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Cottonseed Extract Improves the Function of Osteoblastic MC3T3-E1 Cells

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ABSTRACT We have investigated the effects of cottonseed extract on the proliferation, differentiation and lipopolysaccharide (LPS)-induced production of local factors in murine clonal osteoblastic MC3T3-E1 cells. Ethanol extract of cotton seed (4~63 μg/mL) significantly increased the proliferatin of MC3T3-E1 cells ($p<0.05$). Moreover, cottonseed extract (10~50 μg/mL) caused a significant elevation of alkaline phosphatase (ALP) activity and collagen content in the cells. Lipopolysaccharide (LPS) is a potent stimulator of bone resorption in inflammatory diseases. We examined the effect of cottonseed extract on the LPS-induced production of tumor necrosis factor α (TNF-α) and nitric oxide (NO) in MC3T3-E1 cells. Treatment with cottonseed extract (10~50 μg/mL) decreased the 5 μg/mL LPS-induced production of TNF-α and NO in osteoblasts, suggesting that the antiresorptive action of cottonseed extract may be mediated by decrease in these local factors. This study suggests that cottenseed may contribute to antiresorptive action against osteoblastic cells, resulting in a beneficial effect in promoting the function of osteoblastic cells.

KEYWORDS: cottonseed, osteoblastic MC3T3-E1 cells

INTRODUCTION

Bone volume is maintained by two phases of bone remodeling: the first of these is bone resorption by osteoclasts, and the second is bone formation by osteoblasts (Parfitt 1987). An imbalance between bone formation and bone resorption results in metabolic bone diseases. Severe bone loss owing to excessive bone resorption has been noted in inflammatory diseases such as periodontitis and osteomyelitis, as well as some types of arthritis (Nair et al 1996). Lipopolysaccharide (LPS), a major constituent of Gram-negative bacteria, has been proposed to be a potent stimulator of bone loss in these inflammatory diseases (Nair et al 1996). LPS is known to exert direct effects on bone remodeling, and to stimulate the production of other cytokines (e.g., tumor necrosis factor-α, interleukin (IL)-1, and IL-6) by osteoblasts (Gowen 1992). Recent evidence suggests that LPS induces nitric oxide (NO) production in osteoblastic cells. The production of NO by osteoblasts, or by adjoining endothelial cells in the bone marrow, results in an enhancement of osteoclastic bone resorption (Clemens et al 1994). Toll-like receptor 4 (TLR4) was identified as the signal-transducing receptor for LPS (Poltorak et al 1998). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1Rs. Both TLR4 and IL-1Rs employ common signaling molecules such as myeloid differentiation factor and TNFR-associated factor (Kawai et al 1999). Macrophages, lymphocytes, and osteoblast/stromal cells express TLR4, and generate PGE_2 and proinflammatory cytokines, including TNF-α and IL-1 in response to LPS (Schletter et al 1995). These inflammatory factors also stimulate osteoclastogenesis, either directly or indirectly (Nair et al 1996). Plant chemicals are currently heralded as offering potential alternative therapies for age-related bone resorption (Wetli et al 2005). In this study, we have assessed the effects of cottonseed extract on the proliferation, differentiation, and lipopolysaccharide (LPS)-induced production of local factors in murine clonal osteoblastic MC3T3-E1 cells.

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MATERIALS AND METHODS

Extraction

Cottenseed was purchased commercially (Kyungdong pharmaceuticals, Korea) and the extract of cottenseed (CSE) was obtained by the extraction with 70% ethanol (in water, v/v) at room temperature 3 times. The extract was filtered and concentrated in a rotary evaporator at temperature below 50°C and then freeze dried (yield: 6% w/w dry

weight). All reagents were purchased from Sigma (USA), unless otherwise stated.

Cell cultures

MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured at 37°C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GIBCO Gaithersburg, MD, USA). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell growth

The cells were suspended in medium and plated at a density of 7.0×10^3 cells/well into a 96-well culture dish (Costar, Cambridge, MA). After 24 hr, the medium was replaced with phenol red-free media containing 5% charcoal-dextran-treated FBS (CD-FBS) supplemented with CSE. After 2 days of culture, cell proliferation was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble dark blue formazan reaction product. In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well plate was measured photometrically. MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 5 μg/L and sterilized by passage through a 0.22 mm filter. This stock solution was added (one part to 10 parts medium) to each well of culture plate, and the plate was incubated at 37 °C for 2 h. Dimethylsulfoxide (DMSO) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm. Data are expressed as a percentage of control (100×(OD of control-OD of test)/OD of control).

Alkaline phosphatase (ALP) activity

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS. Then, the cells were cultured with CSE in the presence of 10 mM β-glycerophosphate (β-GP); β-GP was added to initiate in vitro mineralization (Kanno et al 2001). After 3 days, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lyzed with 0.2% Triton X-100 and the lysate was centrifuged at 14,000×*g* for 5 min. The clear supernatant was used for the measurement of ALP activity and protein concentration. ALP activity and protein concentration were determined using an ALP activity assay kit (Wako) and a protein assay kit (Pierce, Rockford, IL), respectively. Data are expressed as a percentage of control (100×(OD of control−OD of test)/OD of control).

Collagen contents

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with α-MEM containing 5% CD-FBS. Then, the cells were cultured with CSE in the presence of 10 mM β-GP for 3 days and cellular collagen content was measured using Sircol Collagen Assay kit (Biocolor Ltd., Northern Ireland). This assay is a quantitative dye-binding method designed for the analysis of collagens extracted from mammalian tissues and cells during *in vitro* culture. The dye reagent binds specifically to the $[Gly\text{-}X\text{-}Y]_n$ helical structure found in mammalian collagens (types I to V).

TNF-α immunoassay

After cells were treated with CSE and 5 μg/mL LPS for 48 hr, TNF-α content in the medium was measured with an enzyme immunoassay system (R&D system Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. In brief, cytokine present is bound by immobilized antibody pre-coated onto a microplate. After washing away any unbound substances using wash buffer (0.05% Tween 20 in PBS, pH 7.2~7.4), an enzyme (streptavidin conjugated to horseradish-peroxidase)-linked polyclonal antibody specific for cytokine is added to the well. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) is added to the wells. The enzyme reaction yields a blue product that turns yellow when stop solution is added. The intensity of the color measured is in proportion to the amount of cytokine bound. Data are expressed as a percentage of control (100×(OD of control−OD of test)/OD of control).

Determination of nitrite production

After cells were treated with CSE and 5 μg/mL LPS for 48 hr, nitrite production, an indicator of nitric oxide (NO) synthesis, was measured in the culture supernatant of osteoblasts, as described previously by Kleinerman et al (1987). Briefly, after mixing 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) with 100 μL culture supernatant, optical density at 540 nm was measured by using a microplate reader. Nitrite concentrations were calculated from the standard curve of sodium nitrite prepared in culture medium. Data are expressed as a percentage of control (100×(OD of control−OD of test)/OD of control).

Statistics

The results are expressed as mean±SEM (n=6). Statistical analysis was performed using one-way ANOVA ($p<0.05$). The analysis was performed using SAS statistical software.

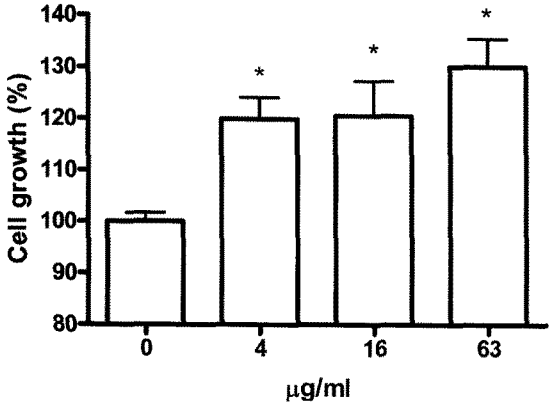

Fig. 1. Effect of CSE on the growth of MC3T3-E1 cells. MC3T3-E1 cells were cultured with α-MEM containing 5% CD-FBS in the presence or absence of CSE for 48 h. Data shown are mean±SEM, expressed as a percentage of control. The control value for MTT assay was 0.247±0.004 OD. *p<0.05 vs. control

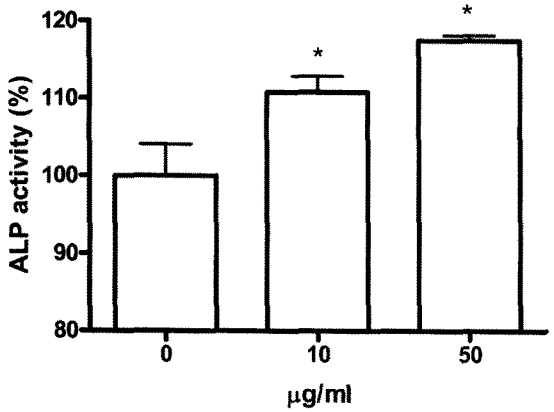

Fig. 2. Effect of CSE on the alkaline phosphatase activity of MC3T3-E1 cells. After the cells reached confluence, the medium was replaced with α-MEM containing 5% CD-FBS in the presence or absence of CSE. Data shown are mean±SEM, expressed as a percentage of control. The control value for ALP activity was 1.88±0.09 Unit/mg protein. *p<0.05 vs. control

RESULTS AND DISCUSSION

The murine clonal osteoblastic MC3T3-E1 cells were isolated via the selection of an alkaline-rich clone from a population of cells maintained under specific sub-culture conditions. This culture system provides a useful model for the quantitative estimation of osteoblast-mediated differentiation *in vitro*. It has been established that osteoblasts generate type I collagen and ALP, which are associated with matrix maturation and mineralization (Stein et al 1990). When the MC3T3-E1 cells were incubated with CSE, MC3T3-E1 cell proliferation was promoted by stimulation with CSE at 4~63 μg/mL (Fig. 1). On the basis of this preliminary observation, we evaluated the differentiation-inducing activities of CSE on MC3T3-E1 cells by assessing the cells for intracellular ALP activity and collagen synthesis. As is shown in Fig. 2

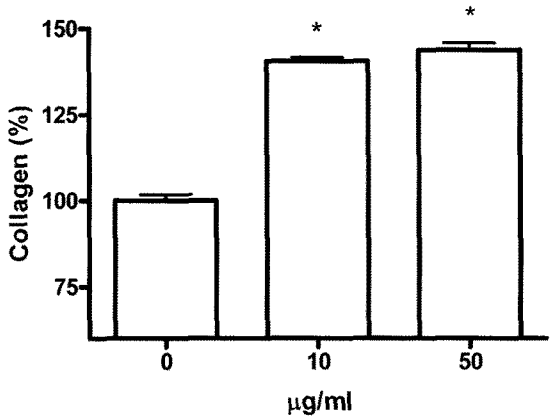

Fig. 3. Effect of CSE on the collagen synthesis of MC3T3-E1 cells. After the cells reached confluence, the medium was replaced with α-MEM containing 5% CD-FBS in the presence or absence of CSE. Data shown are mean±SEM, expressed as a percentage of control. The control value for collagen content was 2.83±0.43 μg per 10^7 cells. *p<0.05 vs. control

and 3, when the cells were cultured in the presence of CSE (10 and 50 μg/mL), we noted a significant increase in the ALP activity and collagen synthesis of the osteoblastic cells (p<0.05), reflecting its anabolic effects. Although the mechanism of action of CSE on osteoblast differentiation remains to be elucidated, the observed increase in ALP activity and collagen synthesis suggests that CSE may act directly on osteoblastic cells in this model.

In order to analyze the inhibitory effect of CSE on bone resorption, we evaluated the effects of CSE on the production of bone resorbing mediators in osteoblasts (Fig. 4). When 5 μg/mL of LPS was added to the cells, the production of TNF-α and NO was significantly increased. However, LPS-induced TNF-α and NO productions were inhibited significantly by CSE treatment (50 μg/mL), which suggests that CSE may modulate the secretion of such mediators by osteoblastic cells to regulate bone resorption.

An important characteristic of the cytokines that regulate bone resorption is their ability to stimulate their own and each other's synthesis in an autocrine and synergistic fashion (Dinarello 1996; Franchimont et al 1997). Owing to the relationships among TNF-α, IL-6, and IL-1β, it is assumed that a small change in TNF-α synthesis after estrogen loss would result in a dramatic increase in the level of all three cytokines. It has been demonstrated that NO performs a crucial function as a paracrine and autocrine mediator of bone cell physiology and in response to diverse stimuli, including cytokine activation, sex hormone deficiency, and mechanical strain. NO production has been detected in primary rat and human osteoblast-like cells and in clonal osteoblast-like cell lines after stimulation with a combination of proinflammatory cytokines (van't Hof and Ralston 2001).

For thousands of years, medicinal plants have been utilized for the treatment of diseases. More recently, interest

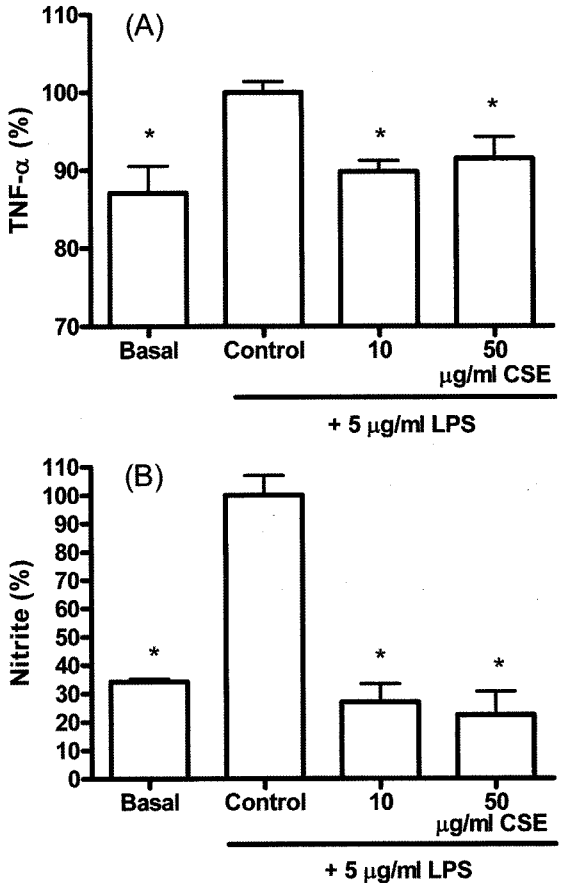

Fig. 4. Effect of CSE on LPS-induced TNF-α and NO production of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or CSE in the presence of 5 μg/mL LPS for 48 h. TNF-α and NO concentration were measured in the conditioned medium. Data shown are mean±SEM, expressed as a percentage of control. Control values of TNF-α and NO production were 7.45±0.17 pg/mL and 47.06±3.27 mM, respectively, per 10^5 cells. Basal means cells without LPS and CSE. *p<0.05 vs. control

in the use of natural products has surged, owing principally to their less severe side effects on the human body, in comparison to synthetic medicinal drugs. Flavonoids are well known for their antioxidant activity, and have been detected in a variety of plants. A large number of flavonoids have been successfully isolated thus far, and have been shown to evidence excellent antioxidant and many other pharmacological activities. Thus far, at least 2000 varieties of flavonoids have been isolated from the plant. Flavonoids that possess antioxidant activity are primarily polyphenolic compounds, and a number of studies have reported on the structure and activity of the flavonoids (Bors et al 2001). The flavonoids contained in cottonseed may contribute to the osteoblast-enhancing activity.

In summary, our results demonstrated that cottonseed extract exerted an accelerating effect on the proliferation and differentiation of bone cells, and inhibited the release of local factors including TNF-α and nitric oxide, both of which are known stimulators of bone resorption. The findings of this study indicate that cottonseed extract may promote the function of osteoblasts and may perform a crucial role in bone remodeling.

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