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A20 overexpression under control of mouse osteocalcin promoter in MC3T3-E1 cells inhibited tumor necrosis factor-alpha-induced apoptosis[1]

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Key words

A20; osteoblast; apoptosis; tumor necrosis factor-alpha

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Abstract

Aim: To construct an A20 expression vector under the control of mouse osteocalcin promoter (OC-A20), and investigate osteoblastic MC3T3-E1 cell line, which stably overexpresses A20 protein prevented tumor necrosis factor (TNF)-alpha-induced apoptosis. **Methods:** OC-A20 vector was constructed by fusing a fragment of the mouse osteocalcin gene-2 promoter with human A20 complementary DNA. Then the mouse MC3T3-E1 cell line, stably transfected by A20, was established. The expression of A20 mRNA and A20 protein in the cells were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis, respectively. To determine the specificity of A20 expression in osteoblast, the mouse osteoblastic MC3T3-E1 cell line and mouse embryo fibroblast NIH3T3 cell line were transiently transfected with OC-A20. The anti-apoptotic role of A20 in MC3T3-E1 cells was determined by Flow cytometric analysis (FACS), terminal dUTP nick endo-labeling (TUNEL) and DNA gel electrophoresis analysis (DNA Ladder), respectively. **Results:** Weak A20 expression was found in MC3T3-E1 cells with the primers of mouse A20. A20 mRNA and A20 protein expression were identified in MC3T3-E1 cells transfected with OC-A20 using RT-PCR and Western blot analysis. Only A20 mRNA expression was found in MC3T3-E1 cell after MC3T3-E1 cells and NIH3T3 cells were transient transfected with OC-A20. A decrease obviously occurred in the rate of apoptosis in the OC-A20 group compared with the empty vector (pcDNA3) group by FACS ($P<0.001$). A significant increase in TUNEL positive staining was found in the pcDNA group compared with OC-A20 group ($P<0.001$). Simultaneously, similar effects were demonstrated in DNA gel electrophoresis analysis. **Conclusion:** We constructed an osteoblast-specific expression vector that expressed A20 protein in MC3T3-E1 cells and confirmed that A20 protects osteoblast against TNF-alpha-induced apoptosis.

Introduction

Bone mass is largely determined by the number of bone forming (osteoblasts) and bone resorbing (osteoclasts) cells present in the basic multicellular units responsible for the regeneration of the adult skeleton[1]. The osteoblast is the cell responsible for the synthesis of collagen and other bone proteins, and also has an important role in the subsequent mineralization of the matrix. Recent evidence has indicated that apoptosis plays a critical role during embryonic limb development, skeletal maturation, adult bone turnover by modeling and remodeling processes, and during fracture healing and bone regeneration. Apoptosis is the most common fate of osteoblasts during physiologic bone remodeling,

thus the control of osteoblast apoptosis will affect the rate of bone formation and bone mass[2–4].

A20 is a cytoplasmic zinc finger protein that inhibits nuclear factor kappaB (NF-kappaB) activity and tumor necrosis factor (TNF)-mediated programmed cell death (PCD)[5–9]. TNF dramatically increases A20 mRNA expression in all tissues. Previous studies have shown that protection against TNF cytotoxicity exists for endothelial cells, hepatocyte, carcinoma cells, and murine embryonic fibroblast cells[10–12].

In 2000, Lee *et al*[13] reported that mice deficient for A20 develop severe inflammation and cachexia, and die prematurely. A20-deficient cells failed to terminate TNF-induced NF-kappaB responses. These cells were also more susceptible than control cells to TNF-mediated PCD. Histological examination of 3- to 6-week-old $A20^{-/-}$ mice revealed severe inflammation and tissue damage in livers, kidneys, and intestines. The osseous parts of joints were thin and bone marrow was filled with inflammatory cells. Histological examination also showed that the trabecular bone was thinner in $A20^{-/-}$ mice than in $A20^{+/+}$ mice. Failure to downregulate NF-kappaB transcriptional activity results in chronic inflammation and cell death. A20 is a potent inhibitor of NF-kappaB signalling, but its mechanism of action is unknown in the study. In 2004, a further study showed that A20 downregulates NF-kappaB signalling through the cooperative activity of its two ubiquitin-editing domains[8]. Boone *et al*[14] also reported mice developed spontaneous inflammation, if mice doubly deficient in either A20 and TNF or A20 and TNF receptor 1, indicated that A20 is also critical for the regulation of TNF-independent signals *in vivo*. However, the mechanism by which A20 is involved in bone loss is still unclear.

In present study, we constructed A20 expression vector under the control of mouse osteocalcin promoter. We then determined the specific expression of A20 in osteoblastic MC3T3-E1 cells using this vector, and investigated whether MC3T3-E1 cells stably over-expressed A20 preventing TNF-alpha-induced apoptosis.

Materials and methods

Construction of OC-A20 vector First, we designed the senseprimer:5'-CATCGCGAGAATTGCTCATCGCAGCC-3' and reverse primer: 5'-CAGGTACCTGCACCCTCCAGC-ATCCA-3' containing the restricted sites of *Nru*I and *Kpn*I. The 1.3 kb DNA fragment of mouse osteocalcin gene 2 promoter, named plasmid *pIIBS*1.3 (a gift from Dr G KARSENTY, Baylor College of Medicine, Houston, Texas, USA), was obtained by PCR using the above primers. The plasmid pcDNA3-A20 containing the human A20 gene was cut with *Nru*I, then restricted with *Kpn*I (TaKaRa BIO, Dalian, China) to remove the CMV promoter fragment[15,16]. The 1030 bp osteocalcin promoter fragment was released from plasmid *pIIBS*1.3 by digesting with *Nru*I and *Kpn*I to create the blunt end. Then osteocalcin promoter and pcDNA3-A20 were ligated to create a new plasmid containing the osteocalcin promoter, which was named A20 expression vector under the control of mouse osteocalcin promoter (OC-A20).

Specific expression of OC-A20 in MC3T3-E1 cells MC3T3-E1 cells (a gift of Dr WB XIA, Beijing Union Hospital, Beijing, China) and mouse embryo fibroblast NIH3T3 cells were grown in a-modified minimal essential medium (a-MEM) supplemented with 10% heat-inactivated FBS and kanamycin (60 μg/mL) (Gibco Invitrogen, Grand Island, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. The day before transient transfection, both kinds of cells were plated on 6-cm-diameter dishes at a density of 4×10^5 cells per dish. MC3T3-E1 cells and NIH3T3 cells were transfected with OC-A20 (5 μg/100-mm dish), respectively, by lipofection according to standard procedures (Invitrogen, Carlsbad, CA, USA). After 8 h, the transfection medium was replaced with fresh medium (10% FBS) overnight. The expression of A20 mRNA of MC3T3-E1 and NIH3T3 cells transfected with OC-A20 was determined by reverse transcription-polymerase chain reaction (RT-PCR).

Cell culture and stable transfection MC3T3-E1 cells were grown in a-MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and kanamycin (60 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed twice weekly. The day before transfection, cells were plated on 6-cm-diameter dishes at a density of 4×10^5 cells per dish. MC3T3-E1 cells were transfected with the A20 plasmid containing mouse osteocalcin gene 2 promoter (5 μg/100-mm dish) by lipofection according to standard procedures described by the manufacturer, and with 5 ug of the plasmid pCMV-pcDNA3 alone to generate control. After 8 h, the transfection medium was replaced with fresh medium (10% FBS) and cultured overnight. The cells were then cultured in α-MEM supplemented with 10% FBS and 60 μg/mL kanamycin and exposed to 400 μg/mL of geneticin G418 (Gibco). After 2 weeks of selection (the medium was replaced with fresh 10% FBS medium each 72 h) and several resistant clones were obtained. The survival clones were subcultured before reaching confluency. Expression of A20 mRNA and A20 protein in the MC3T3-E1 cells was determined by RT-PCR and Western blot analysis, respectively.

Analysis of A20 mRNA endogenous expression in MC3T3-E1 cells Total RNA was isolated from mouse

MC3T3-E1 cells with or without TNF-alpha (1000 u/mL, Peproteck, UK) for 1 h and 2 h, with Trizol Reagent (Gibco Invitrogen, Japan) according to the manufacturer's instructions, and then identified by electrophoresis, and cDNA was synthesized using random primer. For detection of A20, the primers of mice A20 were used (sense primer: 5'-TTTGAGCAATATGCGGAAAGC-3', reverse primer: 5'-AGTTGTCCCATTCGTCATTCC-3'). PCR was performed as follows: denaturing at 94 °C for 1 min, annealing at 62 °C for 1.5 min, synthesis at 72 °C for 2.5 min, for total of 30 cycles. The PCR reaction generated a 480-pb fragment of murine A20 and a 591-bp fragment of murine β-actin (sense primer: 5'-AACGAGCGGTTCCGATGCCCTGAG-3', reverse primer: 5'-TGTCGCCTTCACCGTTCCAGT-T-3')[17]. The RT-PCR products were determined by electrophoresis in 1.5% denaturing agarose gel.

Total RNA extraction, RT-PCR and Western blot analysis of MC3T3-E1 cells-transfected stably with A20 Total RNA of MC3T3-E1 cells-transfected with OC-A20 or pcDNA3-null plasmid DNA was extracted with Trizol Reagent according to the manufacturer's instruction, and then identified by electrophoresis. Random hexamer primers (Promega, Madison, WI, USA) were employed for cDNA preparation using the moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). PCR was performed as follows: denaturing at 94 °C for 1 min, annealing at 62 °C for 1.5 min, synthesis at 72 °C for 2.5 min (for 10 min in last cycle), for total of 30 cycles. The PCR reaction generated a 2370-bp fragment of human A20 (sense primer: 5'-CGGTACCGCACAATGGCTGAACAAGTCCTTCCTC-3', reverse primer: 5'-CGTCTAGAGTTAGCCATACATCT-GCTTGAACTG-3'), and a 591-bp fragment of murine β-actin by previously described primers.

For Western blot, the transfected cells were collected, suspended in suspending buffer (NaCl 0.1 mol/L, Tris-HCl 0.01 mol/L, pH 7.6, egtazic acid 1 mmol/L, aprotinin 1 mg/L, and phenylmethyl sulfonylfluoride (PMSF)(100 mg/L), then 2×SDS loading buffer was immediately added with equal volume, and the mixture was boiled for 10 min. After centrifugation, the lysates were sonicated for 10 s and incubated on ice for 10 min. A sample (30 UL) was resolved on 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred onto a nitrocellulose membrane. The membranes were blocked with 5% fat-free milk and probed with mouse anti-human A20 antibody (1/1000, Oncogene, CA, USA). The blots were washed and exposed to horseradish peroxidase (HRP)-conjugated antimouse IgG secondary antibody (Santa Cruz Biotechnology, USA) and then developed using the enhanced chemiluminescence (ECL) reagent (PerkinElmer, Boston, MA, USA). The molecular mass of the protein was estimated relative to the pre-stained size marker.

Flow cytometric analysis of apoptosis MC3T3-E1 cells transfected stably with OC-A20 and pcDNA3-null were plated on 6-cm-diameter dishes at a density of 4×10^5 cells per dish cells. After the cells were cultured in the presence of 10% FBS medium for 48 h, they were divided into two groups: cells cultured in the presence or absence of TNF-alpha (1000 u/mL)-treated and control groups. After 8 h, cells were harvested, dispersed, fixed in 70% ethanol, and suspended into FACS buffer (1% FBS, 0.05% NaN3 in PBS pH 7.0), and then washed twice in FACS buffer before fixing with 2% paraformaldehyde in PBS for 15 min at 4 °C For reading, cells were suspended in 300 μL FACS buffer and processed with a FACS (Becton Dickinson, Rahway, New Jersey, USA).

TUNEL detection of apoptosis Apoptotic cells were also determined by terminal dUTP nick endo-labeling (TUNEL) staining using an *in situ* cell death detection kit (Roche Diagnostics, Switzerland) according to the manufacturer's protocol. MC3T3-E1 cells ($500/cm^2$) transfected stably with OC-A20 and pcDNA3-null were cultured on Labtek chambers in the presence of 10% FBS medium were treated with TNF-alpha (1000 u/mL; as treatment group) or vehicle (as control group) for 8 h, respectively, and then fixed with paraformaldehyde at room temperature for 25 min. Endogenous peroxidase was quenched with 3% H_2O_2, and the cells were permeabilized with 0.2% Triton X-100, at 4 °C for 5 min and incubated for 1 h at 37 °C with the TUNEL reaction mixture containing the terminal deoxynucleotidyl transferase. Incorporated fluorescein was detected by sheep anti-fluorescein antibody conjugated with horseradish peroxidase. The cells were detected using TUNEL assay using an *in situ* cell death detection kit. TUNEL-positive cells were detected by brown nuclei and nuclear fragmentation. We counted the total of TUNEL positive cells in different groups from four independent experiments. In each experiment group, the TUNEL positive cells were counted from 10 different visual fields in fluorescence microscope, and data from four independent experiments were pooled and are given as the percentage of apoptosis (mean±SD).

DNA ladder analysis MC3T3-E1 cells transfected stably with OC-A20 and pcDNA3-null were stimulated with TNF-alpha (1000 u/mL) for 8 h. Genomic DNA was isolated using the phenol-chloroform extraction method and the DNA were separated by electrophoresis on a 1.5% agarose gel.

Statistical analysis Student's *t*-test was used to assess the statistical significance of differences by the software SPSS11.0. A *P*-value of less than 0.05 was considered to be statistically significant. Data were expressed as mean±SD.

Results

A20 weakly endogenous expression in MC3T3-E1 cells Weak endogenous expression of A20 in MC3T3-E1 cells was analyzed by RT-PCR using mice A20 primer. But A20 mRNA was rapidly induced (within 2h) in MC3T3-E1 cells after TNF-alpha stimulation (Figure1).

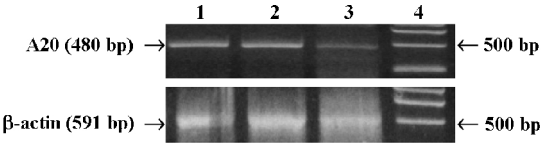

Figure 1. A20 mRNA expression in MC3T3-E1 cells, using the primer of mouse A20. Lane 1: A20 expression in 1 h after TNF-alpha stimulation; Lane 2: A20 expression in 2 h after TNF-alpha stimulation; Lane 3: Weak expression of A20 in MC3T3-E1 cells without TNF-alpha stimulation; Lane 4: 2 kb DNA marker.

Construction of OC-A20 vector and specific expression in MC3T3-E1 cells The new OC-A20 vector containing human A20 gene and mouse osteocalcin gene 2 promoter were digested with *Nru*I and *Kpn*I restriction enzyme, and 1030 bp and 7079 bp DNA fragments were obtained by PCR-RFLP (Figure 2). The identity of the OC-A20 vector was confirmed by DNA sequence analysis (data not shown). After MC3T3-E1 and NIH3T3 cells were transiently transferred with OC-A20, A20 mRNA expression was not found in NIH3T3 cells. A significant expression of A20 mRNA was detected in MC3T3-E1 cells (Figure 3).

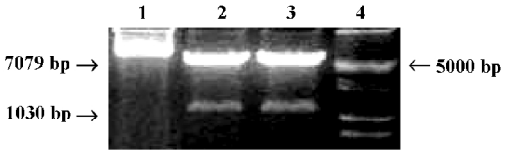

Figure 2. Identification of OC-A20 vector by PCR-RFLP. Lane 1: positive control; Lanes 2 and 3: 1030 bp and 7079 bp DNA fragments of OC-A20 vector was obtained with *Nru* I and *Kpn* I restriction enzyme; Lane 4: 15 kb+2 kb DNA Marker

Establishment of stably transfected cells Using RT-PCR and Western blot analysis, our results showed that the expression of A20 mRNA and A20 protein was significantly higher in MC3T3-E1 cells transfected stably with OC-A20, but the expressions of A20 mRNA and A20 protein were not found in MC3T3-E1 cells transfected with pcDNA3-null (Figure 4).

A20 anti-apoptotic effects Apoptotic cells were quantified as the proportion of cells that had a DNA content of less than 2N(sub-G_1DNA content).As shown in Figure 5, after treatment with TNF-alpha (1000 u/mL) for 8 h, the apoptosis of OC-A20-transfected cells was only 15.4%±1.3%, but significant apoptosis was observed in the non-transfected cells (31.0%±7.5%) and pcDNA3-null transfected cells (32.6%±7.0%) at the same TNF-alpha concentration. Data from six independent experiments were pooled and were given as the percentage of apoptosis. There was a significant difference of apoptosis between the OC-A20-transfected group and pcDNA3-null-transfected group (P<0.001) (Figure 5).

We assessed DNA fragmentation in individual transfected cells by specific labeling of double-strand DNA breaks using the TUNEL methods. TUNEL staining of pcDNA3-null-transfected cells and OC-A20-transfected cells treated with TNF-alpha (1000 u/mL) for 8 h was conducted. Exposure of pcDNA3-null-transfected cells to TNF-alpha showed more DNA fragmentation compared to OC-A20-transfected cells *in vitro*. Data from four independent experiments were pooled and were given as the percent of apoptosis. The difference in apoptosis percent between the OC-A20-transfected group and pcDNA3-null-transfected group was sig-

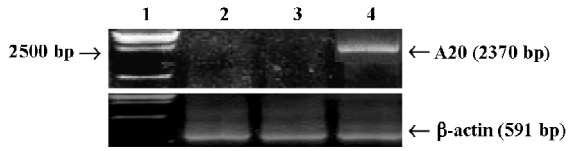

Figure 3. A20 mRNA expression in MC3T3-E1 cells and NIH3T3 cells-transfected with OC-A20, using the primer of human A20. Lane 1: 15 kb DNA Marker; Lanes 2 and 3: no A20 mRNA expression in NIH3T3 cells; Lane 4: A20 mRNA expression in MC3T3-E1 cells. β-actin expression was detected by RT-PCR in MC3T3-E1 and NIH3T3 cells transfected with OC-A20.

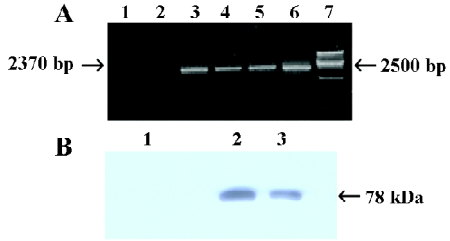

Figure 4. The expression of A20 mRNA and A20 protein in MC3T3-E1 cells, using the primer of human A20. A: Lanes 1 and 2: no A20 mRNA expression in pcDNA3-null-transfected cells; Lane 3-6: A20 mRNA expression in OC-A20-transfected cells. B: Lane 1: no A20 protein expression in pcDNA3-null-transfected cells; Lane 2: positive control; Lane 3: A20 protein expression in OC-A20-transfected cells.

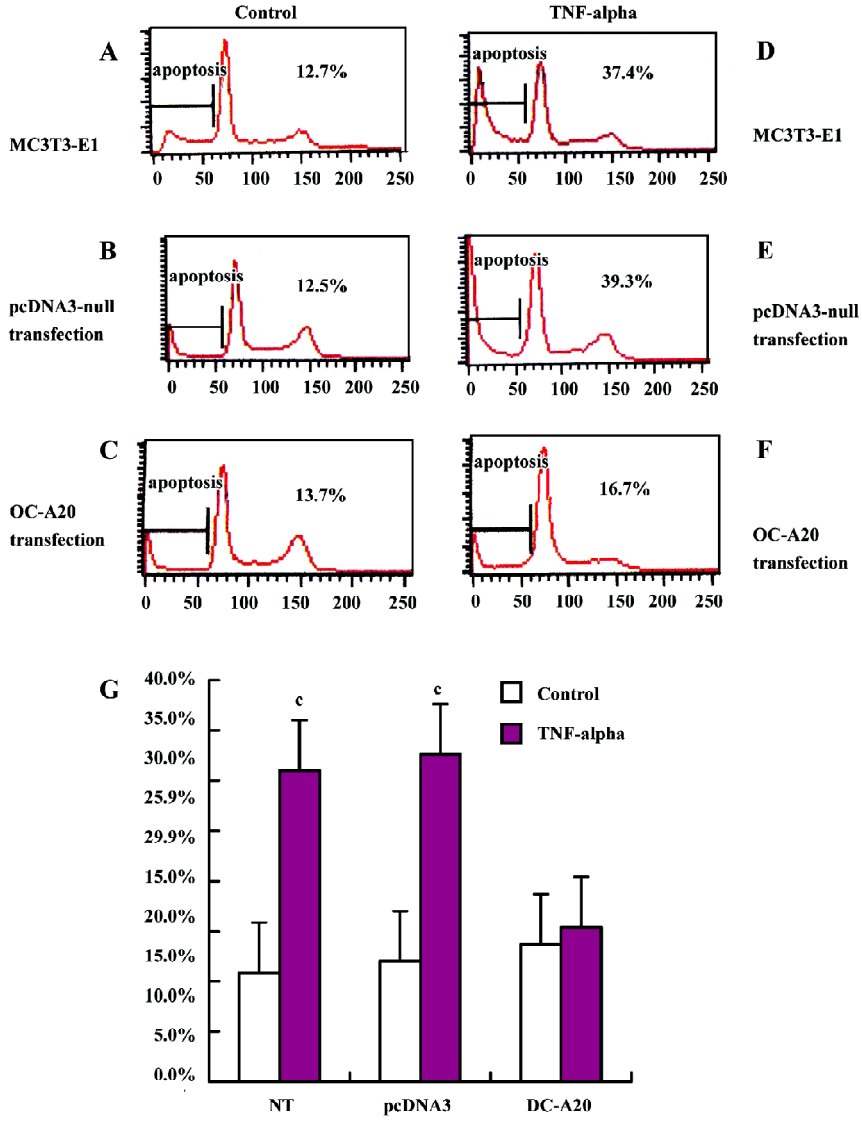

Figure 5. Effect of OC-A20 on the apoptosis of MC3T3-E1 cells. A, B, C: MC3T3-E1 cells, pcDNA3-null-transfected cells and OC-A20-transfected cells were not treated with TNF-alpha, respectively; D, E, F: MC3T3-E1 cells, pcDNA3-transfected cells and OC-A20-transfected cells were treated with TNF-alpha 1000 u/mL for 8 h, respectively. The percentage of apoptotic cells was assayed by FACS using propidum iodide staining. G: Apoptosis percent in non-transfected cells (NT), pcDNA3-null-tansfected cells and OC-A20-transfected cells. $^{c}P<0.001$ *vs* OC-A20-transfected cells in TNF-alpha group.

nificant ($P<0.001$) (Figure 6).

To assay for DNA degradation, DNA was analyzed by agarose gel electrophoresis. TNF-alpha treatment resulted in the formation of a DNA ladder in non-transfected group or pcDNA3-null-transfected group at 8 h, but no ladder was demonstrated in the OC-A20-transfected cells (Figure 7).

Discussion

A20 was originally described as a TNF-alpha-inducible zine finger protein in endothelial cells[18]. Stable overexpression of A20 plays a role of resistance to TNF-induced apoptosis in some cell lines. Several researchers have reported that the antiapoptotic gene A20 protects β cells against apoptosis in islets[15,19,20]. It has also been identified that A20 protects some cell lines against TNF cytotoxicity, such as human breast carcinoma cells, murine fibrosarcoma cells, and murine embryonic fibroblast cells[11,12,21]. Both mouse osteoblast cells and murine embryonic fibroblast cells develop from mesenchymal progenitor. We presume that a similar anti-apoptotic effect of A20 may also exist in osteoblastic cells.

In an attempt to clarify the role of A20 in osteoblasts, we constructed osteoblast-specific A20 expression vector, which induces A20 expression under the control of murine osteocalcin promoter. Ducy and Karsenty[22] have reported that a short promoter fragment of mouse osteocalcin gene 2 (mOG2, -147/+13 fragment) displays osteoblast-specific activity in DNA transfection experiments. They identified that this promoter fragment contains two *cis*-acting elements, which are called OSE1 and OSE2, fulfilling the definition of

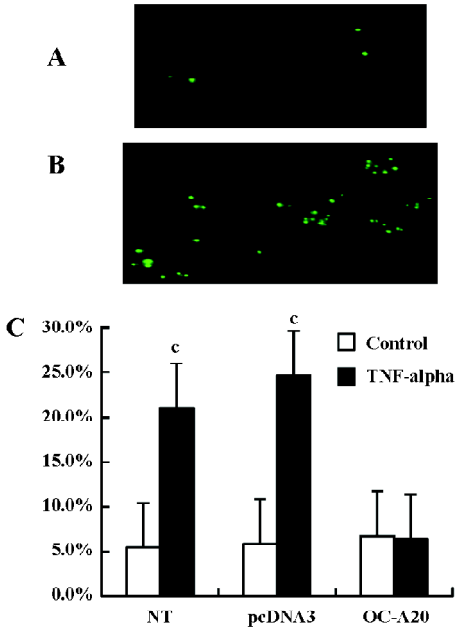

Figure 6. Nuclear damage observed using TUNEL staining in MC3T3-E1 cells *in vitro*. (A) OC-A20-transfected cells treated with TNF-alpha (1000 u/mL) for 8 h , few nuclei were condensed and broken; (B) pcDNA3-tansfected cells treated with TNF-alpha (1000 u/mL) for 8 h, many more cells were positively labeled. Photographs were taken under an Olympus BH-2 fluorescence microscope (×40). (C) Apoptosis percent in non-transfected cells, pcDNA3-tansfected cells and OC-A20-transfected cells. $^cP<0.001$ *vs* OC-A20-transfected cells in TNF-alpha group.

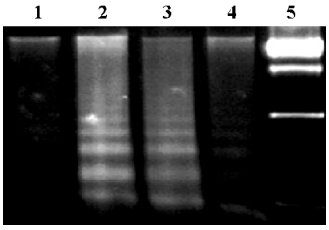

Figure 7. DNA fragmentation in MC3T3-E1 cells treated with TNF-alpha for 8 h. Lanes 1 and 4: only one lane in the OC-A20-transfected cells; Lane 2: DNA ladder in pcDNA3-null-transfected cells; Lane 3: DNA ladder in non-transfected cells. Lane 5: 15 kb DNA marker.

an osteoblast-specific activator of transcription. These two elements may be important at several stages of osteoblast differentiation. They then cloned the mOG2 promoter containing 1.3 kb DNA fragments, named the recombine vector as *pIIBS*1.3[22]. In the present study, we constructed the A20 expression vector under the control of mouse osteocalcin promoter by fusing a 1030-bp fragment of mouse osteocalcin promoter with human A20 complementary DNA. The genomic structure of OC-A20 vector was confirmed through PCR-RFLP and DNA sequence analysis. Using this expression vector, the MC3T3-E1 cells and mouse embryo fibroblast NIH3T3 cells were transiently transfected and the results demonstrated the specific expression of A20 in osteoblastic MC3T3-E1 cells.

Grey *et al*[19] reported on the induced expression of the anti-apoptotic gene A20 in cells of islets in response to IL-beta. But little is currently known about the expression of cytoprotective genes in osteoblast cells. Moreover, to our knowledge, there are no reports regarding the expression of the A20 gene in mouse osteoblast. We raised the question whether osteoblast cells are able to have a protective response to inflammation. In the present study, we found that expression of A20 is rapidly induced in mouse MC3T3-E1 cells in response to TNF-alpha. The rapid expression of A20 in osteoblast suggested that it may be a component of their physiological protective response to injury[23].

Before clarifying the anti-apoptotic effect of A20 in osteoblastic MC3T3-E1 cells, we established the cell line which stably over-expressed A20 protein during osteoblast differentiation. The establishment of MC3T3-E1 cell line stable over-expression A20 can diminish errors in independent experiments of different times. By FACS, TUNEL and DNA ladder analysis, we found that A20 could protect the MC3T3-E1 cells from TNF-alpha induced apoptosis *in vitro*. The mechanism of action has not yet been completely clarified. Previous studies showed that A20 might interfere with TNF-receptor associated death domain (TRADD) binding to the TNF-receptor 1 and might therefore negatively regulate TNF-induced cytotoxicity[5–8,12–14,24].

Apoptosis plays a critical role during bone turnover and bone loss. There are many molecules that are involved in apoptosis, and the fate of the cell depends on how these molecules interact with each other. The functional significance of osteoblast death in the postnatal skeleton and adult skeleton for normal bone homeostasis or in response to pharmacologic agents is not presently well understood. Exploration to determine the selective pathways of apoptosis, which have been identified in other cells and organs, have only been started in the bone. The understanding that A20 protects osteoblasts against apoptosis will be leveraged into potential targets for new therapeutic strategies towards osteoporosis.

At present, the drugs for anti-osteoporotic therapy mainly focus on inhibiting osteoclast function and decreasing bone turnover. Thus the search for drugs that promote differentiation, proliferation and postpone apoptosis of osteoblasts

is still in its infancy. In our study, we have constructed an osteoblast-specific expression vector, which was under the control of mouse osteocalcin promoter, and have confirmed the anti-apoptotic effect of A20 in TNF-alpha-induced apoptosis of osteoblast *in vitro*. Further study is necessary to identify the biological function of A20 *in vivo*. The present study has also established a novel experimental basis for the long-standing goal to exploit and obtain anti-osteoporotic drugs.

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