Effects of IGF-II on promoting proliferation and regulating nitric oxide synthase gene expression in mouse osteoblast-like cell*

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Abstract: Objective: To investigate the effects of insulin-like growth factor II (IGF-II) on promoting cell proliferation, regulating levels of cellular nitric oxide (NO) and mRNA transcriptions of inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) in mouse osteoblast-like cells. Methods: Mouse osteoblastic cell line MC3T3-E1 was selected as the effective cell of IGF-II. After the cells were treated with IGF-II at different concentrations for different time duration, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to examine cell proliferation, and nitrate reductase method was applied to detect NO concentrations in cell culture supernatants and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was employed to determine transcription levels of cellular iNOS and eNOS mRNAs. Results: After the MC3T3-E1 cells were treated with IGF-II at concentration of 1 ng/ml for 72 h, 10 and 100 ng/ml for 24, 48 and 72 h respectively, all the MTT values increased (P<0.05 or P<0.01) with obvious dosage-time dependent pattern. NO levels of the MC3T3-E1 cells treated with 100 ng/ml IGF-II for 48 h, and with 1, 10 and 100 ng/ml IGF-II for 72 h were remarkably lower than that of the normal control, respectively (P<0.05 or P<0.01). After the cells were treated with 100 ng/ml IGF-II for 48 h cellular iNOS mRNA levels were significantly decreased (P<0.01). But the levels of eNOS mRNA in the cells treated with each of the used IGF-II dosages for different time duration did not show any differences compared with the normal control (P>0.05). Conclusion: IGF-II at different concentrations could promote proliferation of mouse MC3T3-E1 cell. This cell proliferation promotion was associated with the low NO levels maintained by IGF-II. Higher concentration of IGF-II could down-regulate iNOS gene expression at the level of transcription but not affect transcription of eNOS mRNA, which might be one of the mechanisms for IGF-II maintenance of the low NO levels in MC3T3-E1 cells.

Key words: Insulin-like growth factor II, Osteoblast, Proliferation, Nitric oxide synthase, Nitric oxide, Regulation


INTRODUCTION

It is well known that the osteoblast is the major functional cell in bone formation. As an important signal molecule participating in cell proliferation and differentiation, nitric oxide (NO) is closely involved in bone remodelling in vivo and in vitro (van’t Hof and Ralston, 2001; Ralston, 1997; Otsuka et al., 1998). Insulin-like growth factors (IGFs), recently identified polypeptides secreted by multiple types of mammal cells, have various bioactivities such as promoting cell proliferation and differentiation (Fournier et al., 1993; Langdahl et al., 1998) and can be divided into two types: IGF-I and IGF-II. Results of previous studies revealed that IGF-II has bioactivities promoting proliferation and differentiation of human osteoblasts and human osteosarcoma cells (Fournier et al., 1993; Langdahl et al., 1998) and regulating cellular NO
levels (Kaliman et al., 1999; Schini et al., 1994; Hill et al., 1999). Especially, this NO level regulating function of IGF-II exhibits certain cell specificity or distinct NO regulating effects for different cells and even the contrary (Kaliman et al., 1999; Schini et al., 1994; Hill et al., 1999). But the effects of IGF-II on proliferation and cellular NO level in osteoblasts and its possible mechanism has so far remained unknown, so it is reasonable for us to speculate that IGF-II may up-or down-regulate cellular NO level in osteoblasts.

In this study, we used mouse osteoblast-like cell line MC3T3-E1 as the effective cell and investigated the effects of IGF-II on promoting proliferation and regulating cellular NO level, inducing expression of inducible NO synthase (iNOS) and endothelial NOS (eNOS) by measuring of their mRNA transcription.

MATERIALS AND METHODS

Cell line and culture

A mouse osteoblast-like cell line MC3T3-E1 was kindly offered by the Department of Medical Microbiology and Parasitology, School of Medicine, Zhejiang University. Medium used for the cell culture was α-minimum essential medium (α-MEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. Two-hundred μl of 1.25×10^6 ml^{-1} MC3T3-E1 cell suspension was inoculated into each of the wells in a 96-well plates and then the plate was pre-incubated at 37 °C in atmosphere condition of 5% CO₂ for 24 h. Medium in the plates was replaced with 250 μl of 2% FBS α-MEM containing different concentrations of recombinant human IGF-II (rhIGF-II) (R & D) with 1, 10 and 100 ng/ml (IGF-II groups) or the same volume of 2% FBS-free α-MEM medium and 20 μl of 5 mg/ml MTT solution (Amresco) and then was continuously incubated at 37 °C for 4 h. The medium in the wells was discarded and 150 μl of dimethyl sulfoxide (DMSO) (Sigma) was then added into each of the wells. The OD₄₀₀ value of each of the wells was detected by spectrophotometry.

Detection of NO concentration

The collected supernatants from MC3T3-E1 cell cultures mentioned above were centrifuged at 2000 rpm for 5 min and the supernatants were then recovered. NO concentrations in the supernatants were detected by using NO Detection kit (JINMEI Biotech Co. Ltd.) based on nitrate reductase method. Nitrate in samples was first reduced into nitrite with nitrate reductase and NO concentration was then obtained through measurement of NO⁻/NO₃⁻ proportion.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA Isolation Kit (TaKaRa) was used to prepare total RNA from the MC3T3-E1 cells mentioned above. RT-PCR Kit (TaKaRa) was used to detect mRNAs of the cellular iNOS and eNOS. The sequences of iNOS primers were (Togari et al., 1998): (F) 5'-CCCTTCCGAAGTTTCGCGACGC-3', (R) 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (497 bp). The sequences of eNOS primers (Laufs et al., 2000): (F) 5'-TCCGCTGCAACGCTGCTGTC-3', (R) 5'-TTCCGCTGCAACGCTGCTGTC-3' (340 bp). The housekeeping gene GAPDH (452 bp) was used to standardize the samples with equal cDNAs and its sequences of primers: (F) 5'-CTGCTCGTCCATGAGGAGGGA-3', (R) 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (497 bp). The sequences of eNOS primers (Laufs et al., 2000): (F) 5'-TCCGCTGCAACGCTGCTGTC-3', (R) 5'-TTCCGCTGCAACGCTGCTGTC-3' (340 bp). The housekeeping gene GAPDH (452 bp) was used to standardize the samples with equal cDNAs and its sequences of primers: (F) 5'-ACCACATGGCTTCCATGAGGAGGGA-3', (R) 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (497 bp). The parameters for RT: 42 °C 50 min, 99 °C 5 min and 5 °C 5 min. The total reaction volume per PCR was 50 μl containing 0.8 μmol/L dNTPs, 0.5 μmol/L for each the iNOS primers, 0.05 μmol/L for each the GAPDH primers, 2.5 U Taq polymerase, 6 μl RT product as template and 10×PCR buffer 5 μl. The parameters for PCR: 94 °C, 5 min, ×1; 94 °C, 50 s, 60 °C, 50 s, 72 °C,