Original Experimental Repositioning of Geranylgeranylacetone to Enhance Bone Remodeling

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Abstract: Drug repositioning (DR) is a strategy to explore new medicinal effects from existing approved drugs whose safety and pharmacokinetics have already been established. We focused on geranylgeranylacetone (GGA), which is known as a heat shock proteins (HSPs) inducing agent. GGA is mainly used as a gastric mucosal protective agent; however, its effects on bone tissues have not been studied. Therefore, we hypothesized that “GGA induces HSPs in osteoblasts thereby promotes cell differentiation”, and administered GGA to MC3T3E-1 cells to examine cell responses. Methods: MC3T3E-1 were cultured in osteogenic medium. After the cultures were established, test cultures were exposed to GGA (GGA group). Cell proliferation, collage synthesis and ALP activity were measured on days 7 and 14 of culture. Alizarin Red S staining was performed on days 21 of culture. Results: On days 14 of culture, cell proliferation and collage synthesis were significantly higher in the GGA group than in the control group (P<0.05). On days 7 and 14 of culture, ALP activity was significantly higher in the GGA group than in the Control group (P<0.05). On days 28 of culture, the Alizarin Red S stained areas were significantly higher in the GGA group than in the control group (P<0.05). Conclusion: GGA promoted the differentiation of MC3T3E-1 in an in vitro cell culture model.

Key words: Calcification, Geranylgeranylacetone, Heat shock protein

Introduction

In drug repositioning (DR), therapeutic agents that have already been approved for human use and whose safety and pharmacokinetic profiles have been confirmed are screened for new medicinal properties and commercial applications\(^3\).\(^4\).

This approach has been applied for decades in the drug development field. However, it is now attracting renewed attention as there are bottlenecks in the drug development pipeline. It may require more than a decade and a substantial budget to bring a novel pharmaceutical agent to market. According to certain estimates, <1% of all drug candidates that have reached the trial stage ever attain clinical use\(^5\). DR could drastically reduce drug development time and costs by repurposing previously approved drugs whose effects and safety have already been confirmed in humans. DR has enabled numerous pharmaceuticals to be prescribed for conditions differing from their original indications. Memantine was originally developed in 1968 as an antiviral drug for influenza treatment. It is now administered for Alzheimer’s disease as it was found to have efficacy against dementia\(^6\). Zonisamide was first formulated in 1974 as an antiepileptic agent. It is currently being applied to manage Parkinson’s disease (PD) because it was serendipitously discovered that zonisamide alleviated motor symptoms in a PD patient receiving it to control epileptic seizures\(^7\).

Here, we focused on the DR of the acyclic isoprenoid geranylgeranylacetone (GGA) which has proven safety and pharmacokinetic profiles. GGA has been administered primarily to protect gastric mucosa. It was the first drug to induce heat shock proteins (HSPs) expression in gastric mucosal cells\(^8\). Several studies investigated its activity in other tissues and anticipated similar HSPs-inducing effects\(^9\). To the best of our knowledge, however, the effects of GGA on osseous tissue have not yet been examined. We hypothesized that GGA induces HSPs expression and promotes differentiation in osteoblasts. We tested our hypothesis using a series of in vitro experiments and explored the cellular responses and phenotypic changes in a MC3T3E-1 mouse osteoblast-like cell line subjected to GGA.

Materials and Methods

The experimental timeline is illustrated in Fig. 1.

Cell cultures

The culture medium consisted of Minimal Essential Medium Alpha Modification (α-MEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% (v/v) fetal bovine serum (FBS; Equitech-Bio Inc., Kerrville, TX, USA), 10⁻³ M dexamethasone (Sigma-Aldrich Corp., St. Louis, MO, USA), 50 μg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich Corp., St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich Corp., St. Louis, MO, USA), and an antibacterial-antifungal agent (Thermo Fisher Scientific, Waltham, MA, USA). MC3T3E-1 cells derived from newborn mouse calvaria were suspended in this solution and cultured in a 10-cm cell culture dish (Corning Inc., Corning, NY, USA) (Fig. 2). Cells were passaged on the third day of culture (day 3) and seeded at a density of 3 × 10⁶ cells well⁻¹. Cultures were maintained in an incubator at 37 °C under a 5% CO₂ atmosphere. The medium was replaced every 3 d.

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GGA preparation and dosage

The GGA solution was prepared by dissolving teprenone (Eisai Co. Ltd., Tokyo, Japan) in 100% ethanol and dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries Ltd., Osaka, Japan), and diluting the mixture in α-MEM (Thermo Fisher Scientific, Waltham, MA, USA) to yield a final concentration of $10^{-4}$ M GGA. The MC3T3E-1 cells in the GGA group were administered the GGA once every 3 d during the study period starting on day 1. Teprenone was omitted from the control solution and 100% ethanol and DMSO were diluted to $10^{-4}$ M in α-MEM. The MC3T3E-1 cells in the control group were treated daily with the aforementioned teprenone-free solution starting on day 1.

Cell proliferation assay

Cell counts were measured on days 7 and 14 with a colorimetric WST-8 assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). Briefly, the tissue in each plate well was gently washed twice with phosphate-buffered saline (PBS). Next, 900 μl culture solution and 100 μl CCK-8 reagent were added to each well. The plate was set in a CO2 incubator for 20 min to allow the chromogenic reaction to proceed. The reaction products were added to a 96-well plate (100 μl well$^{-1}$) and the absorbances were read at 450 nm in a laboratory photometer (Microplate Reader 680; Bio-Rad Laboratories, Hercules, CA, USA). The absorbances were converted to relative cell count changes.

Collagen quantification assay

The collagen content in the cultured tissue was quantified on days 7 and 14 of culture. The tissue was stained with collagen-binding Sirius Red (Polysciences, Inc., Warrington, PA, USA). The collagen content was taken to be proportional to the absorbance of the stained tissue at the characteristic wavelength of the dye. Briefly, the cultured tissue was washed with PBS and fixed in Bouin solution (Polysciences, Inc., Warrington, PA, USA), washed again, and stained for 1 h in Sirius Red (100 mg 100 ml$^{-1}$) dissolved in saturated picric acid solution (Kanto Chemical, Tokyo, Japan). Excess stain was rinsed off with 0.01 M HCl and the Sirius Red was solubilized in 0.1 M NaOH. The absorbance of the tissue at 550 nm was measured in a Microplate Reader 680 (Bio-Rad Laboratories, Hercules, CA, USA).

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity was measured on days 7 and 14 by the $p$-nitrophenyl phosphate substrate-based method. Briefly, 100 μl substrate buffer solution (Lab Assay ALP, Wako Pure Chemical Industries Ltd., Osaka, Japan) was added to 1 ml PBS and the mixture was incubated for 10 min to trigger the chromogenic reaction. The reaction products were added to a 96-well plate (100 μl well$^{-1}$). The ALP activity was quantified as the absorbance measured at 405 nm in a Microplate Reader 680 (Bio-Rad Laboratories, Hercules, CA, USA).

Calcium deposition assay

The cultured tissue was stained with Alizarin Red S (Thermo Fisher Scientific, Waltham, MA, USA) which binds to metal cations. Calcified nodules and their surroundings stained red and calcium deposition was quantified as the total stained area observed in the processed images. Briefly, the cultured tissue was fixed for 10 min in 10% (v/v) neutral buffered formalin (Wako Pure Chemical Industries Ltd., Osaka, Japan) and washed with purified water. The cells were stained in Alizarin Red S solution (pH 4.1–4.3) for 10 min and washed 4× with purified water. The stained area was quantified in ImageJ (National Institute of Health, Bethesda, MD, USA).

Statistical processing

Data for the cell proliferation, collagen quantification, and ALP activity assays were analyzed by two-way ANOVA in order to identify the influences of culture day (day 7 vs. day 14) and group (GGA vs. control). When interactions between these independent factors were observed, one-way ANOVA was performed separately on the data for each culture day. Calcium deposition data were compared by one-way ANO-
Daisuke Yamaguchi et al.: Experimental Repositioning of Geranylgeranylacetone

VA. Statistical significance was set at $P < 0.05$.

Results

Cell proliferation assay
Fig. 3 shows the relative cell counts of both groups measured on days 7 and 14 in terms of absorbance values. By day 14, the GGA-treated cells had proliferated significantly more than those in the control group (ANOVA: $P < 0.05$). However, the difference between treatments was not significant on day 7.

Collagen quantification assay
Fig. 4 shows the amounts of collagen synthesized by each group on days 7 and 14 in terms of absorbance. By day 14, the MC3T3E-1 mouse osteoblast-like cells treated with GGA produced significantly more collagen than those in the control group (ANOVA: $P < 0.05$).

ALP activity assay
Fig. 5 shows the ALP activity on days 7 and 14. The cells treated with GGA exhibited significantly higher ALP activity than those in the control group (ANOVA: $P < 0.05$).

Calcium deposition assay
Fig. 6A and 6B show the cultured tissue stained with Alizarin Red S. Fig. 7 shows the degree of calcification determined by staining intensity on day 28. One-way ANOVA was performed separately for each culture day. The GGA-treated tissue images had significantly more stained pixels than those of the control (ANOVA: $P < 0.05$).

Discussion

Features of the MC3T3-E1 culture model
In general, MC3T3-E1 cells calcify to varying degrees after ~30 d in the presence of 10% (v/v) FBS$^{10,11}$. However, calcium deposits do not form in every cell$^{12,13}$. To ensure that our MC3T3-E1 cells uniformly differentiated into osteoblasts, we supplemented them with 15% (v/v) FBS plus dexamethasone, L-ascorbic acid 2-phosphate, and β-glycerophosphate...
Geranylgeranylacetone (GGA)

GGA was primarily used in gastrotherapy. However, its mode of action was not initially elucidated. Hirakawa et al. performed a comprehensive analysis of its potential mechanisms and concluded that its effects were attributed to HSPs induction\(^{[17]}\). HSPs are temporarily upregulated in response to heat and other stressors\(^{[18,19]}\) and classified by molecular weight such as small HSPs (sHSPs), Hsp60, Hsp70, Hsp90, and Hsp100. These proteins are ubiquitous among organisms ranging from bacteria to humans. Their sequences are well conserved across species and over time\(^{[20-22]}\). Several HSPs known as molecular chaperones are constitutively expressed in normal cells. They are essential for protein folding, repair, transport, and degradation\(^{[23]}\). Tanaka et al. reported that HSPs are anti-inflammatory and GGA could effectively treat conditions such as inflammatory bowel disease (IBD) and duodenal ulcer\(^{[24]}\). Otaka et al. found that GGA inactivates Hsp70 protein chaperones by binding their C termini, activating Heat shock transcription factor 1 (HSF-1), and inducing Hsp70, Hsp90, and Hsp60\(^{[25]}\). Osteoblasts play a central role in bone metabolism. Osteoblasts subjected to thermal, chemical, or mechanical stress synthesize HSPs to regulate cell proliferation and differentiation\(^{[26]}\). However, the effects of GGA on bone tissue have not yet been clarified. We hypothesized that GGA upregulates HSPs and promotes osteoblast differentiation and tested our hypothesis using a series of in vitro experiments. We investigated the responses and phenotypic changes occurring in MC3T3E-1 mouse osteoblast-like cells exposed to GGA. A 10\(^{-5}\) M GGA concentration was applied to all cell cultures as preliminary assays demonstrated that it was the minimum concentration necessary to produce different calcification levels (unpublished data).

Cell growth potential

The GGA group cell count was significantly higher than that of the control group on culture day 14. On day 7, however, MC3T3E-1 counts did not significantly differ among groups. GGA exposure enhanced cell growth potential between culture days 7 and 14. The stark difference in the data between these two days is explained by the fact that osteoblasts grow logarithmically between the proliferative and quiescent stages.

Collagen production

Collagen production was significantly greater in the GGA-treated than the control cells on day 14. Sirius Red detects types I and III collagens\(^{[27]}\). The latter occurs in osteoblast progenitor cells\(^{[28]}\) while the former comprises ~90% of bone matrix\(^{[29]}\). Our findings revealed that GGA promotes MC3T3E-1 differentiation.

ALP activity

ALP activity is an indicator of osteoblast differentiation. ALP degrades pyrophosphates, causes localized increases in phosphate concentration, and fosters the growth of hydroxyapatite crystals\(^{[30]}\). ALP activity was significantly higher in the GGA group than it was in the control group on days 7 and 14. Our results disclosed that the HSPs induced by GGA may have accelerated MC3T3E-1 differentiation.

Calcium deposition

The area of the tissue stained by Alizarin Red S was significantly larger in the GGA group than it was in the control group. The Alizarin Red S staining revealed the tissue area that had undergone calcification. Although calcified nodules eventually formed in all long-term MC3T3E-1 cultures, GGA exposure accelerated this process relative to the untreated control.

The observed increases in collagen production and calcified nodule formation in response to GGA exposure may be explained in several ways. Early in calcification, the osteoblast-like cells secrete matrix vesicles (MVs). Collagen and other extracellular matrix molecules are synthesized around the cells and trigger crystalline hydroxyapatite precipitation in the MVs\(^{[31]}\). The crystals then exit the vesicles, enter the gaps between adjacent collagen molecules, and seed calcium deposition along the collagen fibers\(^{[32]}\). GGA might have promoted this process.

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Conflict of Interest

The authors have declared that no COI exists.

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