Growth without growth hormone: can growth and differentiation factor 5 be the mediator?

Biana Shtaif1,2, Nitzan Dror3, Meytal Bar-Maisels1,3, Moshe Phillip1,2,3, and Galia Gat-Yablonski1,2,3

1Felsentein Medical Research Center, Petach Tikva, Israel, 2Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel, and 3The Jesse Z and Sara Lea Shafer Institute for Endocrinology and Diabetes, National Center for Childhood Diabetes, Schneider Children’s Medical Center of Israel, Petach Tikva, Israel

Abstract

Growth without growth hormone (GH) is often observed in the setup of obesity; however, the missing link between adipocytes and linear growth was until now not identified. 3T3L1 cells were induced to differentiate into adipocytes and their conditioned medium (CM) (adipocytes CM, CMA) was added to metatarsals bone culture and compared to CM derived from undifferentiated cells. CMA significantly increased metatarsals bone elongation. Adipogenic differentiation increased the expression of growth and differentiation factor (GDF)-5, also found to be secreted into the CMA. GDF-5 significantly increased metatarsal length in culture; treatment of the CMA with anti-GDF-5 antibody significantly reduced the stimulatory effect on bone length. The presence of GDF-5 receptor (bone morphogenetic protein receptor; BMPR1) in metatarsal bone was confirmed by immunohistochemistry. Animal studies in rodents subjected to food restriction followed by re-feeding showed an increase in GDF-5 serum levels concomitant with nutritional induced catch up growth. These results show that adipocytes may stimulate bone growth and suggest an additional explanation to the growth without GH phenomenon.

Keywords

Adipocytes, catch up growth, GDF5, metatarsals

History

Received 2 June 2015
Revised 9 August 2015
Accepted 10 August 2015
Published online 9 September 2015

Introduction

While normal growth in children is usually controlled by the growth hormone (GH)-insulin-like growth factor (IGF)-1 axis, it has been recognized that growth can occur also in its absence, especially in clinical situations that involve obesity. Obese children grow at a normal rate despite their low serum GH levels; children with hypopituitarism secondary to craniopharyngioma resection may continue to grow and may even show growth rate acceleration if their weight increases significantly (Geffner, 1996; Lazar et al., 2003; Phillip et al., 2002).

Adipocytes are not only the main site for energy storage, releasing fatty acids when required, but also an exceptionally active secretory tissue, releasing many endocrine and paracrine factors, commonly referred to as adipokines, which affect both peripheral tissues and the central nervous system. Adipokines are involved in diverse physiological processes, including immune response (Pond, 2005), energy homeostasis (Havel, 2002) and bone growth (Berendsen & Olsen, 2014; Maor et al., 2002).

The aim of the present study was to examine the relationship between adipocytes and bone growth and to identify potential mediators of this association. To test this hypothesis we used an in vitro model of 3T3L1 cells induced to differentiate in culture into adipocytes, and metatarsals bone rudiments. The 3T3-L1 pre-adipocyte cell line was originally isolated for its ability to undergo differentiation into adipocytes in vitro. It is now the predominant model for the study of adipogenesis (Green & Kehinde, 1975; Green & Meuth, 1974). DNA microarray studies have been carried out to investigate specific cellular programs in the regulation of gene expression during differentiation of 3T3L1 pre-adipocytes. Soukas et al. (2001) reported that the levels of expression of 1259 transcripts changed threefold or more during 3T3L1 differentiation, and in a similar study, Burton et al. (2004) identified 636 transcripts that were up-regulated at least twofold and 380 transcripts that were down-regulated in adipocytes compared to pre-adipocytes (Burton et al., 2004). Recently, researchers using a 5-plex stable isotope labeling by amino acids in cell culture (SILAC)-based strategy described a temporal profile of nuclear and secreted proteins during adipocyte differentiation (Molina et al., 2009).

The results of our study show that the conditioned medium (CM) of 3T3L1 adipocytes (CMA) stimulates the growth of metatarsal rudiment bones in culture and that growth and differentiation factor (GDF)-5 is the principal mediator in this
setting. This finding adds another nutrition-induced growth-stimulating factor to the currently known battery.

Materials and methods

Materials

Unless otherwise indicated, all chemicals were obtained from Sigma (Rehovot, Israel) or Biological Industries (Beit Haemek, Israel). GDF-5 was obtained from Peprotech Asia (#315-24; Rehovot, Israel); leptin antagonist from Protein Laboratories was a gift from Prof. A. Gertler (Rehovot, Israel) (Eliav et al., 2009); anti–bone morphogenetic protein receptor (BMPR1B) from Santa Cruz Biotechnology (SC-24455; Dallas, TX) and anti-growth and differentiation factor (GDF)-5 from R&D Systems (MAB-8531; Minniepolis, MN).

Metatarsals organ culture

The three middle metatarsal bone rudiments from the hind paws were dissected from Sprague-Dawley rat (Harlan, Israel) fetuses on embryonic day 20–21 under a stereo microscope (Olympus, Tokyo, Japan, SXZ7) and cultured separately (10–12 bones per test group) in 24/48-well culture dishes. Each well contained 0.5/0.2 ml minimal essential media (MEM), respectively, supplemented with 1% fetal bovine serum (FBS), 0.2% bovine serum albumin (BSA), 0.3 mg/ml L-glutamine, 0.05 mg/ml ascorbic acid, 1 mM sodium glycerophosphate, 100 U/ml penicillin and 100 μg/ml streptomycin. The metatarsals were cultured in 5% CO₂ humidified atmosphere (37°C). The medium was changed every other day. The bones were photographed with an Olympus DP71 camera (Olympus) and measured with Image ProPlus software (version 4.5.1.22, Media Cybernetics, Inc., Rockville, MD).

Morphological studies

Metatarsals were fixed in 10% neutral buffered formalin, embedded in paraffin and 6 μm paraffin sections were produced using a microtome (Leica RM2145) from the center of the bones. Morphological staining was performed using Alcian blue, hematoxylin and eosin, as follows: deparaffinization in o-xylene for 20 min, dehydration in 100% ethanol followed by 95% ethanol, 10 min each, followed by a 5-min wash with tap water. Slides were incubated for 3 min in 3% acetic acid and then in Alcian Blue (pH 2.5) for 30 min. After 10 min of washout by tap water, the slides were incubated with hematoxylin stain for 8 min and washed with tap water for 10 min. Eosin stain was added to the slides for 2 min to complete the staining. Slides were photographed with the Olympus DP71 camera.

The hypertrophic zone height, the number of hypertrophic cells per column, the height of hypertrophic cells and the length of the ossification zone were measured using the Image ProPlus software (version 4.5.1.22, Media Cybernetics), essentially as described (Chagin et al., 2010). Two sections of 10–12 bones from each group were analyzed.

Immunohistochemistry

For immunohistochemistry analysis, we used 6 μm sections of the paraffin-embedded metatarsals. The slide-mounted sections were deparaffinized with o-xylene and rehydrated with a graded series of ethanol. Endogenous peroxidases were neutralized with 3% hydrogen peroxide diluted with methanol, for 25 min. The slides were then incubated with a blocking agent (10% non-immune goat serum) for 20 min to block nonspecific binding of the primary antibody, followed by incubation with the primary specific antibody (GDF-5 receptor, BMPR1B) for 1.5 h and then washed three times with PBS. The negative control sections were incubated with non-immune rabbit serum (#08-6199; Zymed). Slides were incubated for 10 min with an enhanced biotinylated secondary antibody that recognizes the primary antibody, and washed three times with phosphate buffered saline (PBS). Enhanced horseradish peroxidase-conjugated streptavidin (HRP-SA) conjugated with aminoethyl carbazole as a substrate was added for another 10 min to allow binding to the biotinylated secondary antibody (Histostatin-SP kit, Zymed Laboratories – Invitrogen, Waltham, MA). Counterstaining was performed with hematoxylin followed by PBS washout. All steps were performed at room temperature.

Induction of 3T3-L1 cell differentiation into adipocytes

3T3-L1 cells were grown in Dulbeco modified Eagle medium (DMEM) supplemented with 10% adult bovine serum (ABS), 4 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin in a 5% CO₂ humidified atmosphere (37°C) to confluence on 100 mm plates. Two days after confluence, cells were treated with 10 μM dexamethasone, 0.25 μM insulin and 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) (differentiation mix) in DMEM supplemented with 10% FBS for 48–72 h followed by 0.25 μM insulin alone in DMEM supplemented with 10% FBS for additional 48–72 h to induce adipocyte differentiation (Somjen et al., 1997). CM was collected after additional 6 days (at day 5 the medium containing 10% FBS was replaced by medium containing 1% or 5% of FBS) (see Supplementary Figure 1). Undifferentiated cells were grown in DMEM supplemented with 10% ABS until confluence. One day prior to cell harvesting the medium was replaced by medium containing 1% or 5% of FBS; both CMA and CMF-contain 5% FBS. Floating cells were eliminated by centrifugation, and the CM was filtered through a 0.22-μm filter and stored at −70°C until use.

Adipocyte staining

Cells were washed three times with PBS and then fixed with 4% paraformaldehyde for 20 min. Oil red O (0.5%) was prepared in isopropanol, mixed with water in a 3:2 ratio, and filtered through a 0.45-μm filter. The fixed cells were incubated with the Oil red O reagent for 30 min at room temperature and washed with water. The stained fat droplets in the cells were visualized under light microscopy and photographed (Supplementary Figure 1) (Molina et al., 2009).

Biochemical analysis of the CM

Free fatty acid content, total protein content and glucose levels in the CMs were measured with the Immulite 2000 (Siemens AG, Munich, Germany).
Mouse growth factor PCR array

Total RNA was extracted from the cells according to the acid guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). The quantity and quality of the RNA were evaluated using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Scientific, Wilmington, DE). Equal amounts of RNA from the 3T3L1 cells before and after differentiation were analyzed with the Mouse Growth Factor RT² Profiler™ PCR Array (PAMM-041A, SaBiosciences, Qiagen, Gaithersberg, MD), which profiles the expression of 84 genes related to growth factors. The panel includes probes for angiogenic growth factors, regulators of apoptosis, and genes involved in cell differentiation, embryonic development, and tissue-specific development. These include probes for the genes encoding members of the bone morphogenetic proteins (BMP) family, fibroblast growth factors (1–22), epidermal growth factor, insulin like growth factors 1 and 2, interleukins, growth and differentiation factors 5, 10 and 11, leptin and others (see Supplementary Table 1). First-strand cDNA synthesis was performed with the RT² FirstStrand Kit (SaBiosciences) using 1 μg of total RNA. This step was preceded by DNase I treatment. Gene expression was measured by qPCR, according to the manufacturer’s instructions, with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Results were confirmed by qPCR reactions performed with specific RT² quantitative PCR primer assay-SYBR Green mouse GDF-5; Hprt1 served as the internal control, as of the five house-keeping genes presented on the array, it showed the least variations (PPM04450A and PPM035596, respectively, SaBiosciences). The following thermal cycling conditions were used: one cycle at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Relative expression was determined using the 2−ΔΔCt method (Livak & Schmittgen, 2001). Each sample was examined in triplicate.

GDF-5 analysis

GDF-5 was measured in the different CMs, and animal serum using ELISA kits (for the CMs and mouse serum: CSB-EL009349MO, Cusabio Biotech, Hubei, P.R. China; for rat serum: SEC101Ra, Cloud-Clone Corp., Houston, TX) according to the manufacturer’s instructions. The sensitivity of the assays as reported by the manufactures are 9.75 and 55 pg/ml, respectively.

Stable isotope labeling by amino acids in cell culture (SILAC)

SILAC was performed in the presence of semi-heavy amino acids for five generations. Once full isotope incorporation has been determined, cells were induced to differentiate. Subsequent SILAC analysis was performed on Tandem mass spectrometry (MS/MS) (The Weizmann Institute of Science, Rehovot, Israel).

Animal studies

Male Sprague-Dawley (SD) rats or ICR mice, 24 days old, were purchased from Harlan (Jerusalem, Israel) and housed individually at the animal care facility of the Felsenstein Medical Research Center. All animals had unlimited access to water. Animals were given 60% of the amount usually consumed by these animals at this age (Even-Zohar et al., 2008; Gat-Yablonski & Phillip, 2008). The food restriction was maintained for 10 days. At that point, animals were divided into two groups: one was kept restricted (RES, n = 6), and the other was given normal chow ad libitum (that induced catch up growth, hence this group was named CU group; n = 6). After 1 day of re-feeding, animals from the two groups were sacrificed by CO2 inhalation; serum was collected and stored at −80°C until further use. Throughout the study, animals were observed daily, and all remained bright, alert and active, with no evidence of any disorder. The Tel Aviv University Animal Care and Use Committee approved all procedures.

Statistical analysis

Studies with CMA and CMF were performed five times. Each experiment on metatarsals was performed at least twice. Results are expressed as mean ± SEM. Student’s T test or one-way analysis of variance (ANOVA) with Tukey post hoc were used to test for significant differences between the groups. The significance level was set at p < 0.05. SPSS v15 software (SPSS Inc., Chicago, IL) was used for statistical analysis of the data.

Results

Effect of CM from adipocytes versus pre-adipocytes on metatarsal growth

The 3T3L1 cells were induced to differentiate with IBMX, dexamethasone and insulin (differentiation mix). Oil red O staining revealed substantial lipid accumulation (Supplementary Figure 1) (Somjen et al., 1997). After 6 days in culture, CM was collected, centrifuged, and filtered, and the supernatant was added in a 1:1 ratio to the metatarsals for up to 14–16 days, with a change of medium every other day. Metatarsal rudiment bones were grown in MEM for up to 14–16 days, with a change of medium every other day. Metatarsal rudiment bones were grown in MEM containing 1% FBS and an equal volume of CM from undifferentiated 3T3L1 cells (fibroblasts; CMF) or differentiated 3T3L1 cells (adipocytes; CMA). A control group cultured in the presence of metatarsal medium alone was always included. Bone length increased significantly in response to CMA compared to CMF from day 8 onwards (p < 0.005; Figure 1). Biochemical analysis of the CM showed an increase in triglyceride content in CMA compared to CMF but no differences in total protein content, or glucose levels were noted (glucose in the range of 84–70 mg/dl, protein 0.3 g/dl in both, triglycerides 146 mg/dl in CMA and 100 mg/dl in CMF).
**Effect of leptin inhibitor on metatarsal growth in CMA**

To determine if the growth-stimulating factor secreted by adipocytes is leptin, 2μg/ml of anti-leptin was added to metatarsals grown in the presence of CMA. This concentration was calculated to be several hundred folds higher than the amount of leptin previously reported to be secreted by 3T3-L1 adipocytes (Norman et al., 2003). No significant effect on growth was observed (relative length increase: 80.97 ± 1.05% for CMA and 84.66 ± 2.26% for CMA+ leptin inhibitor; p = 0.168).

**Identification of the growth-promoting factor**

The growth conditions of the adipocytes had a significant effect on the purification. Normally, adipocytes are grown in the presence of 10% FBS. To achieve better purification of the growth factor, we reduced the content of FBS in the medium. Interestingly, whether the concentration of FBS was 5% or 1%, the cells maintained their morphological appearance with no apparent cell death. However, CMA from cells grown with 1% FBS was significantly less effective in supporting growth than the CMA from cells grown with 5% FBS, with findings essentially similar to those of CMF (data not shown).

Use of SILAC followed by ProteoMiner® columns to purify the relevant protein (Molina et al., 2009), proved unsuccessful, as the best effect on metatarsals’ growth was achieved when the adipocytes were grown in the presence of 5% FBS. Under these conditions, the level of serum albumin was so high that even after treatment with the ProteoMiner® and subsequent gel purification, significant amounts of albumin were present in the extract and hindered the MS/MS analysis of the less abundant labeled proteins.

**Expression array analysis**

We therefore decided to take a molecular approach, using an expression array of growth factors. The results showed an increase in the expression of 19 growth factors in the adipocytes compared to the non-differentiated 3T3L1 cells (the 10 most affected growth factors are shown in Table 1). Given that GDF-5 was the most highly affected factor and that GDF-5 was previously shown to affect the growth and differentiation of skeletal elements (Buxton et al., 2001), we selected it as the focus of our studies.

The results were confirmed with qPCR using a GDF5-specific probe set compared to housekeeping gene Hprt1. GDF5 expression was significantly increased by differentiated adipocytes compared to non-differentiated cells (results of real-time PCR showed 74-fold increase; p < 0.05; average Ct in fibroblasts was 33.36, average Ct in adipocytes 26.2; CT of Hprt1 – fibroblast = 23.25; adipocytes: 22.3). Technical replicates were used for the qPCR, and biological replicates were used for the array and qPCR.

**Effect of GDF-5 on metatarsal growth**

Quantitative analysis of the GDF-5 content of CM revealed that the level of GDF-5 in CMF was below the detection limit (which is reported to be 9.75 pg/ml) of the ELISA kit. The level in the CMA was in the range of 100 pg/ml. We added GDF-5 directly to the culture medium of the metatarsals bone rudiments and followed the effect on bone elongation for several days (10, 100 and 1000 pg/ml; Figure 2). The results showed that GDF5 at concentrations of 100 and 1000 pg/ml significantly stimulated bone growth compared to controls, with maximal effect achieved already at a concentration of 100 pg/ml (which is the level of GDF5 in the CMA). The effect was evident from the beginning of the culture, but was statistically significant from day 6 onwards (Figure 2b; p at the last day <0.0005).

Histological examination of the bones showed that the most affected region was the hypertrophic zone (Figure 3). The height of the hypertrophic zone, the number of cells in each column and the height of the hypertrophic cells were all increased by GDF-5 treatment.

To confirm that the growth stimulating effect is mediated by GDF-5, we incubated CMA with 50 ng/ml of anti-GDF-5 antibody for 2h at RT (this concentration is 500-fold higher than average GDF-5 level in CMA). CMA was then centrifuged, filtered and separated into aliquots. The growth of metatarsals that were incubated with anti-GDF-5 treated CMA was significantly reduced compared to metatarsals grown in the presence of untreated CMA, and was not significantly different from that of the CMF (Figure 4).
Immunohistochemistry of BMPR1

The presence of the receptor for GDF-5, BMPR1B (Nickel et al., 2005), on metatarsal bone was confirmed by immunohistochemistry (Figure 5). Most of the staining was found in the resting and hypertrophic cells as well as in the perichondrium.

GDF-5 in nutritional induced catch up growth in animal models

The body weight of the food restricted animals was \(18.6 \pm 2.0\) and \(57.04 \pm 3.96\) g for mice and rats, respectively; refeeding led to a rapid increase in weight (CU mice \(25.28 \pm 1.025\) g, CU rats \(81.77 \pm 6.53\) g; \(p < 0.001\) between RES and CU groups in each species). Analysis of serum levels of GDF-5 in these animals showed a significant increase in GDF-5 in serum derived from re-fed animals (CU), compared to the level in the RES groups in both mice and rats (Figure 6).

Discussion

Our study clearly shows that adipocyte-secreted GDF-5 directly stimulates metatarsal bone growth in vitro. These findings may suggest an additional link between adipose tissue and longitudinal growth, implied by findings of growth without GH (Geffner, 1996; Lazar et al., 2003; Phillip et al., 2002), occurring mostly in the presence of hyperphagia and obesity.

To investigate the direct connection between adipocytes and growth, we used the metatarsal bone model (De Luca et al., 2000). In this manner, we were able to measure the effects on the length of the long bones in culture while maintaining the cells in their cartilaginous environment, including the extracellular matrix (collagens, proteoglycans, etc.) as well as the interactions between the cells in their different differentiation steps (quiescent, proliferative and hypertrophic zones).

Leptin, known to be secreted by adipocytes, was previously shown by us as well as others to directly stimulate chondrocyte and bone growth (Ducy & Karsenty, 2000; Gat-Yablonski & Phillip, 2008; Gat-Yablonski et al., 2009; Maor et al., 2002; Pelleymouther et al., 1995; Turner et al., 2013). As leptin is secreted by adipocytes, it was our first growth promoting factor candidate, however, using a leptin inhibitor in a concentration of at least 670-fold higher than that secreted by 3T3L1 cells (Norman et al., 2003), we ruled out the possibility that leptin serves as the mediator in this context. These results are supported by the studies of Molina et al. (2009) who also failed to find leptin in the secretome of 3T3L1 and of Norman et al. (2003) who reported 3 ng/ml of leptin in CM of these cells, a concentration that is much lower than that used in our previous in vitro studies [50 ng/ml (Ben-Eliezer et al., 2007) or 500 ng/ml (Maor et al., 2002)].

The growth/differentiation factors (GDFs) are a subfamily of the highly conserved group of bone morphogenetic factors
(BMP), part of the transforming growth factor (TGF)-beta super-family. The TGF-beta super-family of proteins regulates cell differentiation and proliferation by generating a signaling cascade upon binding to serine-threonine kinase receptors. The TGF-beta superfamily, especially TGF-beta itself and the BMPs, consisting of at least 20 proteins, play a central role in the process of cartilage formation (Asahina et al., 1996; Yoon et al., 2006). GDF5, GDF6 and GDF7 form a distinct subgroup of BMPs, all of which are involved in bone and joint formation (Ducy & Karsenty, 2000).

GDF-5, also termed cartilage-derived morphogenetic protein (CDMP) or BMP14, is active during mesenchymal condensation, initiating the initial stages of chondrogenesis by promoting cell adhesion (Buxton et al., 2001) and it can increase the size of skeletal elements (Buxton et al., 2001). GDF-5 was also shown to stimulate proteoglycan production in chondrocyte-like cells, leading to an increase in aggregan and type II collagen gene expression and increased production of proteoglycans (Erlacher et al., 1998). In a large multinational genetic study, a locus near the GDF5 gene on

Figure 3. Histological examination of bones treated with GDF5 at concentrations of 100 and 1000 pg/ml. The height of the hypertrophic zone (A), the number of cells in each column (B), the average height of the hypertrophic cells (C) were all increased by GDF5 treatment. (D) Schematic representation of the different zones. *p < 0.05, **p < 0.01, ***p < 0.001 versus control and *p < 0.01 versus GDF5 1000 pg/ml.

Figure 4. Effect of anti-GDF-5 on CMA induced metatarsals bone growth. Metatarsal rudiment bones were grown in MEM containing 1% FBS and an equal volume of CM from differentiated 3T3L1 cells (CMA, marked as diamonds) or CMA that was pre-incubated with anti-GDF5 (marked as squares). A significant difference can be noted between the two groups (CMA vs. CMA-antGDF5 *p < 0.05). Relative length increase was calculated as the percentage of day ‘0’, the first day of the experiment, results presented are mean ± SEM.
chromosome 20 was found to be associated with final height in humans (Miyamoto et al., 2007). In addition, a specific SNP was found to be associated with increased risk for osteoarthritis (Hinoi et al., 2014a; Reynard et al., 2014).

A deficiency of GDF5 has multiple effects on skeletal tissues, and mutations in the GDF5 gene were shown to cause chondrodysplasia and several types of brachydactyly (Al-Qattan et al., 2015; Everman et al., 2002; Faiyaz-Ul-Haque et al., 2002), acromesomelic chondrodysplasia and DuPan syndrome (Douzgou et al., 2008) in humans, as well as brachypodism in mice. The best characterized experimental model is the GDF-5-deficient brachypod mouse, which has

Figure 5. Immunohistochemical localization of BMPR1B. Findings on immunohistochemical staining for BMPR1B, a GDF5 receptor, were positive in the hypertrophic zone of the growth plate and in the perichondrium (A and C), arrows point to positive staining. Negative controls (B and D). A and B – magnification ×40, scale bar 100 µm; C and D – magnification ×100, scale bar 50 µm.

Figure 6. GDF-5 serum levels in food restricted or re-fed SD rats (A) or ICR mice (B). Twenty-four–day-old animals were subjected to 10 days of 40% food restriction. After 10 days, half of the animals were kept restricted (RES) and half were allowed to re-feed with no restriction for one day (CU). DOI: 10.3109/08977194.2015.1082557 Growth without growth hormone 315
shortened limb bones and reduced growth (Storm et al., 1994) associated with significantly longer phase duration of hypertrophy (Mikic et al., 2004).

Over-expression of GDF-5 in mice led to chondrodysplasia with expanded primordial cartilage, which consisted of an enlarged hypertrophic zone and a reduced proliferating zone, not only in the limbs, but also in the axial skeleton (Tsumaki et al., 1999). GDF-5 was reported to increase the number of chondro-progenitor cells and accelerate chondrocyte differentiation to hypertrophy, essentially as shown here. The histological examination of the bones presented in the current study showed a significant increase in the length of the bones exposed to GDF-5, a significant increase in the hypertrophic cell number and height and the overall length of the hypertrophic zone, with agreement with previous publications (Coleman & Tuan, 2003; Mikic et al., 2004).

GDF-5 binds specifically to BMPR1B, BMPR2 and ACTR2a, forming a hetero-dimeric complex (Chen et al., 2006). We have shown that BMPR1B is present in the metatarsals bones, specifically in the hypertrophic zone, with agreement to its site of action.

GDF-5 was recently found to be expressed during the in vitro differentiation of 3T3L1 cells (Pei et al., 2014) and in vivo during the differentiation of brown and white adipocyte tissue (Hinoi et al., 2014a,b,c). Accordingly, we have found that GDF-5 was secreted to the CMA in a concentration of around 100 pg/ml and that this concentration was enough to cause bone growth in culture. Earlier in vitro studies in the literature were performed with significantly higher concentrations (Chen et al., 2006; Jenner et al., 2007; Seemann et al., 2005; Zeng et al., 2007); for example, a stimulatory effect of GDF-5 on the chondrocyte ATDC5 cells was described with doses of 100 ng/ml (Itoh et al., 1999), 1 µg/ml (Nakamura et al., 1999) or 5 nM (Sammar et al., 2004), which are 100–1000-fold greater than the present study.

Nutritional-induced catch up growth is usually associated with a rapid increase in fat mass suggesting a role for adipocytes in the nutritional-induced growth stimulation. Involvement of GDF-5 in adipocyte-induced growth stimulation is supported by our findings on its increased level in re-fed animals compared to food restricted ones. Increase in food consumption, body weight and leptin levels were previously reported by us in both mice and rats (Even-Zohar et al., 2008; Gat-Yablonski & Phillip 2007), but the observation on the increase in GDF-5 is new. Differences in the level of the growth factors in the blood between mice and rats may be due to difference in the species tested, but in both of them the increase was significant.

This study was limited by our failure to complete the SILAC analysis. The requirement for serum in the CM may sometimes be solved by the use of serum-free medium or by short-term serum-free starvation of the cells. However, in our case, for the cells to secrete the “metatarsal growth factor”, they had to be grown with at least 5% of FBS. We performed SILAC followed by Proteominer® purification and MS/MS analysis essentially as described by Molina et al. (2009). The presence of heavy amino acids in the peptides proved the cellular origin of the corresponding highly abundant proteins as well as the adipogenic nature of the differentiated cells, but failed to identify low abundant proteins owing to the high concentration of serum albumin.

Using an expression array we identified 19 growth factors which expression was increased in adipocytes by at least twofold; of these we analyzed the two most differentially expressed factors: GDF-5 and leptin. We confirmed the effect of GDF-5 by showing reduced growth in the presence of antibodies directed against GDF-5 in the CM. The contribution of additional factors identified by the expression array, as well as those that may have been missed due to the design of the array, may also be important.

Conclusions

This study shows that GDF-5, a skeletal growth factor, is produced and secreted by adipocytes in culture and stimulates the growth of metatarsals in vitro. The level of GDF-5 is also increased under conditions of nutritional catch up growth in vivo. These results may shed new light on cross communication between these closely associated systems, showing that adipocytes may stimulate bone growth. The results add a new potential mediator to the obesity-growth link and may suggest an additional explanation to the growth without GH phenomenon. Further studies are required to investigate the clinical relevance of our findings: whether adipocytes secrete GDF-5 in vivo in humans and the manner by which this stimulates growth. As the clinical toolbox to treat children with short stature is currently very limited, finding additional growth stimulating factors is of utmost importance; our results may suggest that GDF-5 should be further explored as a novel therapeutic agent to treat children with growth abnormalities.

Acknowledgements

The authors are grateful to Dr. Alla Shainskaya from the Biological Mass Spectrometry Department, The Weizmann Institute of Science, Rehovot, Israel, for her help in SILAC and MS/MS analysis and to Gloria Ginzach for editing the paper.

Declaration of interest

All authors state that they have no conflict of interest.

References

Al-Qattan MM, Al-Motairi MI, Al Balwi MA. 2015. Two novel homozygous missense mutations in the GDF5 gene cause brachydactyly type C. Am J Med Genet A 167:1621–1626.

Asahina I, Sampath TK, Haushka PV. 1996. Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or adipocytic differentiation of clonal murine target cells. Exp Cell Res 222:38–47.

Ben-Eliezer M, Phillip M, Gat-Yablonski G. 2007. Leptin regulates chondrogenic differentiation in ATDC5 cell-line through JAK/STAT and MAPK pathways. Endocrine 32:235–244.

Berendsen AD, Olsen BR. 2014. Osteoblast-adipocyte lineage plasticity in vivo. J Bone Joint Surg Am 83-A:S23–S30.

Buxton P, Edwards C, Archer CW, Francis-West P. 2001. Growth/differentiation factor-5 (GDF-5) and skeletal development. J Bone Joint Surg Am 83-A:S23–S30.
Chagin AS, Karimian E, Sundstrom K, Eriksson E, Savendahl L. 2010. Catch-up growth after dexamethasone withdrawal occurs in cultured postnatal rat metatarsal bones. J Endocrinol 204:21–29.

Chen X, Zankl A, Nirooamand F, Liu Z, Katus HA, Jahn L, Tiefenbacher C. 2006. Upregulation of ID protein by growth and differentiation factor 5 (GDF5) through a smad-dependent and MAPK-independent pathway in HUVSMC. J Mol Cell Cardiol 41:26–33.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Coleman CM, Tian RS. 2003. Growth/differentiation factor 5 enhances chondrocyte maturation. Dev Dyn 228:208–216.

De Luca F, Uyeda JA, Mericq V, Mancilla EE, Yanovski JA, Barnes KM, Zile MH, Baron J. 2000. Retinoic acid is a potent regulator of growth plate chondrogenesis. Endocrinology 141:346–353.

Douzou G, Lehmann K, Mingarelli R, Mundlos S, Dallapiccola B. 2008. Compound heterozygosity for GDF5 in Du Pan type chondrodysplasia. Am J Med Genet A 146:2116–2121.

Ducy P, Karsenty G. 2000. The family of bone morphogenetic proteins. Kidney Int 57:2207–2214.

Elinav E, Niv-Spector L, Katz M, Price TO, Ali M, Yacovobitz M, Solomon G, et al. 2009. Pegylated leptin antagonist is a potent orexigenic agent: Preparation and mechanism of activity. Endocrinology 150:3083–3091.

Erlacher L, McCartney J, Piek E, ten Dijke P, Yanagishita M, Oppermann H, Luyten FP. 1998. Cartilage-derived morphogenetic proteins and osteogenic protein-1 differentially regulate osteogenesis. J Bone Miner Res 13:383–392.

Even-Zohar N, Jacob J, Amariglio N, Rechavi G, Potievsky O, Phillip M. 2002. GDF5 signaling in promoting chondrogenesis and apoptosis of ATDC5 cells. J Cell Sci 115:2373–2381.

Faiyaz-Ul-Haque M, Ahmad W, Wahab A, Haque S, Azidi AC, Zaidi SM, et al. 2008. Growth without growth hormone: PI3K/Akt is involved in brown adipogenesis mediated by GDF5 in skeletal growth centers. J Bone Miner Res 17:1034–1043.

Mikic B, Clark RT, Battaglia TC, Gaschen V, Hunziker EB. 2004. Altered hypertrophic chondrocyte kinetics in GDF-5 deficient murine tibial growth plates. J Orthop Res 22:552–556.

Norman D, Isidori AM, Fajrasev C, Caprio M, Chew SL, Grossman AB, Clark AJ, et al. 2003. ACTH and alpha-MSH inhibit leptin expression and secretion in 3T3-L1 adipocytes: Model for a central-peripheral melanocortin-leptin pathway. Mol Endocrinol 200:99–109.

Pei Z, Wang Q, Maw M, Sun C, Luo F. 2014. Dynamic profile and adipogenic role of growth differentiation factor 5 (GDF5) in the differentiation of 3T3-L1 preadipocytes. Biochim Biophys Acta 560:27–35.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 268:540–543.

Phillip M, Moran O, Lazar L. 2002. Growth without growth hormone. J Pediatr Endocrinol Metab 15:1267–1272.

Pond CM. 2005. Adipose tissue and the immune system. Prostaglandins Leukot Essent Fatty Acids 73:17–30.

Reynard LN, Bui C, Syddall CM, Loughlin J. 2014. CpG methylation regulates allelic expression of GDF5 by modulating binding of SP1 and SP3 repressor proteins to the osteoarthritis susceptibility SNP rs143383. Hum Genet 133:1059–1073.

Sommer M, Stricker S, Schwabe OC, Sieber C, Hartung A, Hanke M, Oishi L, et al. 2004. Modulation of GDF5/BRI-b signalling through interaction with the tyrosine kinase receptor Ror2. Genes Cells 9:1227–1238.

Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM. 2001. Distinct transcriptional profiles of adipogenesis in vivo and in vitro. J Biol Chem 276:34167–34174.

Sour K, Huyhn TP, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ. 1994. Limb alterations in brachypondid mice due to mutations in a new member of the TGF-beta superfamily. Nature 369:63–64.

Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM. 2001. Distinct transcriptional profiles of adipogenesis in vivo and in vitro. J Biol Chem 276:34167–34174.

Storm EE, Huyhn TV, Copeland NG, Jenkins NA, Kingsley DM, Lee SJ. 1994. Limb alterations in brachypondid mice due to mutations in a new member of the TGF-beta superfamily. Nature 369:63–64.

Tsumaki N, Tanaka E, Arikawa-Hirasawa E, Nakase T, Kimura T, Thomas JT, Ochi T, et al. 1999. Role of CDMP-1 in skeletal morphogenesis: Promotion of mesenchymal cell recruitment and chondrocyte differentiation. J Cell Biol 144:161–173.

Turner RT, Kalra SP, Wong CP, Philbrick KA, Lindenmaier LB, Boghossian S, Iwaniec UT. 2013. Peripheral leptin regulates bone formation. J Bone Miner Res 28:22–34.
Yoon BS, Pogue R, Ovchinnikov DA, Yoshii I, Mishina Y, Behringer RR, Lyons KM. 2006. BMPs regulate multiple aspects of growth-plate chondrogenesis through opposing actions on FGF pathways. Development 133:4667–4678.

Zeng Q, Li X, Beck G, Balian G, Shen FH. 2007. Growth and differentiation factor-5 (GDF-5) stimulates osteogenic differentiation and increases vascular endothelial growth factor (VEGF) levels in fat-derived stromal cells in vitro. Bone 40:374–381.

Supplementary material available online