Gibberellic acid induces α-amylase expression in adipose-derived stem cells

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Received March 7, 2012; Accepted April 19, 2012

DOI: 10.3892/ijmm.2012.1007

Abstract. Salivary α-amylase is the most important enzyme for oral digestion of dietary starch. Therefore, regeneration of the salivary glands via a tissue engineering approach is clearly required for patients with salivary gland dysfunction. Early during seed germination, the embryo synthesizes gibberellic acid (GA_3), a plant hormone that activates the synthesis and secretion of α-amylase. The purpose of this study was to explore an approach for differentiation of stem cells into salivary glands using GA_3. We isolated adipose-derived stem cells (ASCs), which are positive for mesenchymal stem cell markers (CD73, CD90 and CD105) and possess pluripotency to osteoblasts, adipocytes and neural cells, from human buccal fat pads, which are a readily available source for dentists and oral surgeons. In addition, we investigated the cytotoxicity of GA_3 for human ASCs. GA_3 neither affects cell morphology nor cell viability in a dose- or time-dependent manner. ASCs were incubated with GA_3 to assess mRNA and protein expression of α-amylase by reverse transcriptase-polymerase chain reaction and western blot analyses. α-amylase mRNA expression on 21 days after treatment with GA_3 (1 mM) was seven times greater than that in resting condition (Day 0). While we did not detect α-amylase bands on Day 0, α-amylase protein was detectable 7 days after treatment with GA_3, reaching a maximal level on Day 21. Our results indicated that GA_3 can increase cellular α-amylase expression and that our induction method would be useful for therapeutic application for salivary gland regeneration.

Introduction

In human saliva, α-amylase is the most abundant protein (1), accounting for 40-50% of salivary protein (2), and has the important capacity to rapidly alter the physical properties of starch in the oral cavity (3). Aging or radiation therapy for head and neck cancer leads to severe salivary gland dysfunction and consequential xerostomia (dry mouth syndrome), resulting in hampered speech, dental problems, difficulties with swallowing and food mastication, impaired taste, and nocturnal oral discomfort (4-6).

Mesenchymal stem cells (MSCs) have been isolated from various tissues, such as bone marrow (7), muscle (8), skin (9), and adipose tissue (10). Among them, adipose tissue contains 100- to 300-fold more MSCs than the bone marrow (11). Recent studies have identified adipose-derived stem cells (ASCs) that can differentiate along multiple pathways, including into osteogenic, adipogenic, myogenic, and chondrogenic lineages, if an appropriate environment is provided (12-17). Thus, ASCs have increasingly gained importance due to their abundance in tissues and easy availability for extraction (1).

Plant hormones are small organic molecules commonly used to increase grain production (18,19). Among the hormones, gibberellic acid (GA_3), a plant growth regulator, is used worldwide to increase the growth of fruits, such as strawberries, grapes, and date palm (20) and of some vegetables, such as tomatoes, cabbages, cauliflower, peppers, and olives (21-23). Signal transduction pathways of GA_3 enable aleurone cells to modulate hydrolase production, mainly α-amylase, in response to hormonal and environmental stimuli. These enzymes digest the stored starch and other nutrients in the endosperm to support the growth of young seedlings.

Although GA_3 is widely used in agriculture, its effects on human health have not been well explored. Thus, we focused on the potential effects of GA_3 and demonstrated a novel induction approach that buccal fat pad (BFP)-derived ASCs differentiate into salivation cells with GA_3 treatment.

Materials and methods

Primary culture of human ASCs. BFPs were obtained from healthy donors at Chiba University Hospital, Chiba, Japan. All donors provided written informed consent for a protocol reviewed and approved by the institutional review board of Chiba University. To isolate ASCs, we performed the centrifuge methods described previously (12). Briefly, the adipose tissues were harvested, washed extensively with PBS, minced

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Key words: salivary gland, α-amylase, adipose-derived stem cell, gibberellic acid, buccal fat pad

for 10 min with fine scissors, and enzymatically digested at 37˚C for 40 min with 0.1% collagenase (Wako, Osaka, Japan). An equal volume of control medium (Dulbecco's modified Eagle's medium/F-12; Sigma-Aldrich Co., St. Louis, MO) containing 10% fetal bovine serum (FBS; Sigma Aldrich Co.) and 50 U/ml penicillin and streptomycin (Sigma Aldrich Co.) was then added to neutralize the collagenase. The cell suspension was centrifuged at 1,300 rpm (260 x g) for 5 min to obtain a high-density ASC pellet, which was resuspended in control medium. After being counted using trypan blue, the cells were plated at a concentration of $5x10^5$ cells/100-mm cell culture dishes (BD Biosciences, Franklin Lakes, NJ) and kept in the control medium at 37˚C in 5% CO_2.

Flow cytometric analysis of ASCs. Cultured ASCs were washed twice in cold PBS supplemented with 2% FBS (Sigma-Aldrich Co.) and resuspended to a concentration of about $1x10^6$ cells/antibody test and labeled with anti-human CD73-PE, CD90-FITC, CD105-PerCP, CD31-PE, CD34-PerCP, and CD45-FITC antibodies for 20 min at room temperature in the dark (BD Biosciences). The labeled cells were analyzed using a fluorescence-activated cell sorter (FAC; BD Biosciences). Negative control stains were performed using FITC-, PE- and PerCP-conjugated mouse IgG1 κ isotypes (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Differentiation culture conditions. To induce osteogenic differentiation, ASCs were cultured in an osteogenic differentiation basal medium containing osteogenic supplement (Invitrogen, Carlsbad, CA). After 3 weeks, osteogenic differentiation was evaluated with alkaline phosphatase (ALP) staining (Primary Cell Co., Ltd., Hokkaido, Japan). Adipogenic differentiation of ASCs was induced by adipocyte differentiation basal medium containing an adipogenic supplement (Chemicon International, Inc., Temecula, CA) for 4 weeks. After induction, the cells were stained with Oil Red O (Sigma). To induce neural differentiation, ASCs were grown in neural differentiation medium (Thermo Fisher Scientific, Rockford, IL) for 3 days. The induced cells were subjected to immunocytochemical analysis to assess the expression of nestin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), a neural marker.

GA_3 cytotoxicity. ASCs were seeded at a density of $1x10^4$ cells/60-mm cell culture dishes (BD Biosciences) in the control medium with the indicated concentrations of GA_3 for the indicated time points. The effect of GA_3 cytotoxicity on the numbers of ASCs was determined using phase-contrast microscopy and a trypan blue exclusion test.

Treatment of ASCs with GA_3. The ASCs at 80% confluence were incubated in the control medium with the indicated concentrations of GA_3. ASCs were harvested for extraction of total-RNA and protein at 0, 7, 14, 21 and 28 days after 1 mM GA_3 treatment.

Preparation of cDNA. Total-RNA was isolated using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. cDNA was generated from 5 μg of total-RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) and oligo(dt) primer (Sigma-Genosys, Ishikari, Japan), according to the manufacturer's instructions.

mRNA expression analysis. To evaluate the expression levels of α-amylase in ASCs, real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed. qRT-PCR was carried out with one method using a LightCycler FastStart DNA Master SYBR-Green I kit (Roche Diagnostics GmbH, Mannheim, Germany). The PCR reactions using the LightCycler apparatus were performed in a final volume of 20 μl of a reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR-Green I mix, 3 mM $MgCl_2$, and 1 μM primers, according to the manufacturer's instructions. The reaction mixture was loaded into glass capillary tubes and subjected to an initial denaturation at 95˚C for 10 min, followed by 45 rounds of amplification at 95˚C (10 sec) for denaturation, 62˚C (10 sec) for annealing, and 72˚C (10 sec) for extension, with a temperature slope of 20˚C/sec. Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain size and purity. The transcript amounts for the target genes were estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript amount determined in corresponding samples. The following primers were used: α-amylase, forward, 5'-ATTTTCATGTCGCCCGTTGT-3' and reverse, 5'-CCCATGTGATGGACCAATGTC-3'; GAPDH, forward, 5'-CATCTCTGCCCCCTCTGCTGA-3' and reverse, 5'-GGATGACCTTGCCCACAGCCT-3'.

Protein extraction. The cells were washed twice with cold PBS and centrifuged briefly. The cell pellets were incubated at 4˚C for 30 min in a lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, and 10 mM Tris pH 7.4) with a proteinase inhibitor cocktail (Roche Diagnostics). The protein concentration was measured with the BCA Protein Assay kit (Thermo Scientific).

Evaluation of α-amylase protein expression by western blot analysis. Protein extracts were electrophoresed on 4-12% Bis-Tris gels, transferred to nitrocellulose membranes (Invitrogen), and blocked for 1 h at room temperature in Blocking One (Nacalai Tesque, Kyoto, Japan). The membranes were washed three times with 0.1% Tween-20 in Tris-buffered saline and incubated with anti-human α-amylase (1:100 dilution) and β-actin (1:1,000 dilution) monoclonal antibodies (Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The membranes were washed again and incubated for 1 h at room temperature with a 1:2,500 of goat anti-mouse IgG (H+L) HRP conjugate (Promega, Madison, WI) as a secondary antibody. Finally, the membranes were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) and immunoblotting was visualized by exposing the membranes to ATTO Light-Capture II (ATTO, Tokyo, Japan). Signal intensities were quantitated using the CS Analyzer version 3.0 software (ATTO).

Results

Isolation of ASCs from human BFPs. FACS analysis of BFP-derived ASCs at the fifth passage showed that the cells

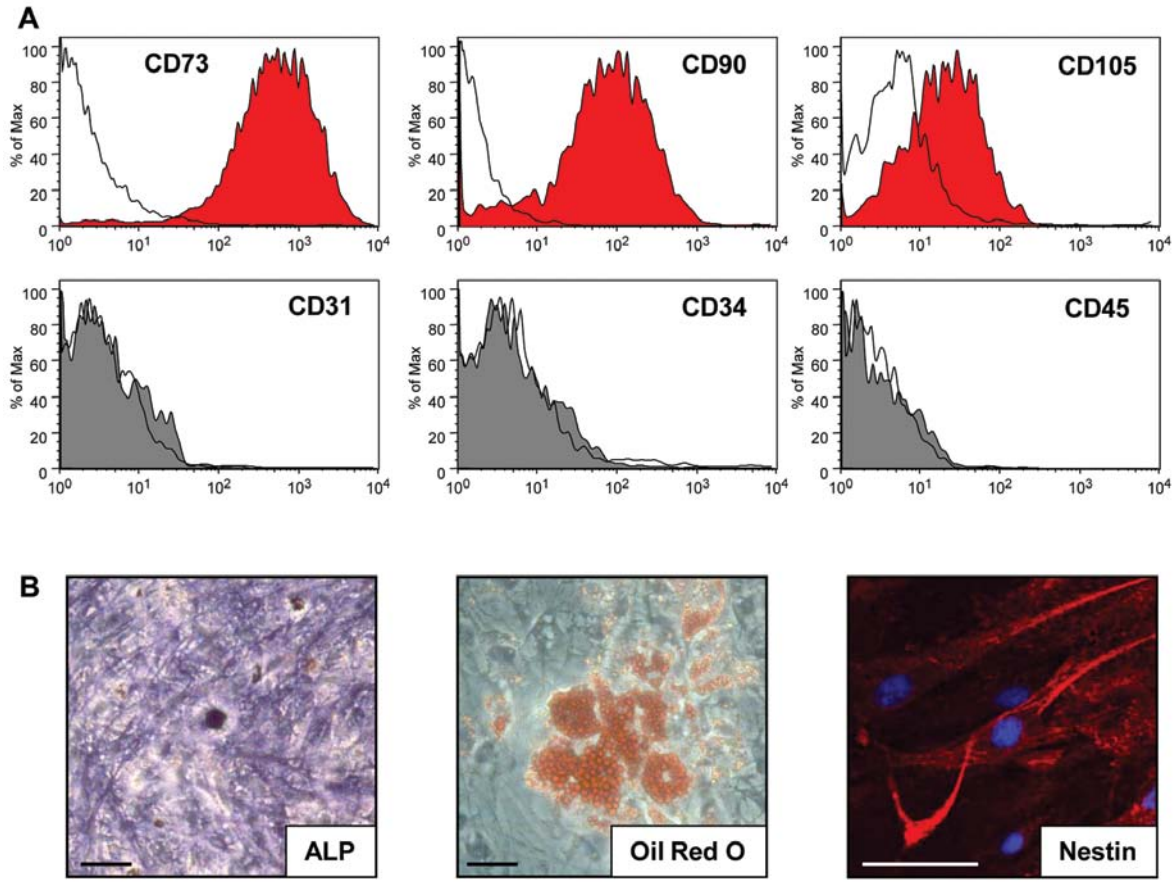

Figure 1. Isolation and characterization of ASCs. (A) The ASCs isolated from BFPs are labeled with antibodies specific to CD31, 34, 45, 73, 90 and 105. The surface phenotype is analyzed by FACS. The ASCs are positive for CD73, CD90 and CD105, cell surface markers for MSCs but do not express hematopoietic stem cell markers including CD31, CD34 and CD45. (B) The cells are cultured for 3 weeks in osteogenic differentiation media. Osteogenic differentiation is seen with ALP staining (left). The cells are cultured for 4 weeks in adipogenic differentiation media. Adipogenic differentiation is shown with Oil Red O (middle). The cells are cultured for 3 days in neural differentiation media. Neural differentiation is seen with immunocytochemistry for nestin (right). Scale bars, 50 μm.

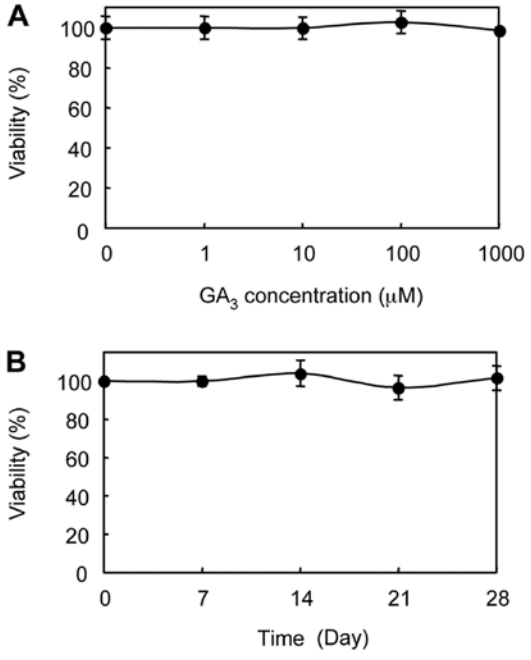

Figure 2. Effect of GA_3 on cell viability. (A) The ASCs are incubated with the indicated concentrations of GA_3 for 72 h. (B) The ASCs are incubated with 1 mM GA_3 for the indicated times. GA_3 does not affect ASC cell viability in a time- or dose-dependent manner. Data are expressed as the means of the percentage of cell viability (%) and ± SEM of the mean.

expressed the cell surface markers, CD73, CD90, and CD105 but not CD31, CD34 and CD45 (Fig. 1A). These results are consistent with the definition that MSCs must express CD73, CD90 and CD105, as suggested by Dominici *et al* (24). ASCs did not spontaneously differentiate during culture expansion. To determine whether ASCs from BFPs can differentiate into various cell types, such as osteoblasts, adipocytes, and neural cells *in vitro*, ASCs were cultured in specific selection media. After 3 weeks in the osteogenic medium culture, the cells differentiated into osteoblasts, which were confirmed with strong ALP staining (Fig. 1B). After 4 weeks in the adipogenic differentiation culture, the cells differentiated into lipid-laden cells that were stained with Oil Red O (Fig. 1B). After 3 days of neural differentiation culture, the ASCs differentiated into neural cells, which were confirmed with immunocytochemistry for nestin (Fig. 1B). These results showed that ASCs from BFPs can multidifferentiate.

Cytotoxicity of GA_3. Ishii *et al* (25) reported that plant hormones are closely related to anticancer therapy. We treated the ASCs with GA_3 to determine the cytotoxic effect. GA_3, up to 1 mM, did not affect the cell viability of ASCs in a dose- or time-dependent manner (Fig. 2). In addition, there were no morphologic changes when we challenged the ASCs with GA_3 (data not shown).

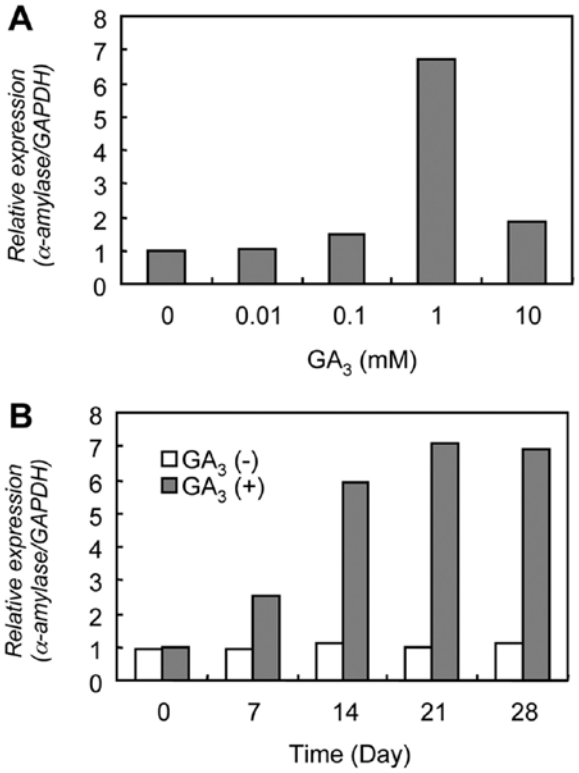

Figure 3. Typical results of expression of α-amylase mRNA in GA_3-treated ASCs. (A) The ASCs are treated with indicated concentrations of GA_3 for 14 days. (B) The ASCs are treated with GA_3 for the indicated periods. Untreated cultures are controls (0 day). α-amylase mRNA reaches a maximum level on Day 21. Quantification of mRNA levels in ASCs by qRT-PCR. The experiments repeated three times with similar results.

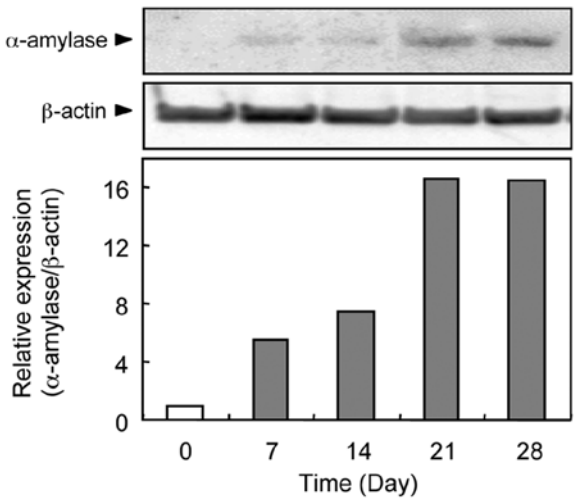

Figure 4. Representative results of expression of α-amylase protein in GA_3-treated ASCs. To investigate α-amylase protein expression in untreated and GA_3-treated ASCs, we performed western blot analysis. Untreated cultures are controls (0 day). α-amylase protein is evident 7 days after treatment with GA_3, reaching a maximal level on Day 21. The experiments were repeated three times with similar results.

Evaluation of α-amylase mRNA expression. The result of qRT-PCR analysis for α-amylase mRNA expression is shown in Fig. 3. Higher α-amylase mRNA expression was found after treatment with 1 mM GA_3 for 14 days. α-amylase mRNA expression reached its maximum on 21 days after 1 mM GA_3 treatment, which was 7-fold than that of resting conditions (0 day).

Evaluation of α-amylase protein expression. We performed western blot analysis to determine the α-amylase protein expression status in the GA_3-treated ASCs. Representative results of western blot analysis for α-amylase protein expression are shown in Fig. 4. We did not detect any α-amylase protein bands under resting conditions (0 day). α-amylase protein became evident 7 days after treatment with GA_3, reaching a maximal level on Day 21.

Discussion

The current study showed that GA_3, a plant growth regulator, plays an important role in regulating α-amylase in BFP-derived ASCs and that the induction method could be an emerging potential therapeutic approach for regenerating salivary glands.

ASCs have been recognized as an efficient source of adult stem cells because of their easy accessibility, minimal morbidity upon harvesting, and abundance of stem cells compared with bone marrow-derived MSCs (11). Moreover, ASCs can be propagated more rapidly, and they retain their mesenchymal pluripotency after multiple passages (15). We isolated ASCs from BFPs, adipose-encapsulated masses in the oral cavity, and revealed that BFP-derived ASCs showed positive MSC markers and pluripotency. BFPs are an easy source for dentists and oral surgeons who treat patients for dry mouth syndrome.

The digestion of dietary starch in humans is initiated by salivary α-amylase, an endo-enzyme that hydrolyzes starch into maltose, maltotriose, and larger oligosaccharides. Salivary α-amylase accounts for 40 to 50% of protein in human saliva and rapidly alters the physical properties of starch. This amylolytic digestion begins during mastication in the oral cavity and continues in the stomach (1-3).

Gibberellins were identified initially in the 1930s as a product of a fungus, which caused excessive shoot elongation. Further studies found that gibberellins are also involved in other processes, e.g., promoting flowering and seed germination (18). One gibberellin, GA_3, accelerates and improves the yield of a wide variety of plants by increasing cell division (18,26). Early in seed germination, the embryo synthesizes GA_3, which diffuses to the aleurone cells in which GA_3 acts as a signal to activate synthesis and secretion of α-amylases and other hydrolases. While GA_3 is widely used in agriculture, only a few experiments have examined the possible toxic effects in mammals. A previous study reported that gibberellin derivatives had strong anticancer activities by inhibiting topoisomerase I activity in rodents (27). To determine the effect of GA_3 on cell viability in ASCs, we carried out a cytotoxic assay of ASCs using several concentrations of GA_3 for a maximum of 28 days. GA_3 never affected cell viability or cell morphology up to 1 mM. However, some groups reported that exposure of GA_3 induced oxidative stress and histopathological changes to rats (28,29). Therefore, further studies with more *in vivo* samples are needed to address the status of α-amylase expression after GA_3 treatment in greater detail.

The aleurone layer of cereal grains is the most widely studied and best characterized system for studying the activity of GA_3. To date, at least one GA_3 receptor is present in the plasma membrane (30) and there is evidence of a number of other components of the pathways, including Ca^{2+} (31,32), lipases (33), cGMP (34), protein phosphatases (35), an endoplasmic reticulum-located Ca^{2+}-ATPase, inositol-1,4,5-triphosphates, and Ca^{2+}/calmodulin (36) at the early stage of GA_3 signal transduction. The GA_3-regulated myb gene, GAmyb, may be a component of the GA_3 response pathway and has been shown to transactivate the α-amylase promoter (37). In the present study, we found that GA_3 regulated α-amylase expression in human ASCs, suggesting that mammalian cells also may have a GA_3 response pathway. Since the mammalian signal transduction pathways of GA_3 are unknown, further studies are required to reveal the pathway for α-amylase expression.

The potential effects of GA_3 on human health have not been explored. This is the first report to show that GA_3 treatment can increase the expression of cellular α-amylase and that our induction method might be a useful therapeutic application for salivary gland regeneration.

Acknowledgements

We thank Dr Hiroshi Mizuno and Dr Morikuni Tobita, Juntendo University, Japan, for helpful discussions and critical review of the manuscript; Lynda C. Charters for editing this manuscript; and Dr Hiroshi Nakajima and Dr Hiroaki Takatori, Department of Molecular Genetics, Graduate School of Medicine, Chiba University, for assistance with the FACS experiments.

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