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IRE1α dissociates with BiP and inhibits ER stress-mediated apoptosis in cartilage development

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ABSTRACT

Bone morphogenetic protein 2 is known to activate unfolded protein response signaling molecules, including XBP1S, BiP and IRE1α. Endoplasmic reticulum stress is induced in chondrogenesis and activates IRE1α signal pathway, which is associated with ER stress-mediated apoptosis. However, the influence on IRE1α and BiP in BMP2-induced chondrocyte differentiation has not yet been elucidated; the molecular mechanism remains unexplored.

In this study, we demonstrate that IRE1α interacts with BiP in unstressed cells and dissociates from BiP in the course of cartilage development. Induction of ER stress-responsive proteins (XBP1S, IRE1α, BiP) was also observed in differentiating cells. IRE1α inhibition ER stress-mediated apoptosis lies in the process of chondrocyte differentiation. Furthermore, knockdown of IRE1α expression by way of the RNAi approach accelerates ER stress-mediated apoptosis in chondrocyte differentiation induced by BMP2, as revealed by enhanced expressions of cleaved caspase3, CHOP and p-JNK1; and this IRE1α inhibition effect on ER stress-mediated apoptosis is required for BiP in chondrogenesis.

Collectively, the ER stress sensors were activated during apoptosis in cartilage development, suggesting that selective activation of ER stress signaling was sufficient for induction of apoptosis. These findings reveal a novel critical role of IRE1α in ER stress-mediated apoptosis and the molecular mechanisms involved. These results suggest that activation of p-JNK1, caspase3 and CHOP was detected in developing chondrocytes and that specific ER stress signaling leads to naturally occurring apoptosis during cartilage development.

1. Introduction

Eukaryotic cells possess several mechanisms to adapt to endoplasmic reticulum (ER) stress and thereby survive. ER stress activates a set of signaling pathways collectively termed as the unfolded protein response (UPR). The UPR includes three molecular branches (inositol requiring enzyme 1, IRE1; PKR-like ER resistant kinase, PERK and activating transcription factor 6, ATF6), which trigger both cell protective and cell death responses. If the cells are exposed to prolonged or strong ER stress, the cells are destroyed by apoptosis. This is called ER stress-induced cell death [1–3]. Recent evidence indicates that ER stress-mediated cell apoptosis has been implicated in the process of development and many diseases, such as cancer, orthopedics diseases, and ischemic reperfusion damage [4–6].

IRE1α is a transmembrane-RNase with an endoplasmic reticulum luminal sensor domain and cytosolic kinase and ribonuclease domains. IRE1α plays a central role in the ER stress response [7,8]. Jonathan reported that IRE1 is involved in the switch between the pro-survival UPR and initiation of cell death pathways during ER Stress. Attenuation of IRE1 can switch the adaptive UPR to apoptosis, and persistent activation of IRE1 increases cell viability upon ER stress [9]. Mammalian IRE1α is activated upon ER stress, and cleaves specific exon–intron sites in the mRNA that encodes the transcription factor X-box-binding protein1 (XBP1). This cleavage initiates an unconventional splicing reaction, which leads to production of an active transcription factor and induction of various adaptive genes [10,11]. Besides, JP Upton et al. [12] reported that IRE1α regulates translation of a proapoptotic protein through terminating microRNA biogenesis recently. They found that ER stress activates IRE1α, and sustained IRE1α RNase activation caused rapid decay of select microRNAs which repressed translation of caspase-2 mRNA, and thus sharply elevated protein levels of this initiator protease of the mitochondrial apoptotic pathway.

Abbreviations: BiP, binding immunoglobulin protein; XBP1S, X-box binding protein1 spliced; UPR, unfolded protein response; BMP2, bone morphogenetic protein 2; IRE1α, inositol requiring enzyme 1α; PERK, PKR-like ER resistant kinase; ATF6, activating transcription factor 6.

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BMP2 (bone morphogenetic protein 2) is one of the most important cytokines and plays several important roles in a variety of cellular functions ranging from embryogenesis, cell growth, and differentiation to bone development and the repair of bone fractures [13,14]. It is known that BMP2 can activate UPR signaling molecules, such as BiP (binding immunoglobulin protein), CHOP (C/EBP homologous protein), ATF4 (activating transcription factor 4) and IRE1α. BiP, a molecular chaperone located in the lumen of the endoplasmic reticulum, is a member of the heat shock 70 protein family. BiP is also an essential component of the translocation into the ER's machinery, as well as plays a role in retrograde transport across the ER membrane of aberrant proteins destined for degradation by the proteasome [15,16]. Jang et al. [17] reported that BMP2 activates UPR transducers, such as PERK, OASIS, and ATF6. BMP2 stimulated ATF6 transcription by enhancing the direct binding of RUNX2 to the OSE2 (osteoblast-specific cis-acting element 2) motif of the ATF6 promoter region.

In our previous study, we found that BMP2 induces mild ER stress during chondrocyte differentiation and activates the IRE1α-XBP1 pathway, then XBP1S in turn enhances chondrocyte hypertrophy through functions as a cofactor of RUNX2. XBP1S controlled growth plate chondrocyte hypertrophy and differentiation. Besides, XBP1S can induce the growth and inhibits ER stress-mediated apoptosis via Erk1/2 signal activation and CHOP attenuation in ER stress [18,19]. However, whether IRE1α can influence the ER stress-mediated apoptosis in BMP2-induced chondrocyte differentiation has not yet been elucidated, especially, the molecular mechanism underlying these processes remains unexplored. In current study, we attempt to clarify the role of IRE1α in ER stress-mediated apoptosis during the course of chondrogenesis; with the special focus on associated molecules of ER stress-mediated apoptosis and chondrocyte differentiation, as well as the molecular events underlying this process.

2. Materials and methods

2.1. Ethics statement

With the approval of the ChongQing Medical University Institutional Animal Care and Use Committee [SYXK 2007-0001, SCXK 2007-0002], all mice were housed under controlled temperatures in a 12 h light/dark cycle with easy access to food and water.

2.2. Plasmids and adenoviruses

To generate the pcDNA3.1(−)-IRE1α and pcDNA3.1(−)-BiP expression plasmids, the corresponding segments were amplified using PCR with the following primers: IRE1α sense: 5′-CGGAATTCATGCCGGCCC GGCGGCTGCTGCTG-3′; IRE1α antisense:5′-CCCAAGCT TGAGGGCGTC TGGAGTCACTGGGGGC-3′; BiP sense: 5′-CGGGATCCATGAAGCTCTC CCTGGTG-3′; BiP antisense: 5′-CCCAAGCTTGGGCAACTCATCTTTTTCTG-3′; the enzyme sites in the primers are underlined. PCR products were inserted into the pcDNA3.1(−) vector.

To generate BiP and IRE1α small interfering RNA (siRNA) expression constructs, siRNA corresponding to the coding sequence of the BiP and IRE1α gene (5′-ATGCCAATGA ACTCTTTCCCTTTT-3′) were cloned into a pSES-HUS vector (an adenoviral shuttle vector expressing siRNA) according to the manufacturer's instructions. Briefly, equimolar amounts of complementary sense and antisense strands were separately mixed, annealed, and slowly cooled to 10 °C in a 50-μl reaction buffer (100 mM NaCl and 50 mM HEPES, pH 7.4). The annealed oligonucleotides were inserted into the SfiI sites of pSES-HUS vector. All constructs were verified by nucleic acid sequencing; subsequent analysis was performed using BLAST software (National Institutes of Health).

Adenovirus IRE1α (Ad-IRE1α) siRNA, Ad-BiP siRNA, and adenovirus encoding IRE1α and BiP were constructed, respectively, using methods described previously [20–22].

All constructs were verified by nucleic acid sequencing; subsequent analysis was performed using BLAST software (available at http://www.ncbi.nlm.nih.gov/blast/).

2.3. Cell culture

The micromass culture was performed as described previously [23]. The ATDC5 cells were briefly trypsinized and then resuspended in DMEM with 10% FBS at a concentration of 10^6 cells per ml, and six drops of 100 ml of cells were placed in a 60-mm tissue culture dish (Becton Dickinson). After 2 h of incubation at 37 °C, 1 ml of DMEM containing 10% FBS and BMP2 protein (300 ng/ml) was added. The media was replaced approximately every 2–3 days. To test the effect of overexpression of IRE1α protein on chondrogenesis, ATDC5 cells were infected with IRE1α expression adenovirus or control GFP adenovirus before micromass culture. To test the effect of knocking down IRE1α on chondrogenesis, ATDC5 cells were transfected with IRE1α siRNA adenovirus (siRNA-IRE1α) or control RFP adenovirus before micromass culture.

Mouse chondrogenic ATDC5 cells were maintained in a medium consisting of a 1:1 mixture of DME medium and Ham's F-12 medium (Flow Laboratories, Irvine, UK) containing 5% FBS (GIBCO BRL, Gaithersburg, MD, USA), 10 mg/ml human transferrin (Boehringer GmbH, Mannheim, Germany) and 30 nM sodium selenite (Sigma Chemical Co., St. Louis, MO, USA), at 37 °C in a humidified atmosphere of 5% CO_2 in air. The ATDC5 cells were seeded at a density of 3×10^5 cells per well in six-well cell-culture plates (Corning, Slangerup, Denmark). To induce chondrogenesis, cells were cultured in the maintenance medium supplemented with 10 mg/ml human insulin (Sigma-Aldrich). The medium was replaced every other day. For adenovirus (Ad-IRE1α or Ad-GFP) infection and siRNA adenovirus (siRNA-IRE1α or siRNA-RFP) infection, same protocol was followed as done with the ATDC5 cells.

2.4. RNA preparation and reverse transcription (RT)-PCR

Total RNA was prepared from ATDC5 cells; control lines were cultured in tissue culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal calf serum and antibiotics using the QIAGEN RNeasy mini kit and reverse-transcribed using oligo(dT) primers with the SuperScript preamplification system (Invitrogen) following the manufacturer's instructions. The following sequence-specific primers were synthesized: 5′-ATGGTGGTGGTGGCAGCCGC-3′ and 5′-GACACTAATCAGCTGGGGAA AGAG-3′ for IRE1α. The following pair of oligonucleotides was used as internal controls: 5′-ACCACAGTCC ATGCCATCAC-3′ and 5′-TCCA CCACCCTGTTGCTGTA-3′ for GAPDH. PCRs were performed for 35 cycles (94 °C 1 min, 60 °C 1 min and 72 °C 1 min) with a final elongation for 10 min at 72 °C. GAPDH was also amplified and employed as an internal control for 35 cycles (94 °C 1 min, 55 °C 1 min and 72 °C 1 min). PCR products were visualized on 1% agarose gels containing 0.1 mg/ml ethidium bromide using ultraviolet light. The identity of each targeted PCR amplification product was confirmed by DNA sequence analysis of gel-purified bands (QIAGEN Inc.)

2.5. Immunoblotting analysis

To examine the expressions of IRE1α and BiP protein in the course of chondrogenesis, total cell extracts prepared from micromass cultures of ATDC5 cells in the presence of 300 ng/ml recombinant BMP2 protein were mixed with 5 × sample buffer (312.5 mM Tris–HCl (pH 6.8), 5% b-mercaptoethanol, 10% SDS, 0.5% bromophenol blue, 50% glycerol). Proteins were resolved on a 10% SDS-polyacrylamide

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