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Effect of Growth Hormone on In Vitro Osteogenesis and Gene Expression of Human Osteoblastic Cells Is Donor-Age-Dependent

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Abstract It has been demonstrated that the effect of GH on bone tissue is reduced with aging. In this study we tested the hypothesis that the action of GH on osteoblastic cells is donor-age-dependent by investigating the effect of GH on the development of osteoblastic phenotype in cultures of cells from adolescents (13–16 years old), young adults (18–35 years old), and adults (36–49 years old). Osteoblastic cells derived from human alveolar bone were cultured with or without GH for periods of up to 21 days, and parameters of in vitro osteogenesis and gene expression of osteoblastic markers were evaluated. GH increased culture growth, collagen content and alkaline phosphatase (ALP) activity in cultures from adolescents and young adults, whereas non-significant effect was observed in cultures from adults. While GH significantly increased the bone-like formation in cultures from adolescents, a slightly effect was observed in cultures from young adults and no alteration was detected in cultures from adults. Results from real-time PCR demonstrated that GH upregulated ALP, osteocalcin, type I collagen, and Cbfa1 mRNA levels in cultures from adolescents. In addition, cultures from young adults showed higher ALP mRNA expression and the expression of all evaluated genes was not affected by GH in cultures from adults. These results indicate that the GH effect on both in vitro osteogenesis and gene expression of osteoblastic markers is donor-age-dependent, being more pronounced on cultures from adolescents. J. Cell. Biochem. 104: 369–376, 2008.

Key words: growth hormone; osteoblastic cells; in vitro osteogenesis; aging; gene expression

Growth hormone (GH) is an important systemic regulator of longitudinal bone growth throughout adolescence phase, whereas in adults GH plays a key role in the maintenance of bone mass by regulating bone remodeling [Laursen, 2004; Ueland, 2004]. The synthesis and secretion of GH reach the peak during the puberty and decline with aging [Corpas et al., 1993]. The therapeutic use of GH has been indicated for both children with bone growth delay and adults with physiological GH deficiency as a replacement therapy to increase bone mineral density (BMD) [Laursen, 2004]. However, the effect of GH replacement on BMD in adults is uncertain. A meta-analysis study reported a non-significant response to GH replacement on BMD [Davidson et al., 2004] and other authors observed a more accentuated effect in young adult than in old patients [Välimäki et al., 1999; Hubina et al., 2004]. Therefore, it is possible to speculate that osteoblasts become less responsive to GH treatment with aging.

Over the years, the cellular bases of GH effects employing in vitro systems with isolated homogenous bone cell populations have been thoroughly studied. The advantages of these

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systems are that the environment can be more carefully controlled and the results are not masked by secondary responses to extra-skeletal regulatory systems [Cooper et al., 1998]. Studies using different cell culture models have demonstrated that GH directly regulates biological functions of the osteoblasts [Slootweg et al., 1988a,b; Kassem et al., 1993, 1994a,b]. GH stimulated proliferation and expression of differentiation markers of both fetal mouse and chicken osteoblasts [Slootweg et al., 1988a,b]. The effects of GH on human osteoblastic cells differ according the cell source and culture conditions. In short-term cultures of osteoblastic cells derived from trabecular bone, GH stimulated proliferation and synthesis of both alkaline phosphatase (ALP) and osteocalcin (OC) [Kassem et al., 1993]. In osteoblasts differentiated from mesenchymal stem cells, GH also stimulated proliferation, but there was no or few inhibitory effect on production of both ALP and OC [Kassem et al., 1994b].

Several genes that encoding osteoblastic markers, including ALP, type-I collagen (COL), osteopontin (OP), and OC have been demonstrated to be expressed at high levels for discrete periods during in vitro osteogenesis [Beck et al., 2003; Zur Nieden et al., 2003] and may be modulated by GH exposure. ALP is expressed in large amounts during osteoblast differentiation and exerts its role in the early stages of osteogenesis [Bellows et al., 1992]. COL is prevalent and comprises 90% of the total organic bone matrix [Olsen, 1996]. At early stages of osteoblast development, OP, a phosphorylated glycoprotein, is secreted and acts linking organic and inorganic phases to promote tissue adhesion [McKee and Nanci, 1996]. OC protein is a member of the Gla protein family, constitutes 1–2% of the total protein of bone extracellular matrix and influences bone mineralization [Hoang et al., 2003]. The core binding factor alpha 1 (Cbfa1) gene is involved with osteoblast differentiation and it is also expressed during in vitro osteogenesis [Ducy et al., 1997; Harada et al., 1999].

Based on different responses to GH by bone tissue from adolescents and adults, we hypothesized that the action of GH on osteoblastic cells is donor-age-dependent. In order to test such hypothesis, we investigated the effect of GH on the development of osteoblastic phenotype in cultures of cells from adolescents (aged between 13 and 16 years), young adults (aged between18 and 35 years), and adults (aged between 36 and 49 years).

MATERIALS AND METHODS

GH Preparation

The GH was purchased from Novo Nordisk A/S (Copenhagen, Denmark) and was directly dissolved in the culture medium at concentrations of 100, 200, and 300 ng/ml. The concentration-response curve was previously designed (1–1,000 ng/ml—data not shown) and the most effective concentrations were used in this study.

Culture of Osteoblastic Cells Derived From Human Alveolar Bone

Human alveolar bone fragments (explants) discarded from oral surgical procedures were obtained from healthy women that were not under hormone therapy replacement using the research protocol approved by the Committee of Ethics in Research from School of Dentistry of Ribeirao Preto, USP, Brazil. Osteoblastic cells were obtained from these explants by enzymatic digestion using type II collagenase (Gibco—Life Technologies, Grand Island, NY) as described by Mailhot and Borke [1998]. These cells were cultured in α-minimum essential medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 50 μg/ml vancomycin (Acros Organics, Gell, Belgium), 20 μg/ml ampicillin (USB Corporation, Cleveland, OH), 0.3 μg/ml fungizone (Gibco), 10^{-7} M dexamethazone (Sigma, St. Louis, MO), 5 μg/ml ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma). Such culture conditions favored the development of the osteoblast phenotype [Coelho and Fernandes, 2000; Rosa and Beloti, 2003]. Subconfluent cells in primary culture were harvested after treatment with 1 mM ethylenediamine tetracetic acid (EDTA) (Gibco) and 0.25% trypsin (Gibco) and subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ) at a cell density of 2×10^4 cells/well in culture medium with or without GH. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and the medium was changed every 3 or 4 days.

Evaluation of In vitro Osteogenesis

The effect of GH (100, 200, and 300 ng/ml medium) was investigated on different parameters of the in vitro osteogenesis using cells from 14 donors: five aged between 13 and 16 years

(adolescents), four aged between 18 and 35 years (young adults) and five aged between 36 and 49 years (adults).

Culture Growth and Viability

To evaluate culture growth and viability, cells were subcultured for 4, 7, and 10 days and enzymatically released (1 mM EDTA—Gibco, 1.3 mg/ml collagenase—Gibco, and 0.25% trypsin—Gibco). Viable and non-viable cells were detected by trypan blue (Sigma) and counted using a haemocytometer (Housser Scientific Company, Horsham, PA). Cell proliferation was expressed as absolute number of cells at days 4, 7, and 10. In addition, it was calculated the doubling time [Patterson, 1979] in hours between 4 and 7 days, which was expressed as a percentage of control. Cell viability was expressed as a percentage of control of the viable cells.

Collagen Content

Collagen content was evaluated at the early stage of matrix maturation, at 7 days, according to a Reddy and Enwemeka [1996] method. The wells were filled with 2 ml of 0.1% sodium lauryl sulfate (Sigma). After 30 min, aliquots containing 1 ml of this solution were lyophilized and resuspended in 100 μl of acetic acid 6 N. Then, the samples were hydrolyzed by autoclaving at 120°C for 30 min. After this procedure, 900 μl of chloramine T (Acros Organics) was added to the hydrolyzed, mixed and the oxidation was allowed to proceed for 25 min at room temperature. Then, 1 ml of Ehrlich's aldehyde reagent was added to each sample, mixed and the color was developed by incubating the samples at 65°C for 20 min. The absorbance was spectrophotometrically measured at 550 nm. The collagen content was calculated from a standard curve, normalized by the total protein content measured by the Lowry method [Lowry et al., 1951], and expressed as a percentage of control.

ALP Activity

ALP activity was evaluated at 7 days. The assay was based on the thymolphthalein released from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica SA, MG, Brazil). Samples from the same solutions used for calculating the collagen content were assayed for measuring ALP activity according to the kit instructions. Briefly, 50 μl of thymolphthalein monophosphate was mixed with 0.5 ml of 0.3 mmol/ml diethanolamine buffer, pH 10.1, and incubated for 2 min at 37°C before the addition of the 50 μl of lysates. After 10 min of incubation at 37°C, 2 ml of 0.09 mmol/ml Na_2CO_3 and 0.25 mmol NaOH was added to allow color development to occur and absorbance was measured at 590 nm. ALP activity was calculated from a standard curve using thymolphthalein, giving a range from 0.012 to 0.40 μmol thymolphthalein/h/ml. Results were calculated and normalized by the total protein content and expressed as a percentage of control.

Bone-Like Formation

At 21 days, cells were fixed, dehydrated through a graded series of alcohol, processed for staining with Alizarin Red S (Sigma) and bone-like formation was evaluated by two methods: image analysis and colorimetric method.

To evaluate the images of mineralized area, ten ×10 microscopic fields of each well were randomly selected and photographed with a high-resolution digital camera (Canon EOS Digital Rebel Camera, 6.3 Megapixel CMOS sensor, with a Canon EF 100 mm f/2.8 macro lens, Canon, Tokyo, Japan). Mineralized area was measured using an image analyzer (Image Tool—University of Texas Health Science Center, San Antonio, TX), and expressed as a percentage of control.

The quantification of staining was also evaluated by a colorimetric method according to [Gregory et al., 2004]. Briefly, 280 μl of 10% acetic acid was added to each well previously stained with Alizarin Red S, and the plate was incubated at room temperature for 30 min under shaking. The attached monolayer was then scraped from the plate with a cell scraper and transferred with 10% acetic acid to a microcentrifuge tube. After vortexing for 30 s, the slurry was overlaid with 100 μl of mineral oil (Sigma), heated to exactly 85°C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000*g* for 15 min and 200 μl of supernatant was transferred to a new microcentrifuge tube. Then, 80 μl of 10% ammonium hydroxide was added to neutralize the acid and this solution was read at 405 nm in 96-well format using opaque-walled transparent-bottomed plates (Fisher Scientific, Pittsburgh, PA) on the plate reader (μQuant, Biotek, Winooski, VT). Results were calculated based on an Alizarin Red S standard curve and expressed as a percentage of control.

Gene Expression Analysis Using Real-Time PCR

Based on our in vitro osteogenesis results, we investigated the GH effect, using the more effective concentration (100 ng/ml), on gene expression of the following osteoblastic markers, ALP, OC, COL, OP, and Cbfa1, at 7 days. These experiments were carried out using cells from other nine donors: three aged between 13 and 16 years (adolescents), three between 18 and 35 years (young adults), and three between 36 and 49 years (adults).

RNA Extraction and Quantitative Real-Time PCR

The primer sequences, the predicted amplicon sizes and the annealing and melting temperatures, were designed using the Primer-Express software (Applied Biosystems, Foster City, CA) are depicted in Table I.

Total RNA from osteoblasts was extracted using the Promega RNA extraction kit (Promega, Madison, WI), according to the manufacturer's instructions. The concentration of RNA was determined by optical density at a wavelength of 260 nm, using the GeneQuant (Amersham Biosciences, Piscataway, NJ). Complementary DNA (cDNA) was synthesized using 2 μg of RNA through a reverse transcription reaction (M-MLV reverse transcriptase, Promega). Real-time PCR quantitative mRNA analyses were performed in an ABI Prism 7000 Sequence Detection System using the SybrGreen system (Applied Biosystems). SybrGreen PCR MasterMix (Applied Biosystems), specific primers and 2.5 ng of cDNA were used in each reaction. The standard PCR conditions were 95°C (10 min) and 40 cycles of 94°C (1 min), 56°C (1 min) and 72°C (2 min), followed by the standard denaturation curve. For mRNA analysis, the relative level of gene expression was calculated in reference to both β-actin expression in the sample and its respectively control using the cycle threshold (Ct) method [Livak and Schmittgen, 2001].

Statistical Analysis

For evaluation of the in vitro osteogenensis, cells from each donor were used separately to carry out each experiment. For each experiment, all evaluated parameters and treatments were assayed in quintuplicate (five replications) and results were expressed as mean ± standard deviation. Statistical analyses were carried out separately for each donor and the presented results are from cultures of one representative donor from each age group. Analysis of variance followed by Duncan test was performed to assess the significance of GH effect on cell number. Kruskal–Wallis test followed by Fischer test were performed to assess the significance of GH effect on all other parameters of in vitro osteogenesis, which were expressed as a percentage of control. Differences at $P \leq 0.05$ were considered statistically significant.

The real-time PCR experiments were carried out in triplicate (three replications for each donor and gene evaluated) and data presented as mean ± standard deviation. Comparisons were conducted using the Kruskal–Wallis test followed by Fischer test and differences at $P \leq 0.05$ were considered statistically significant.

RESULTS

Evaluation of In Vitro Osteogenesis

Culture growth and viability. At 7 days, all concentrations of GH increased, in a similar way, the number of cells from both adolescents

TABLE I. Primers Sequences and Reaction Properties

Target	Sense and anti-sense sequences	T_A (½C)	T_M (°C)	bp
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495
ALP	ACGTGGCTAAGAATGTCATC CTGGTAGGCGATGTCCTTA	60	86	475
OC	CAAAGGTGCAGCCTTTGTGTC TCACAGTCCGGATTGAGCTCA	62	85	150
COL	TGACGAGACCAAGAACTG CCATCCAAACCACTGAAACC	61	84	114
OP	AGACACATATGATGGCCGAGG GGCCTTGTATGCACCATTCAA	58	79	154
Cbfa1	TATGGCACTTCGTCAGGATCC AATAGCGTGCTGCCATTCG	61	83	110

ALP, alkaline phosphatase; OC, osteocalcin; COL, type I collagen; OP, osteopontin; Cbfa1, core binding factor alpha 1; T_A, annealing temperature; T_M, melting temperature; bp, product size.

and young adults, whereas number of cells from adults in all evaluated periods was not affected by GH (Fig. 1A–C). Doubling time, calculated between 4 and 7 days, was reduced by GH in cultures from adolescents and young adults, which indicates an increase in cell proliferation rate (Fig. 1D). No significant effect on the doubling time was observed in cultures from adults (Fig. 1D). Cell viability was not affected by GH in cultures from all donor groups in all evaluated periods and concentrations (data not shown).

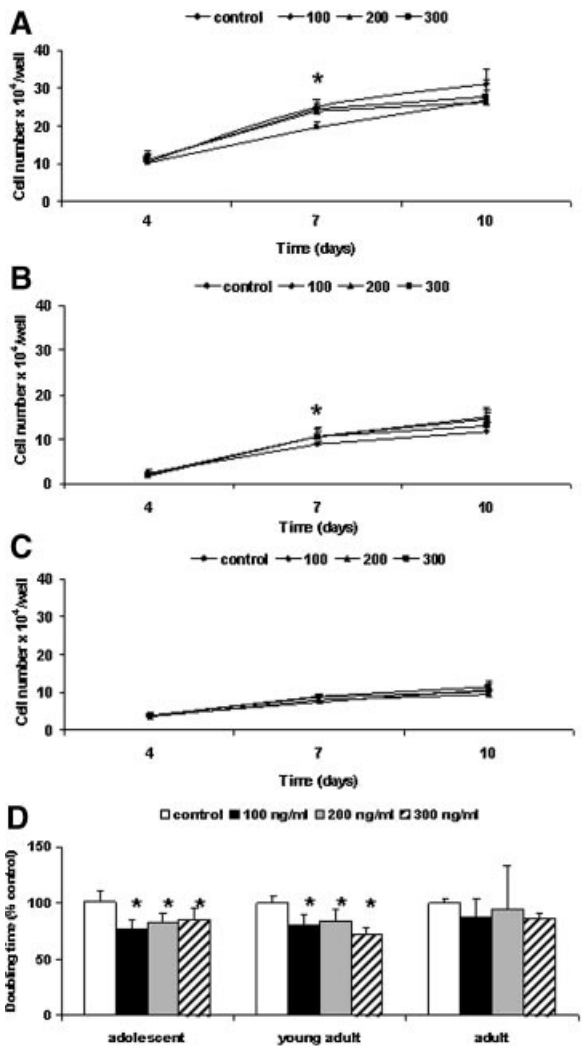

Fig. 1. GH effect on culture growth. GH effect (100, 200, and 300 ng/ml) on number of cells in osteoblastic cultures from adolescent (**A**), young adult (**B**), and adult (**C**) donors. Culture growth was analyzed at different time points (4, 7, and 10 days) and expressed as mean ± standard deviation. GH effect (100, 200, and 300 ng/ml) on doubling time (**D**) in osteoblastic cultures from adolescent, young adult and adult donors, expressed as mean ± standard deviation. $*P < 0.05$ comparing control with GH at different concentrations at the same time point.

Collagen content and ALP activity. Collagen content was increased by all evaluated GH concentrations in cultures from both adolescents and young adults (Fig. 2A). All evaluated GH concentrations, increased in the same way the ALP activity in cultures from adolescents (Fig. 2B). In cultures from young adults, ALP activity was increased by 100 and 200 ng/ml of GH, but no significant effect was observed at 300 ng/ml (Fig. 2B). Both ALP activity and collagen content were not affected by GH in cultures from adults (Fig. 2A,B).

Bone-like formation. At 21 days, cultures from adolescents exhibited considerable increase in bone-like formation induced by GH in all evaluated concentrations as demonstrated by measurement of mineralized area and colorimetric assay (Fig. 3A,B). In cultures from adolescents, the most pronounced effect on mineralized area was induced by 100 ng/ml of GH (Fig. 3A). The colorimetric assay demonstrated similar increase matrix mineralization in cultures exposed to 100 and 300 ng/ml (Fig. 3B). In cultures from young adults, GH at concentrations of 100 and 300 ng/ml increased bone-like formation measured by mineralized area (Fig. 3A) but no significant difference was detected by colorimetric assay (Fig. 3B). Bone-like formation was not affected by GH in cultures from adults (Fig. 3A,B).

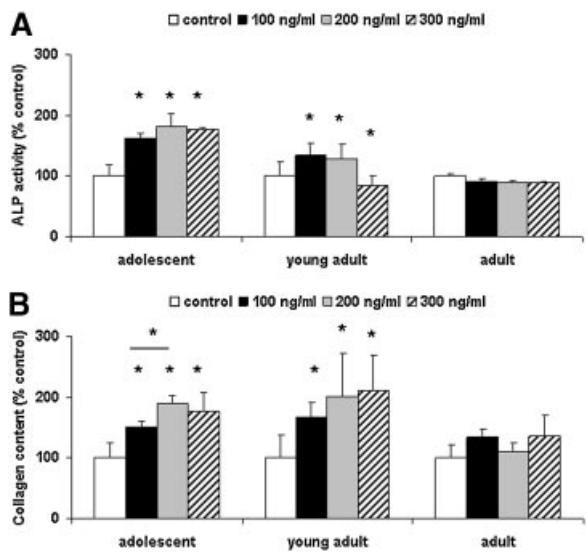

Fig. 2. GH effect on collagen content and ALP activity. GH effect (100, 200, and 300 ng/ml) on collagen content (**A**) and ALP activity (**B**) in osteoblastic cultures from adolescent, young adult, and adult donors at 7 days. Collagen content and ALP activity were expressed as mean ± standard deviation. $*P < 0.05$ comparing control with GH at different concentrations at the same time point.

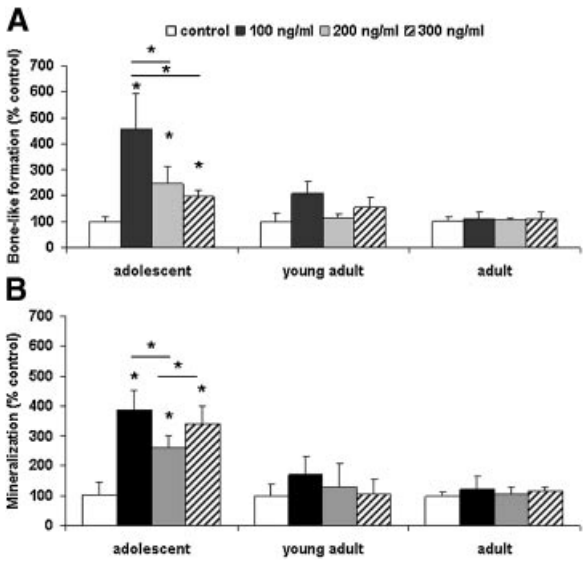

Fig. 3. GH effect on bone-like formation. GH effect (100, 200, and 300 ng/ml) on bone-like formation in osteoblastic cultures from adolescent, young adult, and adult donors, at 21 days. Bone-like formation evaluated by image analysis (**A**) and colorimetric method (**B**). Data were expressed as mean ± standard deviation. $^*P < 0.05$ comparing control with GH on different concentrations at same time point.

Gene Expression Analysis Using Real-Time PCR

Osteoblastic phenotype was confirmed at the transcriptional level by mRNA expression of the genes encoding ALP, OC, COL, OP, and Cbfa1 in cultures treated or not with GH. Results demonstrated that GH induced a significant increase in the expression mRNA for ALP, OC, COL, and Cbfa1 when compared with control in cultures from adolescents (Fig. 4). GH also increased the ALP mRNA expression in cultures from young adults (Fig. 4). OP mRNA expression was not affected by GH in cultures from adolescents (Fig. 4). In cultures from young adults, GH did not affect the mRNA expression for OC, COL, OP, and Cbfa1 (Fig. 4). None of the evaluated genes were influenced by GH in cultures from adults (Fig. 4). The effect of GH on both ALP and OC mRNA expression was consistently greater in cultures from adolescents compared with adults.

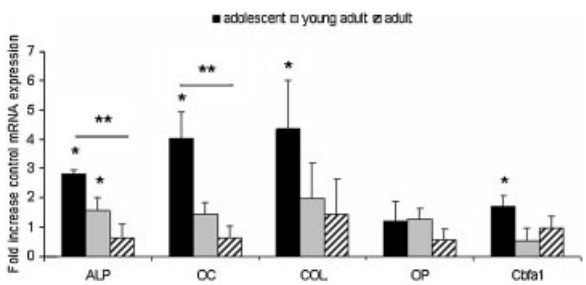

Fig. 4. GH effect on the mRNA expression of osteoblastic markers. GH effect (100 mg/ml) on ALP, OC, COL, OP, and Cbfa1 mRNA expression evaluated at 7 days using real-time PCR. The results are presented as the expression of the target mRNAs normalized to β-actin and to its respective control, mean ± standard deviation. $^*P < 0.05$ comparing with its respective control, $^{**}P < 0.05$, comparison among groups.

DISCUSSION

The present study demonstrated that long-term GH treatment of osteoblastic cells derived from human alveolar bone has an age-dependent effect on key parameters of in vitro osteogenesis, such as culture growth, ALP activity, collagen synthesis and bone-like formation. The GH effect on gene expression of osteoblastic markers, such as ALP, OC, COL, and Cbfa1 corroborated the in vitro osteogenesis findings, also demonstrating an age-dependency. It is worth of note that in general, osteoblastic cell cultures from adolescents were consistently stimulated by GH, whereas from young adults were slightly affected, and from adults were not. Considering that the bone response to GH replacement therapy is gender dependent [Välimäki et al., 1999; Hubina et al., 2004], in this study we used osteoblastic cells derived from only female donors.

It has been demonstrated that the effect of GH on some events of osteogenic cultures development, such as ALP activity, is dose-dependent [Gerland et al., 2000]. Nonetheless, this dose-dependency does not follow the same pattern in the expression of different key parameters of osteoblast phenotype within the same concentration range of GH [Kassem et al., 1993]. In addition, some parameters, such as OC and COL content, are not affected by GH in a dose-dependent way [Kassem et al., 1993]. In our study, the lack of concentration-response to GH on some evaluated parameters could be explained by the use of the most effective concentrations of GH as previously determined (data not shown).

Whereas the GH effect on human osteoblasts has been investigated only in short-term cultures and/or in cultures grown in non-osteogenic medium [Slootweg et al., 1988a; Kassem et al., 1993; Arvat et al., 1998], in the present study, the GH action was investigated in all stages of in vitro osteogenesis (proliferation, matrix maturation, and mineralization) [Beck

et al., 2003] in cultures grown in osteogenic medium. Then, it is possible to speculate that, in our culture conditions, GH would interact with a high proportion of cells exhibiting osteoblastic phenotype. This suggestion is supported by the gene expression of osteoblastic markers in both, control and GH treated cells as showed by real-time PCR results.

Our findings demonstrated that GH stimulated the growth of cell cultures from adolescents and young adults without significant effect on cultures from adults. The age-dependency of the GH effect on cell proliferation was also observed in cultures of human osteoblasts derived from fragments of femoral head bone [Pfeilschifter et al., 1993]. Such mitogenic effect was only observed during the proliferative phase (periods of up to 7 days) of the cultures. As a reciprocal relationship between the decrease in proliferation rate and the induction of cell differentiation was previously related [Owen et al., 1990], the lack of mitogenic effect after 7 days is most likely related to the enhancement of osteoblastic phenotype expression induced by GH.

The age-dependency of the GH effect was also observed on cell differentiation as demonstrated by the collagen content, ALP activity and mineralization assays as well as the mRNA expression for key markers of osteoblastic phenotype (ALP, OC, COL, OP, and Cbfa1). In general this GH stimulatory effect was significantly reduced with donor aging. The enhancement of matrix mineralization induced by GH in cell cultures from adolescents was attenuated in cultures from young adults and abolished in cultures from adults. As the in vitro osteogenesis process is regulated by a cascade of events, the decrease of the GH effect with aging on both bone-like formation and the expression of osteoblastic phenotype markers could be direct related. Additionally, our findings could help to explain the more pronounced effect of GH on BMD observed in young adults compared with adults [Välimäki et al., 1999; Hubina et al., 2004].

Although our results demonstrated the reduction with aging of the GH effect on osteogenic cultures and such reduction has also been observed in in vivo studies [Välimäki et al., 1999; Hubina et al., 2004], the mechanisms involved in this phenomenon are not completely understood. The role of aging on GH signaling has been investigated in GH receptor knockout mice (GHR-KO), which exhibit many phenotypic similarities with humans deficient in GH signaling [Zhou et al., 1997]. A study using GHR-KO with different ages (young, adult, and aged) demonstrated that GH resistance and the consequent reduction in the IGF-I circulatory reduced BMD, bone mineral content and bone area [Bonkowski et al., 2006]. Regarding the positive correlation between GH signaling deficiency and the decrease in hepatic and peripheral IGF-I levels, the findings are inconsistent. Whereas some studies showed that the effect of GH on IGF-I production is reduced with aging, others reported that the serum IGF-I levels remains preserved with aging [Lieberman et al., 1994; Arvat et al., 1998; Johannsson et al., 2000]. It is possible to speculate that the attenuation of the GH effect on osteoblastic cells with aging could involve alterations in the levels of IGF-I, which could act on both autocrine and paracrine pathways.

In conclusion, the present study showed that the stimulatory effect of GH on key parameters of both in vitro osteogenesis and gene expression of osteoblastic markers are donor-age-dependent. Further studies should be carried out to investigate the mechanisms involved in the decrease of response to GH with aging on osteoblastic cells.

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