Kaempferol Attenuates 2-Deoxy-d-ribose-Induced Oxidative Cell Damage in MC3T3-E1 Osteoblastic Cells

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Reducing sugar, 2-deoxy-d-ribose (dRib), produces reactive oxygen species through autoxidation and protein glycosylation and causes osteoblast dysfunction. Kaempferol, a natural flavonoid, was investigated to determine whether it could influence dRib-induced cellular dysfunction and oxidative cell damage in the MC3T3-E1 mouse osteoblastic cell line. Osteoblastic cells were treated with 30 mM dRib in the presence or absence of kaempferol (10−7—10−5 M) and markers of osteoblast function and lipid peroxidation were subsequently examined. Kaempferol (10−5—10−3 M) significantly inhibited the dRib-induced decrease in growth of MC3T3-E1 osteoblastic cells. In addition, treatment with kaempferol resulted in a significant elevation of alkaline phosphatase (ALP) activity, collagen content, and mineralization in the cells. Treatment with kaempferol increased osteoprotegerin (OPG) secretion and decreased malondialdehyde (MDA) contents of MC3T3-E1 osteoblastic cells in the presence of 30 mM dRib. Taken together, these results suggest that kaempferol inhibits dRib-induced osteoblastic cell damage and may be useful for the treatment of diabetes-related bone disease.

Key words kaempferol; osteoblast; MC3T3-E1 cell; oxidative stress; 2-deoxy-d-ribose

Oxidative stress is known to be the major contributing factor for diabetes. The chronic hyperglycemia leads to oxidative stress which is involved in the progression of beta cell deterioration as well as in the development of diabetic complications.1) Osteopenia is also a diabetic complication2) and it causes an increase in bone fracture and a delay in healing of fracture, and affects the quality of life.3) In vitro studies have shown that oxidative stress inhibits osteoblastic differentiation4) and induces osteoblast insults and apoptosis.5) One of the mechanisms of diabetes-related bone disease may be the direct effects on osteoblasts and bone turnover. An imbalance between bone-forming osteoblasts and bone-resorbing osteoclasts leads to the pathogenesis and etiology of certain bone metabolic diseases, including osteoporosis and osteopetrosis.6)

Sugars that contain aldehyde groups that are oxidized to carboxylic acids are classified as reducing sugars, and they produce reactive oxygen species (ROS) through autoxidation and protein glycosylation.7,8) Glucose is the least reactive of the reducing sugars.9) Accordingly, long term exposure to high glucose concentrations is necessary before toxic effects are expressed by cells. 2-Deoxy-d-ribose (dRib) is a reducing sugar with high reactivity with protein.10) Therefore, we chose a dRib as a surrogate for glucose to induce oxidative damage of osteoblasts in vitro. Recent studies have demonstrated that dRib inhibits cellular differentiation and increases oxidative injury in osteoblastic cells.11,12)

Kaempferol is a natural flavonoid that has been independently isolated from Delphinium, Witch-hazel, grapefruit, and other plant sources. In addition, many glycosides of kaempferol, such as kaempferitin and astragalin, have been isolated as natural products from plants. Wattel et al.13) demonstrated that flavonols such as kaempferol decrease osteoclastic bone resorption in vitro by targeting directly mature osteoclasts via a mechanism involving, at least in part, the estrogen receptor (ER). Miyake et al.14) reported a positive effect of kaempferol on the differentiation and mineralization of a murine pre-osteoblastic cell line. Prouillet et al.15) showed that the kaempferol increases the alkaline phosphatase (ALP) activity, and that this effect is dependent upon both the extracellular regulated kinase (ERK) pathway and estrogen receptor (ER) activation.

To investigate whether kaempferol could reduce dRib-induced osteoblast dysfunction and oxidative cell damage, we examined the effects of kaempferol on alkaline phosphatase activity, collagen, mineralization, osteoprotegerin, and lipid peroxidation in osteoblastic MC3T3-E1 cells.

MATERIALS AND METHODS

Cell Culture and Materials Murine osteoblastic MC3T3-E1 cells were cultured at 37 ℃ in a 5% CO2 atmosphere with α-modified minimal essential medium (α-MEM; Gibco BRL, Grand Island, NY, U.S.A.). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. When cells reached confluence, they were harvested using a 0.05% trypsin–0.02% EDTA solution. The cells were then seeded in 6 well plate, grown to 90% confluence, and treated with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate in vitro mineralization.16) Cell culture medium was changed every 2—3 d. After 6 d or 14 d, respectively for the measurement of cell viability, alkaline phosphatase activity, and collagen content, or calcium deposition and osteoprotegerin secretion, the cells were cultured with medium containing dRib and/or kaempferol for 2 d. Kaempferol was purchased from Wako Pure Chemicals (Osaka, Japan). Cell culture reagents and most other biochemical reagents were purchased from Sigma Chemical Co. (MO, U.S.A.) unless otherwise specified.

MTT Assay Cell viability was determined using a col-
orimetric assay based on the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) by viable cells. In brief, a solution of MTT (0.5 mg/ml) was added to the cells and incubated at 37 °C to allow cleavage of the tetrazolium ring by mitochondrial dehydrogenases, resulting in the formation of blue formazan crystals. After 3 h, the residual MTT was carefully removed and the crystals were dissolved by incubation with DMSO for 30 min. The plates were then shaken for 5 min and the absorbance at 570 nm was measured by spectrophotometry.

Alkaline Phosphatase Activity At the time of cell harvesting, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were then lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14000×g for 5 min. The cleared 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 (Somang Co., Korea) and a Bradford (Bio-Rad, Hercules, CA, U.S.A.) assay kit, respectively.

Collagen Contents Cellular collagen content was measured using a 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 specifically binds to the [Gly-X-Y] helical structure found in mammalian collagens (types I to V).

Mineralization (Calcium Deposition Assay) Staining with Alizarin Red S is a standard method used to visualize the nodular pattern and calcium deposition of osteoblast cultures in vitro. At harvest, the cells were fixed with 70% ethanol for 1 h and stained with 40 mM Alizarin Red S (pH 4.2, Sigma) for 10 min with gentle shaking. Alizarin Red S stains calcium dark red and any surrounding tissue yellow. To quantify the bound dye, the stain was solubilized with 1 ml 10% (w/v) cetylpyridinium chloride for 15 min with shaking and shielded from light. The absorbance of the solubilized stain was subsequently measured at 561 nm.

Measurement of Osteoprotegerin Osteoprotegerin (OPG) acts as a soluble secreted receptor for receptor activator of NF-κB ligand (RANKL) that prevents it from binding to and activating RANK (osteoclast differentiation and activation receptor) on the osteoclast surface, thus inhibiting osteoclast development. The abundance of OPG in culture medium was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) assay kit (Biomedical Technologies Inc., U.S.A.).

Intracellular Lipid Peroxidation (Malondialdehyde Assay) The binding of thiobarbituric acid to malondialdehyde-bis-(dimethylacetal) 1,1,3,3-tetramethoxypropan (MDA) formed during lipid peroxidation results in a chromogenic complex. The OXItek Thiobarbituric acid reactive substances (TBARS) Assay kit (ZeptoMetrix Co., New York, U.S.A.) was used to determine the degree of lipid peroxidation, which increases as a result of oxidative stress. In this study, an MDA standard was used to construct a standard curve.

Statistical Analysis All results are expressed as a mean±S.E.M. (n=6). Statistical analysis was performed using one-way ANOVA, and a p value <0.05 was used as a threshold for statistical significant. Statistical analysis was performed using SAS statistical software (SAS Inc., NC, U.S.A.).

RESULTS AND DISCUSSION

In the present study we investigated the effects of kaempferol on dRib-induced osteoblastic dysfunction and oxidative damage using a mouse osteoblastic MC3T3-E1 cell culture model. Confluent MC3T3-E1 cells were cultured with differentiation-inducing medium and subsequently incubated in medium containing kaempferol (10⁻⁸—10⁻⁵ M) in the presence or absence of 30 mM dRib for 2 d. As shown in Fig. 1, no toxic effects were observed after incubation with kaempferol (10⁻⁸—10⁻⁵ M) in untreated cells. This result indicated that kaempferol alone was not toxic at the concentrations used in this study. When the cells were treated with kaempferol in the presence of 30 mM dRib, kaempferol increased the survival of MC3T3-E1 cells compared to the dRib-treated control, suggesting that kaempferol suppresses dRib-induced cytotoxicity. The anti-oxidant N-acetyl-cysteine (NAC) was used to investigate the mechanism of dRib-induced cell damage. Pretreatment with 5 mM NAC almost reversed the dRib-induced cytotoxicity. These findings suggested that dRib-induced cytotoxicity was most likely due to oxidative stress-induced effects. To exclude the possibility that dRib-induced cell damage was caused by increased osmolarity, we tested the effect of 30 mM mannitol on cell viability. There was no significant inhibitory effect on cell viability of MC3T3-E1 cells (data not shown).

To investigate osteoblast function, ALP activity, collagen content, and calcium deposition were determined. Compared with control cells, the presence of dRib significantly decreased cellular ALP activity, collagen content, and nodule mineralization (Figs. 2—4). Bone ALP is a glycoprotein localized in the plasma membrane of osteoblastic cells and a useful marker of bone formation. Alterations in this activity have been observed in osteoporosis and other metabolic bone diseases. High levels of ALP activity are shown in both pre-osteoblasts and osteoblasts in vivo and in differentiating osteoblasts in vitro. In addition, connective tissue cells expressing high levels of ALP can be induced to deposit mineral in vitro. Osteoblast cells play a major role in bone formation and produce type 1 collagen, which is the most abundant pro-
tein in the bone matrix, serves an early marker of osteoblast differentiation, and is the major organic component of mineralized bone matrix. The present study demonstrated that the reducing sugar dRib had a profound inhibitory effect on osteoblastic differentiation; however, when osteoblasts were treated with kaempferol (10^{-9}–10^{-7} M) in the presence of 30 mM dRib, significant increases in ALP activity and collagen content were observed. Moreover, kaempferol (10^{-9}–10^{-7} M) supplementation in the culture medium significantly increased nodules mineralization compared with a dRib-treated control.

OPG, a member of the tumor necrosis factor receptor (TNF-R) superfamily, is produced by mature and immature osteoblastic cells and is an essential inhibitor of osteoclast differentiation, activity, and survival. OPG acts as a decoy receptor by blocking the interaction of RANKL, necessary for osteoclast formation and function, with its functional receptor RANK, thereby inhibiting osteoclastogenesis. Indeed, the increase in osteoclast numbers as well as the associated development of bone disease, osteolytic lesions, and decrease in bone volume can be prevented by treatment with recombinant OPG, demonstrating its importance in vivo. In order to determine the regulator of osteoclast differentiation in osteoblasts, we examined the production of OPG in the presence of dRib and kaempferol (Fig. 5). Kaempferol (10^{-9}–10^{-7} M) stimulated the production of OPG in presence of dRib, which might have affected the induction of osteoclast differentiation and function by affecting the production of OPG in the osteoblastic cells. However, no alteration was detected in the amount of osteoprotegerin released into the culture medium, using either control or dRib treatment. It cannot be excluded the possibility that the peak osteoprotegerin expression was missed, or the osteoprotegerin concentration detected in the culture medium reflects the total amounts released from the cultures.

Reducing sugars induce lipid peroxidation in membranes by producing ROS. The oxidative stress caused by dRib in osteoblast-like cells was evaluated by measuring production of malondialdehyde (MDA), an abundant aldehyde that forms as a result from lipid peroxidation, which we used as an indicator for generation of ROS. In this study, after 2 d of incubation with 30 mM dRib, the MDA level was greatly increased, which again was suppressed when 10^{-9}–10^{-7} M kaempferol was added to the media. Also, NAC prevented dRib-induced MDA production. This result indicates that kaempferol can act as an antioxidant and protect osteoblastic cells from oxidative cell damage, which may promote bone recovery under pathologic conditions. The addition of antioxidants reverses the negative effects exerted by high glucose on extracellular matrix proteins and cell growth. These studies are supported by our previous report that NAC, a potent antioxidant, protects HIT-T15 pancreatic beta cells against oxidative cell damage caused by...
dRib.10) Furthermore, recent studies demonstrated that the flavonoids, hesperetin and myricetin prevented dRib-induced dysfunction and oxidative damage in osteoblastic cells.11,12) In summary, the data presented here suggest that kaempferol inhibits dRib-induced dysfunction of osteoblastic cells and may exert its anti-resorptive effects on bone, at least in part, by stimulating OPG expression in osteoblastic cells. Moreover, kaempferol can act as a biological antioxidant in an experimental cell culture system, which may promote bone recovery in diabetes-related bone diseases.

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Fig. 6. Effect of Kaempferol on Lipid Peroxidation in MC3T3-E1 Cells in the Presence of dRib
MC3T3-E1 cells were cultured with an increasing concentration of kaempferol and 5 mM NAC in the presence or absence of 30 mM dRib. The data are expressed as the mean±S.E.M. of five independent determinations. * Significantly different from control (p<0.05).