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Inhibitory effects of mevastatin and a geranylgeranyl transferase I inhibitor (GGTI-2166) on mononuclear osteoclast formation induced by receptor activator of NFκB ligand (RANKL) or tumor necrosis factor-α (TNF-α)

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Abstract

We have previously reported that the statin mevastatin (compactin) reversibly inhibits the fusion of TRAP-positive mononuclear preosteoclasts (pOCs) into multinucleated osteoclasts and disrupts the actin ring in mature osteoclasts through the inhibition of protein prenylation. Protein geranylgeranylation, specifically, is known to be required for pOC fusion and for the function and survival of mature osteoclasts. However, it has not been determined whether protein geranylgeranylation is involved in early differentiation of osteoclasts (pOC formation). The current study shows that statins and the geranylgeranyl transferase I inhibitor GGTI-2166 inhibit the pOC formation induced by RANKL or TNF-α in cultures of both mouse marrow-derived macrophage-colony-stimulating factor (M-CSF) dependent monocytes (MD cells) and the mouse monocyte cell line RAW 264.7 (RAW cells). Mevastatin, 0.1–0.6 μM, inhibited the formation of pOCs induced by receptor activator of nuclear factor-κB ligand (RANKL) or tumor necrosis factor (TNF-α) in both cell cultures. The inhibitory effects of mevastatin were overcome by the addition of mevalonate, farnesyl pyrophosphate or geranylgeranyl pyrophosphate. GGTI-2166 inhibited TRAP activity induced by RANKL or TNF-α in both cell cultures and prevented the incorporation of [^{3}H]all-*trans* geranylgeraniol into prenylated proteins in RAW cells. However, the farnesyl transferase inhibitor FTI-2153 did not inhibit TRAP activity although FTI prevented the incorporation of [^{14}C]mevalonate into farnesylated proteins in RAW cells. *Clostridium difficile* cytotoxin B (toxin B) inhibited pOC formation induced by RANKL or TNF-α in both cell cultures. The inhibitory effects of statins and GGTI-2166 on pOC formation may result from the inhibition of the geranylgeranylation of G-proteins, such as Rho or Rac, suggesting that the geranylgeranylation of these proteins is involved in the early differentiation of progenitor cells into pOCs.

Keywords: Osteoclast differentiation; Statin; GGTI; FTI; Geranylgeranylation; Small G-protein

1. Introduction

Osteoclasts (OCL) are multinucleated cells (3–100 nuclei per cell) that play a critical role in bone remodeling. Osteoclasts are formed by the fusion of TRAP-positive mononuclear preosteoclasts (pOCs), which differentiate from hematopoietic precursors [1]. Osteoclasts, when activated, exhibit bone-resorbing activity [2–4].

Abbreviations: pOC, preosteoclast; RANKL, receptor activator of NFκB ligand; M-CSF, macrophage colony-stimulating factor; GGTI, geranylgeranyl transferase inhibitor; GGOH, geranylgeraniol; FTI, farnesyl transferase inhibitor; TRAP, tartrate-resistant acid phosphatase; TNF, tumor necrosis factor; GTP, guanosine triphosphate; HMG-CoA, hydroxymethylglutaryl coenzyme A; MEM, minimal essential medium; MD, marrow derived M-CSF dependent monocytes; MTT, methyl thiazole tetrazolium

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Small GTP-binding proteins (G-proteins) of the Ras superfamily (e.g., Ras, Rho, Rab, and cdc-42) play important roles in cell proliferation, differentiation, structural organization, and apoptosis [4–8]. The G-proteins Rab, Rho, and Rac are known to regulate the function of mature osteoclasts [9–12]. The activity of these proteins is regulated by a critical step in post-translational processing, the addition of isoprenoid farnesyl and geranylgeranyl groups [13–15].

Nitrogen-containing bisphosphonates inhibit multinucleated osteoclast formation, suppress osteoclastic bone resorption and induce apoptosis in mature osteoclasts [16–19]. Recently, nitrogen-containing bisphosphonates have been shown to prevent the production of isoprenyl diphosphates, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), through inhibition of farnesyl diphosphate synthase, an enzyme in the cholesterol synthesis pathway [20]. FPP and GGPP are substrates for prenyl protein transferases that prenylate small GTP-binding proteins such as Rab, Rho, Rac, and cdc-42. Therefore, it has been suggested that the inhibitory effects of nitrogen-containing bisphosphonates on bone resorption result from the decrease in the prenylation of these proteins in mature osteoclasts. In other studies, the post-translational prenylation of small GTP-binding proteins was shown to be involved in the effects of nitrogen-containing bisphosphonates [16,21,22].

Statins, specific HMG-CoA reductase inhibitors that inhibit the synthesis of mevalonate, have similar effects on osteoclasts to those of nitrogen-containing bisphosphonates. We have shown previously that the statin mevastatin (compactin) reversibly inhibits the fusion of pOCs into multinucleated osteoclasts, disrupts the actin ring in mature osteoclasts, and suppresses bone-resorption activity without promoting apoptosis in the osteoclasts [23]. The inhibition of mevalonate synthesis also prevents the synthesis of its downstream intermediates, FPP and GGPP. Like the nitrogen-containing bisphosphonates, statins also exert these effects on osteoclasts by preventing the prenylation of small GTP-binding proteins. Recently, it has been shown that a geranylgeranyl transferase I inhibitor (GGTI), but not a farnesyl transferase inhibitor (FTI), inhibits the formation, function and survival of multinucleated osteoclasts in bone marrow cultures [16]. These findings suggest that the inhibitory effect of nitrogen-containing bisphosphonates and statins on osteoclasts result from the decrease of geranylgeranylation of prenylated G-proteins.

Although statins and GGTI inhibit OCL formation in bone marrow cell cultures or cocultures of bone marrow cells and osteoclasts/stromal cells, it has not been determined whether statins and GGTI affect the differentiation of osteoclast precursor cells into pOCs. In this study, we determined the effects of mevastatin, GGTI-2166 (GGTase I specific inhibitor), and FTI-2153 (FTase specific inhibitor) on pOC formation induced by receptor activator of NF-κB ligand (RANKL) or tumor necrosis factor-α (TNF-α) in cultures of mouse marrow-derived M-CSF dependent monocytes (MD cells) or the mouse monocyte cell line RAW 264.7 (RAW cells). We show that statins and GGTI inhibit pOC formation induced by RANKL or TNF-α in both cell culture models. Furthermore, we show that *Clostridium difficile* cytotoxin B (toxin B), which inactivates small Rho family proteins such as Rac, Rho and cdc-42, inhibits the effect of RANKL or TNF-α to induce early differentiation of these two types of cells into pOCs. The results suggest that the inhibitory effects of statins and GGTI on bone resorption are partially due to the inhibition of pOC formation, and that geranylgeranylation of G-proteins such as Rho or Rac may be required for pOC formation.

2. Materials and methods

2.1. Materials

Farnesyl pyrophosphate, geranylgeranyl pyrophosphate, squalene, fast violet LB salt and naphthol AS-MX phosphate were purchased from Sigma Chemical Co. Mevalonic acid lactone was purchased from Wako Pure Chemicals Co. Mevastatin (compactin) was a generous gift from Dr. A. Endo (Biopharm Research Laboratories). Lovastatin and simvastatin were generous gifts from Sankyo Co. Recombinant murine M-CSF and recombinant human RANKL (sRANKL) were from R&D Systems and Pepro Tech EC Ltd., respectively. Recombinant murine TNF-α was purchased from R&D Systems. [^{3}H]all-*trans* geranylgeraniol (GGOH) and [^{14}C]mevalonolactone were from Amersham Radiochemicals Ltd. All other reagents were obtained from Sigma unless otherwise stated.

2.2. Cell culture

The mouse monocyte cell line RAW 264.7 (RAW cells) was obtained from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM L-glutamine (α-MEM + 10% FBS) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.3. Preparation of MD cells from bone marrow cells

Bone marrow cells were isolated from 5 to 7-day-old CD-1 mice as described previously [24]. Tibiae and femora were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut off and the marrow was forced out into a petri dish containing α-MEM + 10% FBS. The marrow suspension was filtered with a fine mesh sieve to remove bone particles and carefully agitated with a plastic Pasteur pipette to obtain a single-cell suspension. The bone marrow cells were washed twice, resuspended in α-MEM + 10% FBS, and incubated for 24 h in M-CSF

(5 ng/ml) at a density of 1×10^6 cells/ml in 100 mm dishes. After 24 h, non-adherent cells were harvested, washed, and resuspended (1×10^6/ml) in α-MEM + 10% FBS. These non-adherent cells were used as MD cells for osteoclast formation experiments.

2.4. Osteoclast formation

MD cells (1×10^6/ml) were added (100 μl/well) to 96-well plates. After 24 h, an additional 100 μl of α-MEM + 10% FBS containing cytokines (M-CSF, 20 ng/ml and RANKL, 100 ng/ml) were added to each well. The cells were then cultured for 1–3 days. RAW cells were seeded on 96-well plates (3×10^3 cells/well). After 24 h, an additional 100 μl of medium containing RANKL was added to each well. Osteoclast formation was evaluated by measuring TRAP activity in the fixed cells and by counting the number of TRAP-stained cells.

2.5. TRAP activity and cytochemistry

TRAP activity was measured by a modification of a previously-described method [25]. Cells were fixed with 10% formalin and 95% ethanol, and then dried. To measure TRAP activity in solution, dried cells were incubated in 100 μl citrate buffer (50 mM, pH 4.6) containing 10 mM tartrate and 5 mM *p*-nitrophenylphosphate for 20–30 min. After incubation, the enzyme reaction mixture was removed and added to 100 μl of 0.1N NaOH in a 96-well plate. Absorption was measured at 410 nm with a microplate reader (Molecular Devices). TRAP staining was carried out as described previously. In brief, the fixed cells were reacted for 30 min in 0.1% sodium acetate buffer (pH 5) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate and 1 mg/ml fast violet LB salt. After washing with water and drying, TRAP-positive cells were counted as pOCs and OCLs.

2.6. Cell viability

Cell viability was measured by MTT assay. MD cells were cultured with or without inhibitors for 2 days in the presence of 20 ng/ml M-CSF and 100 ng/ml RANKL. RAW 264 cells were cultured with or without inhibitors for 3 days in the presence of 100 ng/ml RANKL. After culture, MTT was added, followed by incubation at 37 °C for 1 h in a CO_2 incubator. Supernatants were carefully removed and dissolved with DMSO. Absorbance at 570 nm was measured with a microplate reader.

2.7. Incorporation of [^{3}H]GGOH and [^{14}C]mevalonolactone into prenylated proteins in RAW cells

Detection of prenylated proteins in RAW cells was performed by modification of the method described by Coxon et al. [16]. For incorporation of [^{3}H]GGOH (25 mCi/ml) or [^{14}C]mevalonolactone (52.0 mCi/ml) into prenylated proteins, RAW cells (2×10^5 cells) were seeded in 6-well plates. After 12 h culture, the cells were depleted of mevalonate by incubation for 5 h in α-MEM + 10% FBS containing 5 μM mevastatin. The cells were then washed with α-MEM + 10% FBS and incubated for 40 h in α-MEM + 10% FBS containing 5 μM mevastatin, 30 μCi/ml [^{3}H]GGOH or 6 μCi/ml [^{14}C]mevalonolactone, and GGTI-2166 or FTI-2153, respectively. The cells were washed with ice cold PBS and then resuspended in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 10 μg/ml aprotinin, 100 μM sodium orthovanadate in PBS). Fifty micrograms of protein from each sample was separated by electrophoresis on a 12% polyacrylamide-SDS gel. The gel was fixed and then dried, and labeled proteins were visualized by exposing the gel to imaging film (Eastman Kodak Co. for 4 days at −80 °C for [^{14}C]mevalonate-labeled cells or for 8 days for [^{3}H]GGOH-labeled cells.

3. Results

3.1. Statins inhibit pOC formation

To determine the effects of statins on pOC formation, we used both MD cells from bone marrow cells and RAW cells. As shown in Figs. 1 and 2, multinucleated OCLs were not formed in 2-day cultures of MD cells (Fig. 1A and Fig. 2B and F) or in 3-day cultures of RAW cells (Fig. 1B). However, as shown in Fig. 1C and D, RANKL dramatically stimulated TRAP activity in the cultures of both MD cells (within 2 days) and RAW cells (within 3 days). The number of TRAP positive pOC cells formed in the cultures of MD cells correlated with TRAP activity (compare Fig. 1C and E). Therefore, in further experiments, the effect of statins on pOC formation was determined by measuring TRAP activity. Mevastatin inhibited the increase of TRAP activity induced by RANKL in both cell cultures, (Fig. 3B and D), but had no effect on the growth and survival of MD cells (Fig. 2E and F and Fig. 3A) or RAW cells (Fig. 3C), as detected by MTT assay or cell counting. The morphology of mevastatin-treated MD cells was different from that of the control cells. The fibroblast-like morphology that was characteristic of the untreated cells changed to a rounded shape after treatment with mevastatin (compare Fig. 2D and E).

3.2. Mevastatin reversibly blocks the action of RANKL, but not that of M-CSF, during mononuclear osteoclast formation of bone marrow cells

The effects of mevastatin on the actions of M-CSF and RANKL on TRAP activity were determined in MD cells. When cells were incubated with M-CSF in the presence of

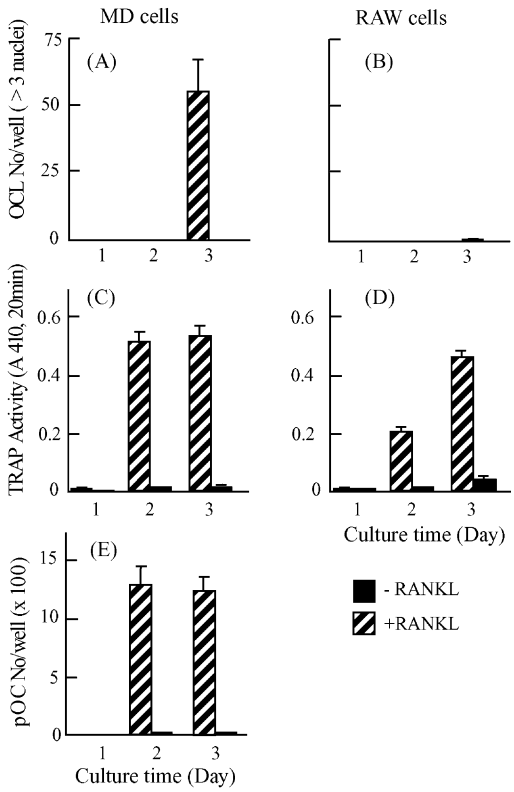

Fig. 1. Osteoclastogenesis in MD (A, C and E) and RAW 264.7 (B and D) cell cultures. (A and B) Number of TRAP-positive OCL (containing more than three nuclei), (C and D) TRAP activity (see Section 2), (E) number of TRAP-positive pOC number. MD cells derived from bone marrow cells and RAW 264.7 cells were cultured in the presence (hatched bars) or absence (solid bars) of RANKL (100 ng/ml). After culture for the indicated number of days, the cells were fixed and stained for TRAP, and their TRAP activity was determined. TRAP-positive cells were counted. Values are expressed as the mean ± S.D. of responses from three cultures.

mevastatin for 2 days and then cultured in the presence of M-CSF and RANKL in the absence of mevastatin for another 2 days, the observed TRAP activity (Fig. 4, 3rd column) was nearly the same as that of cells that had not been treated with mevastatin (Fig. 4, 2nd column). These results suggest that mevastatin does not affect M-CSF dependent MD cell growth. However, when MD cells were cultured for 2 days with M-CSF in the absence of mevastatin, and then washed and incubated for 2 days with M-CSF, RANKL and mevastatin, the TRAP activity was inhibited by about 60% (Fig. 4, 4th column). These results indicate that mevastatin blocks the action of RANKL.

3.3. Mevalonate, FPP and GGPP, but not squalene, overcome the effects of compactin on pOC formation

Inhibition of HMG-CoA reductase by mevastatin blocks the synthesis of mevalonate (MVA) from hydroxymethylglutaryl-CoA. This suggests that mevalonate or one of its downstream metabolites is required for the differentiation of progenitor cells into pOCs. Therefore, we investigated whether mevalonate and its downstream metabolites (GGPP, FPP and squalene) are involved in the effects of mevastatin on the early differentiation of MD cells and RAW cells into pOCs. Mevalonate, FPP, GGPP or squalene alone had no effect on TRAP activity (data not shown). However, mevalonate completely prevented the inhibitory effects of mevastatin on TRAP activity and FPP or GGPP also partially prevented the inhibition, whereas squalene had no effect (Fig. 5). These results suggested that MVA or its downstream metabolites, FPP and GGPP, but not squalene, are required for pOC formation. These observations further suggest that mevastatin inhibits RANKL-induced pOC formation via a pathway involving GGPP or FPP.

3.4. GGTI, but not FTI, inhibits pOC formation

Because GGPP and FPP are substrates of FTase and GGTase, respectively, the inhibitory effect of mevastatin may result from the inhibition of one or both of these enzyme activities. Therefore, we determined the effects of the cell permeable inhibitors, GGTI-2166 and FTI-2153, specific inhibitors of GGTase I and FTase [26], respectively, on TRAP activity induced by RANKL in MD cells and RAW cells. GGTI-2166 dose dependently inhibited TRAP activity induced by RANKL in both RAW cells (Fig. 6A) and MD cells (Fig. 6B), whereas FTI-2153 did not inhibit TRAP activity in either cell type (Fig. 6A and B). Similar to what was observed for mevastatin, neither GGTI-2166 nor FTI-2153 affected cell survival) (data not shown). MD cells treated with GGTI-2166 acquired a rounded shape, similar to that observed when cells were treated with mevastatin (Fig. 2G). We confirmed the effects of the inhibitors on geranylgeranylation in RAW cells by labeling with [^{3}H]GGOH and on geranylgeranylation and farnesylation by labeling with [^{14}C]mevalonolactone. Electrophoretic analysis of whole cell lysates from RAW cells that had been labeled with [^{3}H]GGOH showed a minor set of proteins of approximately 21–26 kDa, which represent geranylgeranylated small GTP-binding proteins (Fig. 7A). [^{14}C]mevalonolactone-labeled cells showed minor but discrete proteins of approximately 60–70 kDa, which represent farnesylated lamin B and prelamin A, as well as a more prominent set of proteins of 21–26 kDa (Fig. 7B), which represent farnesylated and geranylgeranylated small G-proteins. GGTI-2166 (3 μM) significantly inhibited the incorporation of [^{3}H]GGOH into geranylgeranylated proteins (Fig. 7A), and FTI-2153 (5 μM) completely inhibited the incorporation of [^{14}C]mevalonolactone into lamin proteins, which are only farnesylated (Fig. 7B). Although FTI-2153 inhibited farnesylation of proteins, it did not affect pOC formation. These results suggest that the inhibitory effect of GGTI on pOC formation is specific to the prevention of geranylgeranylation of small G-proteins, and that geranylgeranylation of small G-proteins is required for formation of pOCs from progenitor cells.

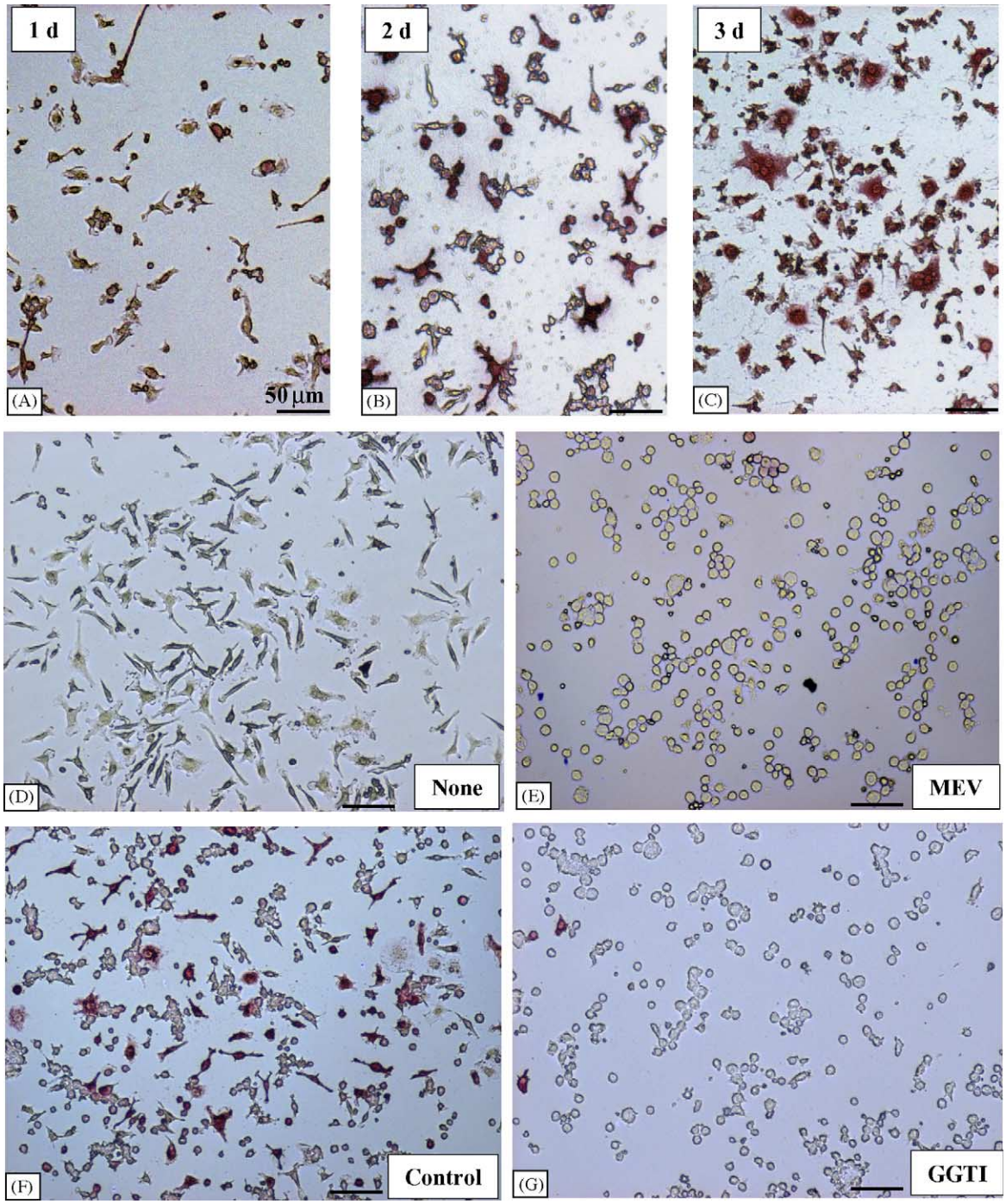

Fig. 2. Photomicrographs of MD cells cultured in the presence of 50 ng/ml RANKL. After culture for the indicated number of days, the cells were stained for TRAP. (A–C) Osteoclast formation in the presence of 100 ng/ml RANKL: 1 day (A), 2 days (B), and 3 days (C). (D–G) Osteoclast formation in the absence or presence of RANKL with mevastatin (Mev) or GGTI: no treatment (D), 0.4 μM mevastatin plus RANKL (E), RANKL alone (F), 5 μM GGTI-2166 plus RANKL (G).

3.5. Toxin B inhibits the pOC formation induced by RANKL in both MD cells and RAW cells

Clostridium difficile cytotoxin B is known to inactivate the Rho family small G-proteins, Rho, Rac and cdc-42 [27]. We determined the effects of toxin B on pOC formation in both cell models and found that toxin B dose dependently inhibited TRAP activity induced by RANKL in both cultures (Fig. 8A and B). Toxin B did not affect the viability of the RAW cells at any of the concentrations tested; however, in the MD cells, the highest toxin B concentration, 3 ng/ml, produced a 35% decrease in the MTT assay (data not shown). The morphological change in cells treated with toxin B was very similar to that obtained with mevastatin or GGTI (data not shown). These results suggest that at least a subset of the small G-proteins such as Rho or Rac that are inactivated by toxin B are involved in pOC formation.

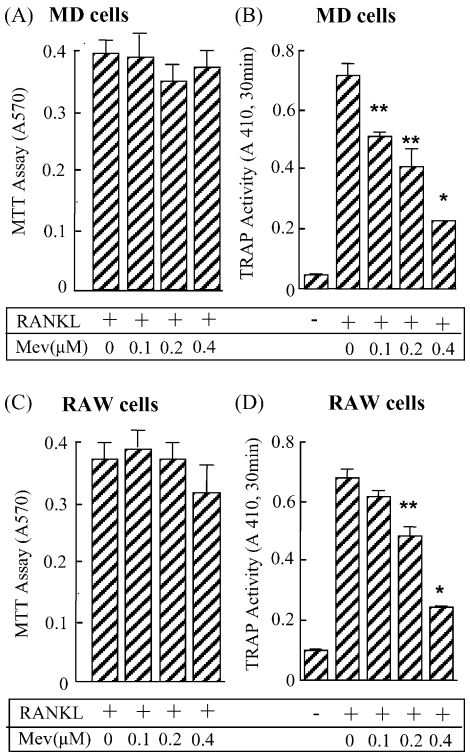

Fig. 3. Effect of mevastatin on survival and TRAP activity in MD and RAW cell cultures. (A) MD cells were cultured with mevastatin in the presence of 100 ng/ml RANKL for 2 days, and (B) RAW cells were cultured in the presence of 100 ng/ml RANKL for 3days. After culture, cells in some wells were assessed for survival by MTT assay (A, C) whereas cells in other wells were fixed and TRAP activity was determined (B, D). Values are expressed as the mean ± S.D. of responses from three cultures. $^{*}P < 0.01$ vs. mevastatin 0 μM; $^{**}P < 0.05$ vs. mevastatin 0 μM.

3.6. Mevastatin, GGTI, and toxin B inhibit pOC formation induced by TNF-α

In addition to RANKL, TNF-α also has the potential to induce OCLs in bone marrow hemapoietic cell cultures

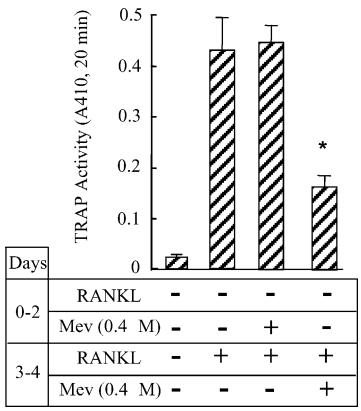

Fig. 4. Effect of mevastatin on the RANKL, but not the M-CSF effect on TRAP activity in MD cell cultures. MD cells were cultured in the presence of 20 ng/ml M-CSF with or without 0.4 μM mevastatin, as indicated, for 2 days and then in the presence of M-CSF with or without RANKL and with or without 0.4 μM mevastatin for an additional 2 days, as indicated. After culture, the cells were fixed and their TRAP activity was determined. Values are expressed as the mean ± S.D. of responses from three cultures.

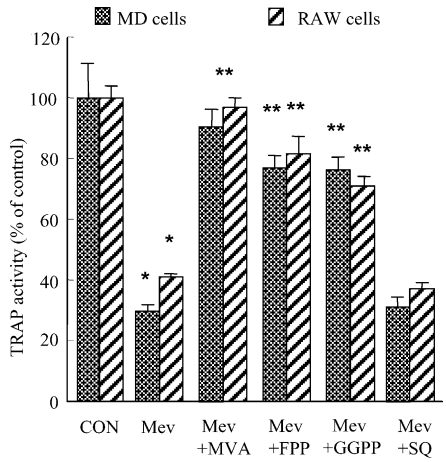

Fig. 5. Mevalonate, FPP and GGPP, but not squalene, overcome the effect of mevastatin on the TRAP activity in both MD cells and RAW cells. (A) MD cells (2 days) or (B) RAW cells (3 days) were cultured in the presence of 50 ng/ml RANKL and 0.4 μM mevastatin (Mev) with or without 50 μM mevalonate (MVA), 10 μM FPP, 10 μM GGPP, or 100 μM squalene (SQ). The cells were fixed and TRAP activity was determined. Values are expressed as the mean ± S.D. of responses from three cultures. $^{*}P < 0.01$ vs. CON; $^{**}P < 0.01$ vs. mevastatin.

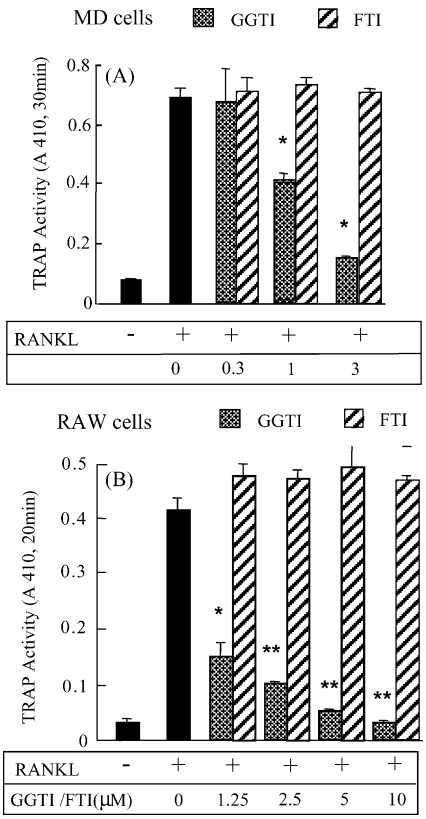

Fig. 6. GGTI-2166, but not FTI-2153, inhibits TRAP activity in RAW cells and MD cells. (A) MD were cultured in the presence of 20 ng/ml M-CSF and 100 ng/ml RANKL with or without GGTI-2166 or FTI-2153 for 2 days. (B) RAW cells were cultured in the presence of 100 ng/ml RANKL with GGTI-2166 or FTI-2153 for 3 days. At the end of the culture period, the cells were fixed and their TRAP activity was determined. Values are expressed as the mean ± S.D. of responses from three cultures. $^{*}P < 0.01$ vs. GGTI 0 μM; $^{**}P < 0.001$ vs. GGTI 0 μM.

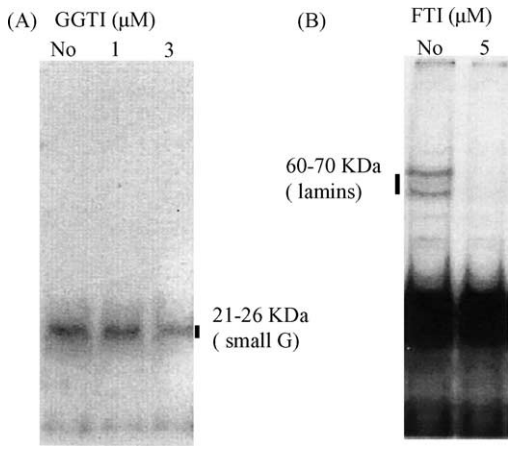

Fig. 7. Effects of GGTI-2166 and FTI-2153 on the incorporation of [^{3}H]GGOH and [^{14}C]mevalonolactone into the prenylated proteins in RAW cells. (A) Incorporation of [^{3}H]GGOH into geranylgeranylated proteins. (B) Incorporation of [^{14}C]mevalonolactone into geranylated and farnesylated proteins. RAW cells (2×10^5) were incubated for 5 h in α-MEM + 10% FBS containing 5 μM mevastatin. The cells were then washed with α-MEM and incubated for 40 h in α-MEM + 10% FBS containing 5 μM mevastatin and (A) 30 μCi/ml ^{3}H-GGOH in the presence of 1 or 3 μM GGTI-2166, or (B) 6 μCi/ml [^{14}C]mevalonolactone in the presence of 5 μM FTI-2153. The cells were washed with ice cold PBS and then resuspended in lysis buffer. Fifty micrograms of total protein from each sample was separated by electrophoresis on a 12% polyacrylamide-SDS gel. The gel was fixed and then dried, and labeled proteins were visualized by exposing the gel to an imaging film 4 days at −80 °C for [^{14}C]mevalonate-labeled cells or for 8 days for [^{3}H]GGOH-labeled cells.

[28,29]. We determined whether mevastatin, GGTI, and toxin B inhibit pOC formation in both cell cultures. Mevastatin, GGTI, and toxin B each inhibited TRAP activity induced by TNF-α (Fig. 9A and B). These results suggest that these inhibit a common pathway induced by RANKL and TNF-α and that the geranylgeranylation of proteins is the common pathway for pOC formation.

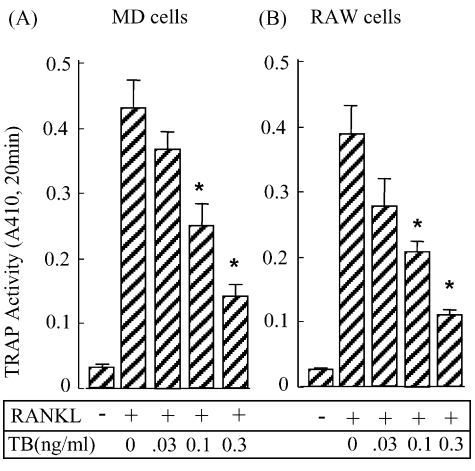

Fig. 8. Effect of toxin B on the TRAP activity in both MD and RAW cell cultures. (A) MD cells (2 days) or (B) RAW cells (3 days) cells were cultured in the presence of 100 ng/ml RANKL with toxin B (0.03–0.3 ng/ml). After culture, the cells were fixed and TRAP activity was determined. Values are expressed as the mean ± S.D. of responses from three cultures. $^*P < 0.01$ vs. toxin B 0 ng/ml.

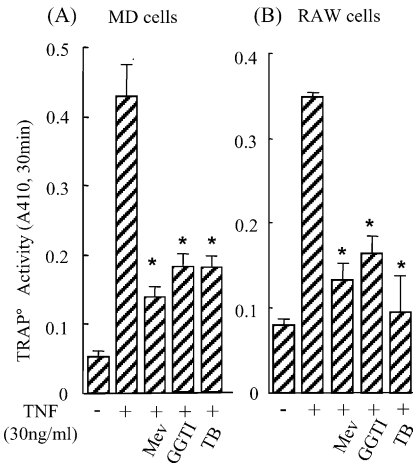

Fig. 9. Effect of mevastatin, toxin B and GGTI on the osteoclast formation induced by TNF-α. (A) MD (2 days) or (B) RAW (3 days) were cultured in the presence of 30 ng/ml TNF-α with 0.4 μM mevastatin (Mev), 0.3 ng/ml toxin B (TB) and 1 μM GGTI. After cultures, the cells were fixed and their TRAP activity was determined. Values are expressed as the mean ± S.D. of responses from three cultures. $^*P < 0.01$ vs. TNF-α.

4. Discussion

In the current study, we have demonstrated that statins inhibit pOC formation induced by RANKL or TNF-α in cultures of osteoclast progenitor cells. MVA or its downstream metabolites FPP and/or GGPP prevented the effects of statins on pOC formation, suggesting that protein prenylation is involved in pOC formation. The geranylgeranyl transferase I inhibitor GGTI-2166 also inhibited pOC formation and the incorporation of labeled mevalonate into prenylated proteins. Furthermore, toxin B, which inactivates the Rho family small G-proteins, Rho, Rac, and cdc-42, inhibited pOC formation. Thus, the inhibitory effects of statins on pOC formation may result from the inhibition of the geranylgeranylation of one or more of these G-proteins. This suggests that the geranylgeranylation of these proteins plays an important role in the early differentiation of progenitor cells into mononuclear pOCs.

Osteoclast-like multinucleated cells (OCLs) can be differentiated in vitro from cocultures of mouse bone marrow cells and calvarial osteoblastic cells by treatment with osteotropic factors such as 1α,25-dihydroxyvitamin D_3, prostaglandin E_2, interleukin-1 (IL-1) or parathyroid hormone [2,3]. Osteoblasts or stromal cells are the target cells for these factors in bone. Recently, an essential factor provided by osteoblasts or stromal cells, has been identified and named osteoclast differentiation factor (ODF)/osteoprotegerin ligand (OPGL)/tumor necrosis factor-related activation-induced cytokine (TRANCE)/receptor activator of nuclear factor-κB ligand (RANKL) [30,31]. It was shown that RANKL induces OCL formation in cultures of MC cells and RAW cells without requiring osteoblasts or stromal cells [32]. In previous studies, statins were found to inhibit OCL formation in cocultures containing osteoblasts or stromal cells together with the marrow cells

[17]. GGTI-298 also was shown to inhibit OCL formation in bone marrow cell cultures that included stromal cells [16]. We also recently reported that statins inhibit the fusion of pOCs into OCLs in cocultures [23]. From these studies, it was not clear which cells in the cocultures were affected by these compounds. By using MC and RAW cell cultures, we were able to eliminate the potential influence of indirect effects mediated through osteoblastic or stromal cells. Furthermore, when MC and RAW cells were treated with RANKL or TNF-α for 2 or 3 days, respectively, there were no multinucleated OCLs formed in either cell culture. Therefore, to investigate the effects of statins on differentiation of osteoclast precursor cells into pOCs, MC cells derived from bone marrow and RAW cells were treated directly with RANKL and TNF-α. Statins inhibited the differentiation of MD and RAW cells into pOCs. Viability of MD and RAW cells and M-CSF dependent cell growth were not affected by statins or GGTI. These results suggest that statins and GGTI-2166 directly affect the action of RANKL and TNF-α on osteoclast precursor cells, and that the inhibition of early differentiation of osteoclasts by statins contributes to the inhibitory effects of statins on bone resorption.

Statins inhibit survival of mature multinucleated osteoclasts, induce morphological changes in activated osteoclasts, and inhibit bone resorption by osteoclasts [16,17]. We previously reported that mevastatin inhibits bone resorption by inducing morphological changes in activated osteoclasts without affecting survival of osteoclasts [23]. The effect of statins on mature osteoclasts could be completely reversed by mevalonate and GGPP, suggesting that the effects of statins result from the inhibition of protein geranylgeranylation [17,23]. It was noted that GGTI-298 induced the disruption of actin rings in the osteoclasts and inhibited bone resorption, but FTI did not [16]. In the current study, statins and GGTI also induced morphological changes in progenitor cells and the inhibitory effect of statins on the pOC formation in both MD and RAW cell cultures was completely reversed by mevalonate, but only partially reversed by GGPP and FPP. GGTI-2166, but not FTI, inhibited the pOC formation. These results suggest that the mechanism of action of the statin on osteoclast progenitor cells involves inhibition of protein geranylgeranylation, indicating a molecular mechanism similar to that established for mature osteoclasts.

Isoprenylation of small G-proteins results in their localization to specific membranes, where they are activated by GEFs. The inhibitory effects of the statins on osteoclasts, including pOC formation may be mediated through their effects on isoprenylation of these proteins. The G-proteins Rab, Rho and Rac are involved in the organization of the actin cytoskeleton (the ringed structure comprised of actin) and in bone resorption [9–12,33]. In our study, the pOC formation induced by RANKL or TNF-α was inhibited by toxin B, which inactivates Rho, Rac and cdc-42 family proteins. This suggests that pOC formation, as well as osteoclast function, are regulated by these G-proteins. Which G-proteins are involved in the pOC formation induced by RANKL or TNF-α from MD cells and RAW cells has not been determined. Further studies are necessary to identify the specific G-protein(s) involved in pOC formation.

Statins have received attention as possible agents for the treatment of osteoporosis due to their effects to stimulate the production of bone morphogenetic protein-2 in osteoblasts [34]. A limited number of studies have shown beneficial effects on bone mineral density [35,36] or fracture risk [37,38]. It is possible that in addition to the positive effects on bone formation, the inhibitory effects of statins on osteoclastogenesis could contribute to their beneficial effects on bone. This possibility is supported by a study of postmenopausal women receiving a statin, in whom C-telepeptide, a marker of bone resorption, was significantly decreased [39]. However, the statins currently in clinical use were developed to target to the liver, and thus the amounts accumulating in bone may be inadequate to elicit consistent beneficial effects. Development of statins with improved targeting to bone will allow a more thorough assessment of their efficacy for the treatment osteoporosis and for determination of the relative contributions of stimulatory effects on anabolic functions and inhibitory effects on osteoclastogenesis to the in vivo effects on statins on bone.

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