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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
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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

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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 1.25×10^4 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% CO_2 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% FBSα-MEM without IGF-II (control groups), and then the plates were continuously incubated for 8, 24, 48 and 72 h, respectively. After stopping incubation, the supernatants of cultures in 7 repeated wells were collected for NO detection and cells in the same wells were used for MTT examination, and cells in the rest of the 8 repeated wells were used for total RNA preparation.

Detection of cell proliferation

Cell proliferation was detected by MTT method which determines cell proliferation through measuring the absorbent value at OD_{490} based on the fine positive linear correlation between OD_{490} value and cell numbers. The MC3T3-E1 cells in 96-well plates above were washed twice with 0.01 mol/L PBS (pH 7.4). Into each of the wells in the plates was added 200 μl of 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 OD_{490} 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_2^-/NO_3^- 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′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′, (R) 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′ (497 bp). The sequences of eNOS primers (Laufs *et al*., 2000): (F) 5′-TTCCGGCTGCCACCTGATCCTAA-3′, (R) 5′-AACATATGTCCTTGCTCAAGGCA-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′-ACCACAGTCCATGCCATCAC-3′, (R) 5′-TCCACCACCCTGTTGCTGTA-3′ (452 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,

1 min, ×28; 72 °C, 8 min, ×1. For detecting eNOS mRNA, the reagents, reaction volume and RT-PCR parameters were the same as those for iNOS mRNA detection, except of 0.02 μmol/L for each the GAPDH primers and 34 amplification cycles. RT-PCR products were detected by electrophoresis in 3% agarose gel (Sigma) pre-stained with 0.5 μg/ml ethidium bromide. The target fragments were quantitatively analyzed by density scanning technique using Bio Imaging System (Synoptics Ltd. USA).

Data analysis

All values obtained were expressed as the mean±*SD*. *T*-test and one-way analysis of variance was used to statistically analyze the data.

RESULTS

Effect of IGF-II on promoting MC3T3-E1 cell proliferation

After MC3T3-E1 cells were treated with 1 ng/ml IGF-II for 72 h, 10 or 100 ng/ml IGF-II for 24 h, 48 h and 72 h, respectively, the MTT values were remarkably increased (P<0.05 or P<0.01) in a marked time-dosage-dependant manner (Fig.1).

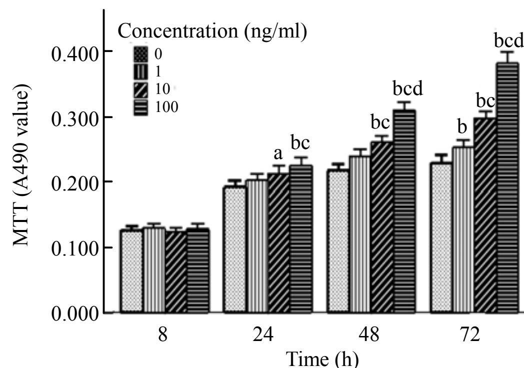

Fig.1 Effects of IGF-II with different concentrations and treatment duration on MC3T3-E1 cell proliferation. *n*=7, $\bar{x}$ ±*s*. [a]*P*<0.05 vs the control group; [b]*P*<0.01 vs the control group; [c]*P*<0.01 vs the 1 ng/ml IGF-II group; [d]*P*<0.01 vs the 10 ng/ml IGF-II group

Effect of IGF-II on NO levels of MC3T3-E1 cells

Compared with the control group, NO level of the MC3T3-E1 cells treated with 100 ng/ml IGF-II for 48 h was remarkably decreased (P<0.01), and obviously lower NO levels were observed in the MC3T3-E1 cells treated with 1, 10 or 100 ng/ml IGF-II for 72 h (P<0.01, P<0.05 and P<0.01, respectively) (Fig.2 and Fig.3).

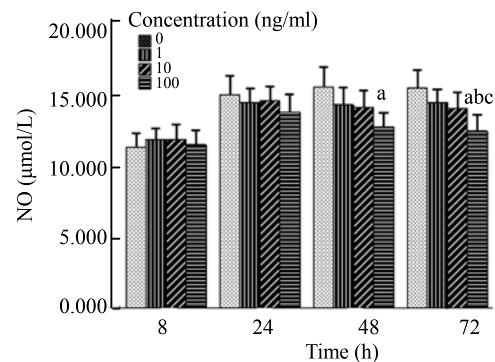

Fig.2 Effects of IGF-II at different concentrations and treatment durations on NO levels of MC3T3-E1 cells. *n*=7, $\bar{x}$ ±*s*. [a]*P*<0.01 vs the control group; [b]*P*<0.01 vs the 1 ng/ml IGF-II group; [c]*P*<0.01 vs the 10 ng/ml IGF-II group

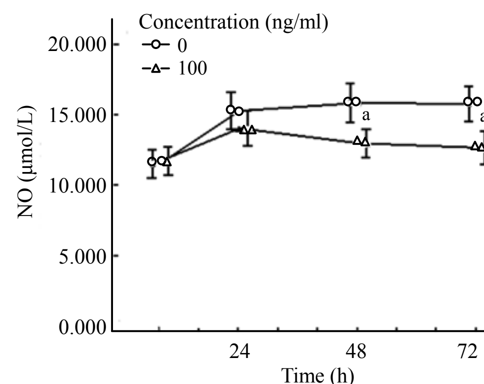

Fig.3 Effects of 100 ng/ml IGF-II for different treatment durations on NO levels of MC3T3-E1 cells. *n*=7, $\bar{x}$ ±*s*. [a]*P*<0.01 vs the control group

Effects of IGF-II on iNOS and eNOS mRNA levels of MC3T3-E1 cells

iNOS mRNA levels of the MC3T3-E1 cells treated with 100 ng/ml IGF-II for 48 h were obviously lower than those of the control group and 1 ng/ml IGF-II group (P<0.01). After being incubated for 72 h, iNOS mRNA levels of the MC3T3-E1 cells treated with 100 ng/ml IGF-II were remarkably lower than

those of the control group, 1 ng/ml and 10 ng/ml IGF-II groups (P<0.01) (Fig.4 and Fig.5). eNOS mRNA levels of the MC3T3-E1 cells treated with different dosages of IGF-II and time durations were similar to those of the control group (P>0.05) (data not shown).

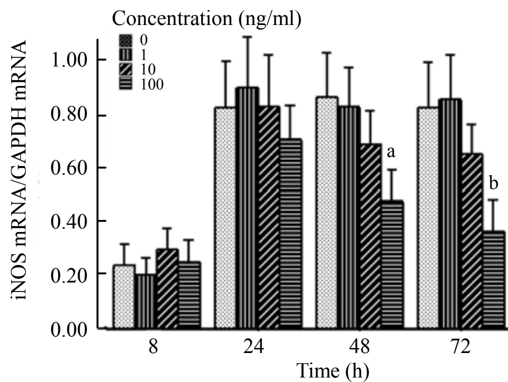

Fig.4 Effects of IGF-II with different concentrations and treatment durations on iNOS mRNA levels of MC3T3-E1 cells (iNOS mRNA/GAPDH mRNA). n=4, $\bar{x}\pm s$. [a]P<0.01 vs the control group and the 1 ng/ml IGF-II group; [b]P<0.01 vs the control group and the 1 and 10 ng/ml IGF-II groups

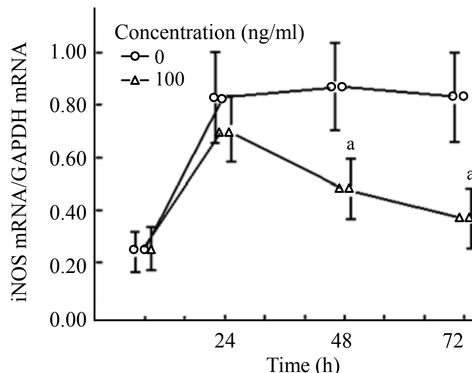

Fig.5 Effects of 100 ng/ml IGF-II for different treatment durations on iNOS mRNA levels of MC3T3-E1 cells. n=4, $\bar{x}\pm s$. [a]P<0.01 vs the control group

DISCUSSION

IGF-II is one of the most abundant growth factors in bone matrix and the effects of IGF-II on osteoblasts are being closely studied (Fournier *et al.*, 1993; Langdahl *et al.*, 1998). Over the past decade, the mitogenic effect of IGF-II and its mechanisms have been extensively investigated. However, its regulating effect on cellular NO was noticed only recently. NO is a very small lipophilic molecule which diffuses and reaches the cytoplasmic components, and results in the activation of diverse biological function, and IGF-II had a distinct effect on NO and iNOS gene expression in different cell types (Kaliman *et al.*, 1999; Schini *et al.*, 1994; Hill *et al.*, 1999). Our present study, is the first study showing that IGF-II downregulates the production of NO in the mouse preosteoblastic cell line, MC3T3-E1, via inhibiting mRNA transcription of iNOS gene. And in agreement with the general view, IGF-II has been shown to be a mitogenic factor, at concentration range of 1−100 ng/ml, IGF-II can obviously promote MC3T3-E1 proliferation in a dosage- and time-dependent manner.

NO exerts biphasic effects on osteoblasts: at moderately low concentrations NO can maintain normal proliferation or promote proliferation, while at high concentrations, can inhibit proliferation and induce apoptosis (Kanamaru *et al.*, 2001; Mancini *et al.*, 2000; Armour *et al.*, 2001a). In our studies, 1−100 ng/ml IGF-II, especially 100 ng/ml IGF-II, with 48 h and 72 h treatment enable the MC3T3-E1 cells to maintain lower NO levels compared to the controls, and significantly decrease NO levels compared to the control group. These lower NO levels occurred in parallel with the IGF-II-induced cell proliferation, indicating that maintenance of moderate low NO level benefits cell proliferation. Extracellular regulated kinase (ERK), a key molecule in the mitogen-activated protein kinase (MAPK) pathway, can mediate signals of cell proliferation and differentiation, and moderate level of NO can activate ERK (Robinson and Cobb, 1997; Jessop *et al.*, 2002). IGF-II exhibits high-affinity binding to IGF-I receptor (IGF-IR), which can start the MAPK pathway (Vincent and Feldman, 2002). Thus, the mechanism of IGF-II promoting MC3T3-E1 cell proliferation probably involves activations of the ERK and MAPK pathway.

No expression or low expression of iNOS in osteoblast was found under basal conditions. But activated iNOS induces a great quantity of NO. It was reported that NO production, bone mineral density

decrease and osteoblast apoptosis in iNOS gene knockout mice were much lower than those in experimental inflammation-mediated osteoporosis mice (Armour *et al*., 2001a). High levels of inducible NO were cytotoxic to osteoblasts and even inhibited bone formation (Mancini *et al*., 2000; Armour *et al*., 2001a). Significantly high levels of NO in lesions of localised bone destruction (Mancini *et al*., 2000), rheumatoid arthritis (Gonzalez-Gay *et al*., 2004), and in periodontal lesions, a disease accompanied by alveolar bone loss (Batista *et al*., 2002) were also found. Thus, inducible NO is harmful. On the contrary, eNOS seems to play an important role in increasing osteoblast activity and promoting bone formation. For example, eNOS gene knockout mice had defective bone formation (Aguirre *et al*., 2001; Armour *et al*., 2001b). In this study, we found that 100 ng/ml IGF-II continuously inhibited mRNA transcription of MC3T3-E1 cell iNOS gene, whereas eNOS mRNA levels of the MC3T3-E1 cells treated with the different concentrations of IGF-II in all the tested durations were similar to those of the normal control. These data indicated that the mechanism of IGF-II activity promoting MC3T3-E1 cell proliferation was associated with downregulation of iNOS mRNA transcription and block excessive NO production but not affect normal transcription of MC3T3-E1 cell eNOS gene. Therefore, effective inhibition of inducible NO production may represent a beneficial therapeutic strategy. The bioactivities of IGF-II to promote proliferation and inhibit inducible NO production in osteoblasts might have therapeutic potential for localised bone destruction associated with inflammatory bone diseases such as rheumatoid arthritis and periodontal disease.

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