP70 can promote hMSC osteogenesis, possibly through activation of the ERK signaling pathway.

2. Materials and methods

2.1. Reagents and cell culture

HSP70 was purchased from Boston Biochem, Inc. (Cambridge, MA 02139, USA). Human mesenchymal stem cells (hMSCs) were purchased from Cyagen Biosciences, Inc. (Guangzhou, China). hMSCs were cultured in culture flasks in hMSC growth medium (MSCGM) (Cyagen Biosciences, Inc., Guangzhou, China) in an incubator at 37 °C with 5% CO_2. The hMSC growth medium consisted of hMSC basal medium, 10% fetal bovine serum, and 1% penicillin–streptomycin. Subculture was performed at a density of 5000 cells/cm^2. Passage 3–7 hMSCs were used in this study.

2.2. Osteogenic differentiation and cell treatment

Osteogenesis of hMSCs was induced by osteogenic differentiation medium (ODM) (Cyagen Biosciences, Inc., Guangzhou, China) consisting of hMSC osteogenic differentiation basal medium, 10% fetal bovine serum, 1% penicillin–streptomycin, 100 nmol/L dexamethasone, 10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate. After cells reached confluence, the culture medium was aspirated off and ODM was added. The ODM was changed every three days. Meanwhile, eHSP70 was added to the ODM at concentrations ranging from 6.25 ng/ml to 200 ng/ml.

2.3. Cell viability assay

hMSCs were inoculated in 96-well plates at 5×10^3 cells per well in an incubator at 37 °C with 5% CO_2. Twenty-four hours later, various concentrations of HSP70 (12.5, 25, 50, 100, and 200 μg/ml) were added to each well with five other duplicates, and cells were incubated overnight. Cell viability was measured at the indicated time points using a cell counting kit (Dojindo Laboratories, Shanghai, China) according to the manufacturer's instructions.

2.4. Alkaline phosphatase (ALP) activity

After hMSCs were treated with ODM and HSP70 at different concentrations, plates were washed with phosphate-buffered saline (PBS), and the cells were lysed with lysis buffer consisting of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100. ALP activity was determined using the ALP activity assay (Beyotime, China) according to the manufacturer's instructions. Briefly, the conversion of colorless p-nitrophenyl phosphate to colored p-nitrophenol was measured after 3 and 7 days of culture in osteogenic media at 405/650 nm.

2.5. Mineralization assay

Calcium deposits were determined after 6, 8, and 11 days of culture by Alizarin Red S (ARS) staining. hMSCs were fixed in 70% ethanol for 10 min and stained with 0.5% ARS (pH 4.1) for 10 min at room temperature. Then, hMSCs were washed three times with double distilled water (ddH2O). Orange red staining indicated the position and intensity of calcium deposits. Calcium deposits were extracted with 10% cetylpyridinium chloride (CPC, Sigma) and quantified by measuring the OD of the extract at 550 nm.

2.6. RNA extraction and real-time PCR

Total RNA was extracted from hMSC samples on days 3 and 7 during osteogenesis using TRIzol Reagent (Takara, Japan), and first-strand cDNA was synthesized using PrimeScript™ RT Master Mix (Takara, Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed using the Step One Plus Real-Time PCR System (Applied Biosystems, USA). Amplification conditions were as follows: an initial denaturation at 95 °C for 5 min, and then 45 cycles at 95 °C for 5 s and 60 °C for 30 s.

2.7. Western blotting

Cells were harvested and equal amounts of protein were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels for 2 h at 100 V and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was blocked with 5% skim milk for 1 h at room temperature. After washing three times with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST), the membranes were incubated with anti-ALP (Santa Cruz, 1:800), anti-SP7 (Abcam, 1:1000), anti-BMP2 (Santa Cruz, 1:1000), anti-ERK (CST, 1:1000), anti-p-ERK (CST, 1:1000), or anti-β-actin (Santa Cruz, 1:2000) antibodies at 4 °C overnight. After washing in TBST four times (5 min each), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit) for 1 h at room temperature. After washing five times with TBST, we detected proteins using enhanced chemiluminescence (ECL) blotting reagents according to the manufacturer's instructions. X-ray film was placed in the cartridge for development and fixed after exposure for 5–10 min. Band intensity was quantified using Bandscan software.

2.8. Microarray analysis

Whole genome expression was analyzed after a 3-day culture of hMSCs with 200 ng/ml HSP70. A total microarray analysis was performed using a Human Genome U133 Plus 2.0 Array (Affymetrix) at the Shanghai Biochip Company. The microarray data have been deposited in Gene Expression Omnibus under accession code GSE71080.

2.9. Data processing and pathway analysis

We used the Affymetrix Human U133 Plus 2.0 microarray, which interrogates over 47,000 transcripts. Raw data were normalized by the MAS 5.0 algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA). Differentially-expressed genes (DEGs) were determined by comparison with the control group. Pathway analysis was carried out using the DAVID Functional Classification tool and the open source pathway resources of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/).

2.10. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA). The significance level was $P < 0.05$.