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Screening of flavonoids for effective osteoclastogenesis suppression

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ABSTRACT

Flavonoids are natural compounds derived from plants and some of them have been shown to inhibit osteoclast formation, implicating their potential use for the treatment of osteoporosis. Conventionally, the screening of antiosteoclastic agents is a tedious process that requires visual counting of the number of osteoclasts produced. The purpose of this study was to establish an easier and faster method for screening the antiosteoclastogenic flavonoids by using an enzyme assay. Tartrate-resistant acid phosphatase (TRAP) is a marker enzyme of the osteoclast. Results obtained demonstrated that cellular TRAP activity tended to correlate with the number of osteoclasts formed. However, the secreted TRAP activity was actually responsible for the resorption activities of the functional osteoclasts. Consequently, the effectiveness of antiosteoclastogenic agents was screened for by assessing their inhibition on receptor activator of NF-κB ligand (RANKL)-induced TRAP secretion. The half-inhibitory concentrations of flavonoids on TRAP secretion were employed as indices to compare the effectiveness of various flavonoids. The effective flavonoids also exhibited similar inhibitory potencies in the pit-formation analysis. This protocol provides a rapid analysis to screen for effective antiosteoclastogenic agents.

Osteoporosis is a major skeletal disorder during aging, especially during the postmenopausal life of women. The pathogenesis of osteoporosis is due to the imbalance of bone remodeling—the increase in bone resorption and the decrease in bone formation. Estrogen deficiency is one of the major factors causing postmenopausal osteoporosis [1]. However, hormone replacement therapy might be a risk factor for breast and ovarian cancers for a certain population of women [2,3]. Consequently, the screening of phytoestrogen and/or estrogen receptor modulatory candidates is anticipated in novel drug discovery for the management of postmenopausal osteoporosis [4,5].

Flavonoids are naturally occurring compounds found in all vascular plants and have potential biological activities such as antioxidant, antimicrobial, anti-inflammatory, etc. [6–10]. Soy isoflavones such as genistein and daidzein, for example, are reported to inhibit osteoclast formation and reduce bone loss [11]. This antiosteoclastogenic activity is possibly due to the weak estrogenic activities of their metabolites. Flavonoids with weak estrogenic activities are called phytoestrogens [10]. To date, over 4000 naturally occurring flavonoids have been identified [6]. The compound diversity of flavonoids provides a chemical library to be screened for candidates for antiosteoclastogenic drugs.

Osteoclasts are the key participant in bone resorption. They are a class of specialized multinucleated cells formed by the fusion of monocyte/macrophage progenitors. Cytokines, including macrophage colony-stimulating factor (M-CSF)[2] and receptor activator of nuclear factor κB ligand (RANKL), essentially stimulate the formation of osteoclasts, termed osteoclastogenesis. M-CSF is required for macrophage maturation, whereas RANKL is the key differentiating factor for osteoclastogenesis [12,13]. The maturation of osteoclasts includes the expression of tartrate-resistant acid phosphatase (TRAP; EC 3.1.3.2) activity in the fused polykaryon cells. There are two forms of TRAP: TRAP 5a and TRAP 5b. Both isoforms are found in osteoclasts, macrophages, and dendritic cells; however, TRAP 5b is secreted only by osteoclasts, while TRAP 5a is secreted by macrophages and dendritic cells [14]. This leads to the use of TRAP 5b as a bone resorption marker and TRAP 5a as an inflammatory marker. In clinical practice, the measurement of TRAP 5b levels in patients' sera has been used to predict the turnover rate of their bone

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[2] *Abbreviations used:* TRAP, tartrate-resistant acid phosphatase; cTRAP, cellular TRAP; sTRAP, secretory TRAP; PBS, phosphate-buffered saline; NP, naphthol AS-MX phosphate disodium salt; FR, fast red violet LB salt; pNPP, 4-nitrophenyl phosphate disodium salt hexahydrate; RANKL, receptor activator of nuclear factor κB ligand; M-CSF, macrophage colony-stimulating factor.

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[15,16]. In the in vitro culture of osteoclasts, TRAP activity might be detected in the cells as well as in the cell culture medium [17–20]. In this study, the secretory TRAP is named "sTRAP" and the cellular TRAP named "cTRAP."

Calculating the multinuclear TRAP-positive cells ($\geqslant$3 nuclei) has been the conventional method of evaluating the formation of osteoclasts [21]. However, these methods are time-consuming and thus not suitable for drug screening. To facilitate the analysis, some investigators analyzed TRAP activities to predict osteoclastogenesis. Both cTRAP and sTRAP were analyzed either biochemically or immunochemically by different investigators [18,20]. However, the corelationship between TRAP activities (both cTRAP and sTRAP) and the number of osteoclasts or the resorption activities had not been examined or compared in detail. Further, the use of a TRAP activity assay for screening of antiosteoclastogenic agents also had never been performed. This study evaluated these parameters and developed a protocol to screen for antiosteoclastogenic agents by measuring their sTRAP activities. The half-inhibitory concentrations of flavonoids on TRAP secretion (sTRAP IC_{50}) of each flavonoid was calculated based on its potency to inhibit 50% of osteoclastogenesis, the control sTRAP activity. This method may rapidly and accurately evaluate the efficacy of antiosteoclastic agents/drugs and facilitate the mass screening for antiosteoclastic agents/drugs in the pharmaceutical industry.

Materials and methods

Materials

Six- to eight-week-old female C57BL/6JNarl mice were purchased from the National Laboratory Animal Center (National Applied Research Laboratories, Taipei, Taiwan). The Animal Care and Use Committee of Taipei Medical University (TMU; No. LAC-96-0041) approved all animal care and experimental procedures. RAW 264.7 cells were from the Biosource Collection and Research Center (Hsinchu, Taiwan). Recombinant murine M-CSF and soluble RANKL were purchased from PeproTech (Rocky Hill, NJ, USA). Flavonoids were gifts from Professor Y.C. Chen (Graduate Institute of Medical Sciences, TMU, purchased from INDOFINE Chemical Co., Hillsborough, NJ, USA). The flavonoids used in this study include F1, kaempferol; F2, morin; F3, baicalein; F4, quercetin; F5, flavone; F6, 5-methoxyflavone; F7, 6-hydroxyflavone; F8, 7-hydroxyflavone; F9, hesperetin; F10, naringenin; F11, taxifolin; F12, naringin; F13, hesperidin; F14, rutin; F15, myricetin; F16, baicalin; F17, genistein; F18, daidzein; and F19, 7-methoxyflavone. Horse serum, fetal bovine serum (FBS), α-MEM, antibiotics, and Alamar blue reagent were from Invitrogen (Camarillo, CA, USA). Dextran-coated charcoal, trypsin–EDTA solution (0.25% (w/v) trypsin and 0.1% (w/v) EDTA), phosphate-buffered saline (PBS), naphthol AS-MX phosphate disodium salt (NP), fast red violet LB salt (FR), sodium tartrate dibasic dihydrate, 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP), and other analytical grade chemicals were from Sigma–Aldrich (St. Louis, MO, USA). Charcoal-stripped FBS (CS-FBS) was prepared according to the manufacturer's instructions (Sigma–Aldrich).

Culture of MBMMφ and RAW 264.7 cells

The isolation and cultivation of mouse MBMMφ cells were based on methods of Takahashi et al. [21], Weischenfeldt et al. [22], and Wang et al. [23] with modification. In brief, 6- to 8-week-old female C57BL/6J mice were sacrificed by cervical dislocation, and the tibias and femurs were aseptically removed. Bone marrow cells were obtained by flushing the tibias and femurs with α-MEM containing 2× antibiotics. The suspended bone marrow cells were pelleted by centrifugation at 800*g* for 5 min and then resuspended in α-MEM containing 10% (v/v) heat-inactivated horse serum and 2× antibiotics. The cells were resuspended by repeated pipetting, passed through a 70-μm cell strainer to make a single-cell suspension, and then supplemented with 100 ng/ml M-CSF. Bone marrow cells obtained from about two mice were seeded in a 15-cm dish. The cells were cultured at 37 °C in a humidified incubator with 5% (v/v) CO_2 for 5 days to reach confluence. To purify MBMMφ cells, nonadherent cells were removed by washing with PBS. The adherent cells were collected using a two-step detachment protocol described by Wang et al. [23]. The cells were first treated with trypsin–EDTA solution for 30 min to remove nonmacrophage cells such as fibroblasts from the adherent layer without detaching the mature macrophages. The remaining attached cells were treated with trypsin–EDTA solution again for another 30 min to loosen the attachment and then detached with a cell scraper and collected as MBMMφ cells. For the long-term storage of MBMMφ cells, the detached cells were washed, pelleted, resuspended in freezing medium (consisting of 90% (v/v) FBS and 10% (v/v) dimethyl sulfoxide), and cryopreserved at −150 °C. The viability of thawed frozen cells was about 80%. Unless otherwise stated, the recovered MBMMφ cells were cultured in α-MEM with 10% (v/v) heat-inactivated CS-FBS, 10 ng/ml M-CSF, and antibiotics. RAW 264.7 cells were cultured in the same medium without M-CSF. Both MBMMφ and RAW 264.7 cells exhibited the positive surface markers Mac-1/CD11b, MOMA-2, and F4/80 by flow cytometric analysis or immunocytochemistry (data not shown).

Differentiation, characterization, and quantitation of osteoclasts

For osteoclastogenesis, the thawed MBMMφ or RAW 264.7 cells were seeded in 96-well plates at a density of 2000 cells/well in sextuplicate and cultured at 37 °C in a humidified incubator with 5% (v/v) CO_2 for 5 or 2 days, respectively. The culture medium for MBMMφ cells was the basic α-MEM (α-MEM–10% (v/v) heat-inactivated CS-FBS–antibiotics) supplemented with 10 ng/ml M-CSF, while RAW 264.7 cells were grown in the basic α-MEM only. The medium was then replaced with the differentiation medium made of the basic α-MEM with 10 ng/ml M-CSF and 10 ng/ml RANKL, for MBMMφ cells, or 10 ng/ml RANKL only for RAW 264.7 cells. The cells were allowed to differentiate for another 3–4 days, and the formation of osteoclasts was characterized by TRAP staining according to Technical Bulletin 445 (BD BioSciences, Bedford, MA, USA), with modification. Briefly, the cells were fixed with 4% (w/v) formaldehyde at room temperature for 15 min, rinsed with normal saline, and stained with the TRAP staining buffer (97 mM sodium acetate, pH 4.5, 48.5 mM sodium tartrate, 0.1% (v/v) Triton X-100, 0.1 mg/ml NP, 0.3 mg/ml FR) at 37 °C for 5–10 min. After being washed twice with normal saline, the stained cells were evaluated microscopically. The TRAP-positive multinucleated cells (nuclei $\geqslant$3) were counted as osteoclasts.

The measurement of cTRAP and sTRAP activity in osteoclasts

For cTRAP activity assay, the differentiated osteoclasts described above were fixed with 4% (w/v) formaldehyde at room temperature for 15 min. After the cells were washed with normal saline, the assay was initiated by adding 50 μl per well of cTRAP assay buffer (0.1 M sodium acetate, pH 4.5, 30 mM sodium tartrate, 0.1% (v/v) Triton X-100, 2.5 mg/ml pNPP) at 37 °C for 7 min. At the end of incubation, the plate was placed on ice and immediately the reacted cTRAP assay buffer was transferred into a new plate prefilled with 50 μl of 0.5 N NaOH per well. The absorbance of the final mixture at 405 nm was measured in a microplate reader (Synergy H1 m Hybrid Multi-Mode microplate reader; BioTek Instruments, Winooski, VT, USA), as cTRAP activity.