Effects of (+)-Catechin on the Function of Osteoblastic Cells

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Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are candidate cytokines which are produced by osteoblastic lineage cells and promote osteoblast apoptosis, osteoclastogenesis and bone resorption. Here, we examined the effect of (+)-catechin, one of the most common grape flavonols, on osteoblastic MC3T3-E1 cells. (+)-Catechin caused a significant elevation of cell survival at 10⁻² and 10⁻⁴ M and alkaline phosphatase activity at 10⁻⁵ M. Also, treatment with (+)-catechin (10⁻³ M) decreased bone-resorbing cytokines (TNF-α and IL-6) production and apoptosis in osteoblasts. Our data indicate that the reduction of bone-resorbing cytokines and apoptosis in osteoblasts by (+)-catechin may result in the prevention and therapy for osteoporosis and inflammatory bone diseases.

Key words catechin; osteoblast; apoptosis; tumor necrosis factor-α; interleukin-6

Osteoblasts, typically located on bone-lining surfaces, are physically positioned to significantly influence bone resorption. Bone resorption may be further enhanced in areas of inflammation, notably arthritis and periodontal disease. Osteoblasts and bone marrow stromal cells produce bone-active cytokines such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6), which are critical for osteoclast formation and bone resorption. These cytokines have been suggested to mediate the effects of many stimulators of bone resorption such as parathyroid hormone (PTH) and IL-1. In vitro study demonstrated TNF-α acts on murine osteoblasts and induces apoptosis of these cells. These findings indicate that cytokines could modulate the life span of osteoblasts via apoptosis, thus regulating bone metabolism in certain pathologic conditions such as periarticular osteoporosis found in patients with rheumatoid arthritis. Regulation of apoptosis is considered an important mechanism for controlling the number of monocytes available. IL-6 promotes the recruitment of osteoclast precursors and their subsequent differentiation into mature osteoclasts. In calvaria models, addition of IL-6 induced bone resorption, and increased numbers of active osteoclasts. These effects are enhanced by IL-6 inducing agents, such as IL-1β and TNF-α, and are diminished by treatment with antibody to IL-6. In addition, elevated serum IL-6 levels have been reported in osteoporotic woman and offer a further indication of an association between IL-6 and bone resorption in the adult human population.

Observational studies show that fruits and vegetables protect against chronic diseases. These associations are stronger for red wine consumers, which are potentially related to the high content of polyphenols in this beverage. Several studies have attributed the protective action of red wine to the presence of antioxidant phenolic compounds, especially flavonoids. Flavonoids, secondary plant metabolites with antioxidant activity, are potentially protective compounds. (+)-Catechin is one of the major polyphenolic flavonoids present in red wine, which has been shown to have antioxidant properties in different biological systems. Stoss et al. have reported the evidence for a possibly beneficial influence of (+)-catechin in some cases of osteogenesis imperfecta clinically. Moreover, it has been demonstrated that a pretreatment with (+)-catechin renders embryonic mouse calvaria in culture resistant to the action of bone resorbing agents, either PTH or retinoic acid, and the resorption induced in calvaria by a pretreatment with PTH or retinoic acid is inhibited by a further culture with (+)-catechin. While (+)-catechin clearly has significant skeletal effects, the paracrine mediators of (+)-catechin action on bone are at present unclear and the cellular mechanism by which (+)-catechin exerts an inhibitory effect on bone resorption remains to be elucidated.

In the present study, we examined the effect of (+)-catechin on the viability and apoptosis of osteoblastic MC3T3-E1 cells and assessed cytokine (TNF-α and IL-6) secretion by immunoassay.

MATERIALS AND METHODS

Cell Culture Osteoblastic MC3T3-E1 cells (purchased from RIKEN Cell Bank, Tsukuba, Japan) were cultured in plastic dishes containing alpha-minimum essential medium (α-MEM, Sigma, St. Louis, MO, U.S.A.) plus 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, U.S.A.) in a CO₂ incubator (5% CO₂ in air) at 37 °C and subcultured every 3 d to become nearly confluent. Then cells were transferred to fresh medium containing 0.3 g/l bovine serum albumin (BSA, Sigma, St. Louis, MO, U.S.A.) with (+)-catechin (Sigma, St. Louis, MO, U.S.A.) dissolved in dimethylsulfoxide (DMSO) and incubated for 48 h (final DMSO concentration ≤0.05% (v/v)). For experiments evaluating the effects of (+)-catechin on cytokine-stimulated production of IL-6 and apoptosis, the medium was replaced with fresh medium containing (+)-catechin with or without 10⁻¹⁰ M TNF-α (Roche molecular biochemicals, Germany), and the incubation was continued for further 48 h. The conditioned medium was collected and centrifuged at 4000 × g for 4 min at 4 °C to remove cell debris.

Cell Viability Assay (MTT Assay) Mitochondrial function was assayed by the ability of viable cells to convert soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MO, U.S.A.) into an insoluble dark blue formazan reaction product. In the bulk cell photo-

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metric MTT assay, the bulk conversion of MTT in the well of a 24-well plate was measured photometrically. MTT was dissolved in DPBS at a concentration of 5 g/l and sterilized by passage through a 0.22 μm 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 4 h. 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.

Alkaline Phosphatase (ALP) Activity The cells were treated with (+)-catechin (10^{-5} M) in 6-well plate. After washing twice with DPBS, the cells were scraped into a 1 ml of 10^{-2} M Tris–HCl (pH 7.6) buffer containing 1 g/l Triton-X on ice, and centrifuged. An aliquot of the supernatant was used for the determination of ALP activity by measuring the release of p-nitrophenol from p-nitrophenylphosphate.

Analysis of Apoptosis in Cultured Cells Cells were seeded at a density of 2×10^4 cells in 24-well culture plate, cultured for 2 d to become nearly confluent. Then cells were cultured with (+)-catechin (10^{-3} M) in the presence and absence of 10^{-10} M TNF-α for 48 h. For quantitation of apoptosis, the cell death detection kit (Roche molecular biochemicals, Germany) was used according to the manufacturer’s instruction. After the incubation, the cells were lysed and intact nuclei were pelleted by centrifugation. An aliquot of the supernatant was transferred to a streptavidin-coated well of a microtiter plate. Subsequently, a mixture of anti-histone-biotin and anti-DNA-peroxidase was added and incubated for 2 h. During the incubation period, the anti-histone antibody bound to the histone component of the nucleosomes and simultaneously fixed the immunocomplex to the streptavidin-coated microplate via its biotinylated. Additionally, the anti-DNA-peroxidase antibody reacted with the DNA component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically with substrate.

Cytokine Immunoassays The cytokines (TNF-α and IL-6) in the medium was measured with a cytokine enzyme immunoassay system (R&D system Inc., Minneapolis, MN, U.S.A.).

Statistics All experiments were performed three or four times and the results are expressed as mean±S.E.M. (n=6). Differences between the means were calculated using one-way analysis of variance (ANOVA). The significant level was set at p<0.05.

RESULTS

When osteoblastic MC3T3-E1 cells were treated with (+)-catechin (10^{-10}—10^{-4} M), cell viability was increased dose-dependently up to 143% of basal value at 10^{-4} M (Fig. 1) and the 10^{-5} M (+)-catechin significantly increased the ALP activity of MC3T3-E1 cells (Fig. 2). We assessed the conditioned medium from MC3T3-E1 cell cultures for TNF-α levels using ELISA (Fig. 3). Constitutive TNF-α levels were 43.56 pg/10^4 cells in the conditioned medium, and dramatically decreased by 32% of control value following treatment with (+)-catechin (10^{-3} M). We quantitated the apoptosis induced by serum removal, or TNF-α (10^{-10} M) using one-step sandwich immunoassay (Fig. 4). When MC3T3-E1 cells were treated with TNF-α (10^{-10} M), apoptosis was increased 127% significantly compared with the basal state. Addition of 10^{-5} M (+)-catechin to MC3T3-E1 cells had no significant effect on apoptosis in the basal state but significantly inhibited TNF-α induced apoptosis by 33%. The secretion of IL-6 in osteoblastic MC3T3-E1 cells was quantitated by a specific ELISA (Fig. 5). MC3T3-E1 cells synthesized IL-6 constitutively, but at very low levels. However, when 10^{-10} M TNF-α was added in cells, production of IL-6 was increased dramat-
Nutritional and pharmacological factors are needed to prevent bone loss with aging. Catechins, one of the six classes of flavonoids, are found particularly in fruits, but black chocolate contains the highest levels of catechins. Catechins are absorbed into the bloodstream, exhibit antioxidant and free radical scavenging activity, and act as antihepatic, antiviral, and anticarcinogenic agents.\textsuperscript{10} Free radical scavenging activity, and act as antihepatic, antiviral, and anticarcinogenic agents.\textsuperscript{10}

Chocolate contains the highest levels of catechins. Catechins are believed to play a role in mineralization. Therefore, we examined the effect of (+)-catechin on bone metabolism, in this study we employed cell culture systems. ALP is the most widely recognized biochemical marker for osteoblastic activity. Although its precise mechanism of action is poorly understood, this enzyme is believed to play a role in mineralization. Therefore, we examined the effect of (+)-catechin on the survival and ALP activity of osteoblastic MC3T3-E1 cells. The present study demonstrates that (+)-catechin can increase survival and ALP activity in osteoblastic MC3T3-E1 cells \textit{in vitro}, indicating its anabolic effect. Although Yamaguchi and Ma\textsuperscript{11} reported that catechin had no effect on ALP activity in the femoral-diaphyseal and -metaphyseal tissues, our findings support the view that (+)-catechin can stimulate osteoblastic bone formation. Also, our observation is in agreement with the finding of a previous study suggesting that resveratrol, which is a phenolic compound found in the berry skins of most grape cultivars, directly stimulates cell proliferation and differentiation of osteoblasts.\textsuperscript{12} (+)-Catechin has been shown to stabilize collagen molecules, rendering them less soluble and susceptible to degradation by collagenases.\textsuperscript{13} It is known from \textit{in vitro} experiments to increase the resistance of collagen to the action of collagenase, an effect that is apparently not due to intact catechin but to some of its oxidation products.\textsuperscript{13} It may thus be speculated that in bone tissue, (+)-catechin or its derivatives may gain access and bind to the thin layer of unmineralized collagen that separates resting osteoblasts from the mineralized matrix and prevent its removal by osteoblast-secreted collagenase. This could be sufficient to inhibit the whole process of bone resorption by preventing osteoclast activation. Thus, Delaisse \textit{et al.}\textsuperscript{9} suggested that (+)-catechin, by acting upon bone collagen, could well render bone tissue less prone to resorption.

It is known that some bone-resorbing agents like TNF-\(\alpha\) act on osteoblasts and stimulate apoptosis in osteoblasts.\textsuperscript{14} Our findings from these \textit{in vitro} studies indicate that (+)-catechin (10\(^{-5}\) M) inhibited apoptotic cell death, suggesting that (+)-catechin seems to promote survival by inhibiting apoptosis. In bone cells, inflammatory cytokine TNF-\(\alpha\) has been shown to play an important role in the local control of bone remodeling. TNF-\(\alpha\) inhibits bone formation and has been found to inhibit collagen synthesis and ALP activity in osteoblasts.\textsuperscript{9} Catechin-induced inhibition of apoptotic cells may be resulted from the decrease in production of TNF-\(\alpha\) and IL-6 in the cells as shown in Figs. 3 and 5. Sato \textit{et al.}\textsuperscript{15} demonstrated that when MC3T3-E1 cells were treated with a low dose (1 ng/ml) of TNF-\(\alpha\), ALP activity was decreased, but cell growth was slightly affected. Hill \textit{et al.}\textsuperscript{10} reported that TNF-\(\alpha\) dose-dependently (10\(^{-14}\)—10\(^{-9}\) M) decreased the survival of primary mouse osteoblast and induced osteoblast apoptosis. This suggests that factors exerting a catabolic action on osteoblasts may also induce apoptosis while, conversely, agents with an anabolic action may promote survival. In the present study, apoptosis was increased when MC3T3-E1 cells were treated with TNF-\(\alpha\) (10\(^{-10}\) M) whereas apoptosis significantly decreased when cells were treated with (+)-catechin (10\(^{-5}\) M). This finding suggests that (+)-catechin may reduce bone loss induced by the inflammatory cytokine TNF-\(\alpha\), in conditions such as rheumatoid arthritis, tumor osteolysis, and periodontal disease. Meanwhile, epigallocatechin-3-gallate (EGCG) which is contained with a large amount in tea leaves as compared to (+)-catechin, was reported to produce apoptosis of human chondrosarcoma cells.\textsuperscript{17} Presumably, the mechanism by which (+)-catechin acts on osteoblast is different from that of EGCG in cancer cells.

TNF-\(\alpha\) has been reported to stimulate IL-6 production and its secretion through sphingosine 1-phosphate following activation of protein kinase C in cultured osteoblasts.\textsuperscript{10} The pre-
sent study demonstrates that (+)-catechin inhibit the secretion of TNF-α and IL-6 in osteoblastic MC3T3-E1 cells, but inhibition of IL-6 by catechin was smaller than that of TNF-α. From these results, it may be speculated that the decreased TNF-α and IL-6 production caused by (+)-catechin in osteoblasts results in increased bone mass via modulation of bone resorption as well as apoptosis in osteoblasts.

In conclusion, (+)-catechin has a direct stimulatory effect on osteoblast growth and inhibits both TNF-α-induced apoptosis and inflammatory cytokines production in cultured osteoblastic MC3T3-E1 cells in vitro. These data support the hypothesis that part of antiresorptive action of (+)-catechin is mediated by decreased bone-resorbing cytokine production by osteoblasts. Our data also indicate that the reduction of TNF-α and apoptosis in osteoblasts by (+)-catechin results in the prevention and therapy for osteoporosis and inflammatory bone diseases.

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