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Osteoinductive effects of glyceollins on adult mesenchymal stromal/stem cells from adipose tissue and bone marrow

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ABSTRACT

Background: While current therapies for osteoporosis focus on reducing bone resorption, the development of therapies to regenerate bone may also be beneficial. Promising anabolic therapy candidates include phytoestrogens, such as daidzein, which effectively induce osteogenesis of adipose-derived stromal cells (ASCs) and bone marrow stromal cells (BMSCs).

Purpose: To investigate the effects of glyceollins, structural derivatives of daidzein, on osteogenesis of ASCs and BMSCs.

Study Design: Herein, the osteoinductive effects of glyceollin I and glyceollin II were assessed and compared to estradiol in ASCs and BMSCs. The mechanism by which glyceollin II induces osteogenesis was further examined.

Methods: The ability of glyceollins to promote osteogenesis of ASCs and BMSCs was evaluated in adherent and scaffold cultures. Relative deposition of calcium was analyzed using Alizarin Red staining, Bichinchoninic acid Protein Assay, and Alamar Blue Assay. To further explore the mechanism by which glyceollin II exerts its osteoinductive effects, docking studies of glyceollin II, RNA isolation, cDNA synthesis, and quantitative RT-PCR (qPCR) were performed.

Results: In adherent cultures, ASCs and BMSCs treated with estradiol, glyceollin I, or glyceollin II demonstrated increased calcium deposition relative to vehicle-treated cells. During evaluation on PLGA scaffolds seeded with ASCs and BMSCs, glyceollin II was the most efficacious in inducing ASC and BMSC osteogenesis compared to estradiol and glyceollin I. Dose-response analysis in ASCs and BMSCs revealed that glyceollin II has the highest potency at 10 nM in adherent cultures and 1 μM in tissue scaffold cultures. At all doses, osteoinductive effects were attenuated by fulvestrant, suggesting that glyceollin II acts at least in part through estrogen receptor-mediated pathways to induce osteogenesis. Analysis of gene expression demonstrated that, similar to estradiol, glyceollin II induces upregulation of genes involved in osteogenic differentiation.

Conclusion: The ability of glyceollin II to induce osteogenic differentiation in ASCs and BMSCs indicates that glyceollins hold the potential for the development of pharmacological interventions to improve clinical outcomes of patients with osteoporosis.

Abbreviations: β-actin, beta-actin; ALP, alkaline phosphatase; ASCs, adipose-derived stromal/stem cells; BMD, bone mass density; BMI, body mass index; BMSCs, bone marrow-derived mesenchymal stem cells; BSA, bovine serum albumin; c-FOS, FBJ murine osteosarcoma viral oncogene homolog; CDS-FBS, charcoal dextran stripped fetal bovine serum; CCM, complete culture media; FBS, fetal bovine serum; ICI187,280, Fulvestrant; IGF1, insulin-like growth factor 1; OP, osteopontin; P/S, penicillin streptomycin; PBS, phosphate buffered saline; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RUNX2, runt-related transcription factor 2; SERM, selective estrogen receptor modulator.

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Introduction

Osteoporosis is a pathological condition characterized by reduced bone strength, compromised bone architecture, and increased susceptibility to fractures, which results in significant morbidity and mortality. An estimated 9 million osteoporosis-related fractures occurred in 2000: 1.6 million hip fractures, 1.7 million forearm fractures, and 1.4 million vertebral fractures. The greatest number of fractures occurred in Europe, followed by the Western Pacific, Southeast Asia, and the Americas, which together accounted for 96% of all fractures. Total disability-adjusted life years (DALYs) lost in 2000 was 5.8 million worldwide. Thus, osteoporosis represents a substantial source of morbidity and mortality, and therefore, continued development of therapeutics is essential (Johnell and Kanis, 2006).

Osteoporosis is defined pathologically by a shift in the balance of bone remodeling to favor resorption over regeneration. Therapeutic compounds for osteoporosis are divided into two groups: anti-resorptive drugs and anabolic drugs. Anti-resorptive drugs such as bisphosphonates, selective estrogen receptor modulators (SERMs), tissue-specific estrogen complex (Bazedoxifene-estrogen), denosumab, cathepsin K inhibitors, and integrin antagonists, reduce the breakdown of bone during normal remodeling and reduce bone loss by limiting osteoclast activity. While these drugs decrease the disease progression and limit the severity of osteoporosis, the use of anabolic agents will further reduce bone turnover and restore normal bone remodeling. The only anabolic agent currently approved for the treatment of osteoporosis is teriparatide (PTH 1-34). However, restrictions have been placed on teriparatide limiting the length of use to 18–24 months due to increased incidence of osteosarcoma in rats treated with teriparatide. Another anabolic drug is estradiol, which has been shown to reduce the development of osteoporosis and increase BMD through both anti-resorptive and anabolic mechanisms. However, estradiol is no longer recommended as a treatment for osteoporosis due to population studies demonstrating a correlation between hormone replacement therapy and an increased risk of developing breast and endometrial cancers (Schairer et al., 2000; Weiderpass et al., 1999).

Development of safe and efficacious anabolic drugs is necessary to regenerate the bone lost to osteoporosis and to improve the quality and strength of bone. Recently, phytoestrogens have drawn significant attention as these compounds have shown the capacity to inhibit the bone resorption activity of osteoclasts, to stimulate osteogenic differentiation, and to increase maturation of bone marrow-derived mesenchymal stem cells (BMSCs) and osteoblasts (De Wilde et al., 2004). Previous studies have also shown that these compounds have the ability to induce osteogenesis *in vivo*, both in animal and human subjects (Marini et al., 2007; Picherit et al., 2000). Significantly, induction of osteogenesis by phytoestrogens, such as daidzein and genistein, occurs through estrogen signaling pathways without increasing cancer risk (Lamartiniere et al., 2002). In several studies, daidzein and genistein have even shown an inhibitory effect on ovarian and breast cancer cells (Gercel-Taylor et al., 2004; Jin et al., 2010).

The promise of daidzein and genistein as candidate anabolic therapies for osteoporosis has led to research focusing on the effects of daidzein and genistein derivatives on bone. In a recent paper, it was demonstrated that ASCs and BMSCs treated with daidzein analogs underwent enhanced osteogenesis compared to estradiol, daidzein, or genistein (Strong et al., 2014). This study provides support for exploring the therapeutic potential of daidzein derivatives for the treatment of osteoporosis.

Glyceollins, structural derivatives of daidzein, are produced de novo in soybean [*Glycine max* (L.) Merr] (Fabaceae) (checked with http://www.theplantlist.org) plants in response to stress signals such as fungal infections. Therefore, glyceollins are likely to share physiological properties with isoflavones like daidzein and genistein, and studies have suggested that glyceollins may have osteoinductive effects due to structural similarity with daidzein. Glyceollins have also been shown to have promising anti-estrogenic effects on breast cancer and ovarian cancer cells (Salvo et al., 2006; Burow et al., 2001). Current literature supports that glyceollins may have estrogenic and anti-estrogenic effects at different concentrations (Kim et al., 2010). Glyceollins may also have varying effects with individual tissues and are likely SERMs with the capacity to antagonize estrogen receptors in the breast and ovary and to act as estrogen receptor agonists in bone. Thus, glyceollins have several characteristics that make them ideal potential candidates for anabolic therapy for osteoporosis.

In the current study, the effects of glyceollins on ASCs and BMSCs both in adherent cultures and in a three-dimensional scaffold model were assessed by measuring calcium deposition and gene expression. Although previous studies have focused on BMSCs as the precursor to osteoblasts, recent studies indicated that ASCs have the potential to differentiate into osteoblasts, therefore ASCs were included in this study (Gimble et al., 2007). ASCs may be ideal candidates for tissue engineering and regenerative medicine applications, due to their ease of isolation and their abundance within adipose tissue. In addition to traditional adherent cultures, a scaffold model was chosen for three-dimensional assessment of glyceollins on BMSC and ASC-seeded scaffolds. The current study demonstrates that glyceollins have the potential to regenerate bone in patients with osteoporosis by inducing ASCs and BMSCs to undergo osteogenesis.

Materials and methods

Type 1 collagenase, bovine serum albumin (BSA, fraction V), calcium chloride, β-glycerol phosphate, dexamethasone, ascorbate-2-phosphate, Alizarin Red s, estradiol, and poly(lactic-co-glycolic acid) (PLGA) were purchased from Sigma (St. Louis, MO, USA).

Isolation of glyceollins

Glyceollins I and II were isolated from soybean *G. max* plants using a procedure developed at the Southern Regional Research Center (U.S. Department of Agriculture-Agricultural Research Service, New Orleans, LA). The glyceollins were specifically isolated from Asgrow Monsanto soybean seeds (Lot # HE3S8D21).

The procedure of isolation involved slicing 1 kg of *G.* max seeds and inoculating them with *Aspergillus sojae.* After 3 days of inoculation, glyceollins were extracted from the seeds using 1 l of methanol (Aldrich Chemical Co., Milwaukee, WI, USA), and preparative scale high-performance liquid chromatograph (HPLC) was performed on a 600E System Controller (Waters, Milford, MA, USA) with a UV-VIS 996 detector (Waters) using two 25-mm 10-µm particle size µBondapak C18 radial compression column segments (Waters) combined by an extension tube. Elution was performed at a flow rate of 8.0 ml/min with a solvent system of acetonitrile (Aldrich Chemical Co.) and water (Millipore, Billerica, MA) as follows: 5% acetonitrile for 10 min, 5% acetonitrile to 90% acetonitrile in 60 min, and holding at 90% acetonitrile for 20 min. Injection volume was 20 ml. The fraction containing the mixture of glyceollins was concentrated under vacuum and freeze-dried.

For isolation of individual glyceollins I and II, semipreparative HPLC was utilized with an ODS-2 (10 µm 500-mm) column (Whatman, Maidestone, UK) and a flow rate of 3.0 ml/min with the solvent system of acetonitrile (Aldrich Chemical Co.) and water (Millipore) as follows: 5% acetonitrile for 15 min, 5% acetonitrile to 90% acetonitrile in 40 min, and holding at 90% acetonitrile for 20 min. The presence and purity of glyceollins I and II (98% and 96% purity, respectively) were confirmed by multiple analyses