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Growth Hormone Has Dual Stage-Specific Effects on the Differentiation of 3T3-L1 Preadipocytes[1]

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Reports vary on the role of growth hormone (GH) in adipocyte differentiation. In this study, we showed that GH exerted dual effects depending on the stage of differentiation, using a serum-free culture of 3T3-L1 preadipocytes. GH promoted the differentiation when added to the medium during differentiation-inducing treatment with a hormone cocktail, but apparently suppressed it when added after the treatment. Only the suppressive effect was observed in the presence of 10% fetal bovine serum (FBS). Immunodepletion study showed that GH contributes to the differentiation-promoting activity of FBS. Insulin-like growth factor-1 could not replicate either the stimulative or the suppressive effect of GH. Stimulation of differentiation by GH involved the enhanced expression of mRNA of middle to late adipocyte markers. Among the key regulators of adipogenesis, peroxisome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer binding protein (C/EBP) α, but not C/EBPβ, were stimulated for mRNA expression by GH added during the treatment with hormone cocktail. The stimulation of adipogenesis by GH was indeed due to the increase in the ratio of differentiated cells, though GH also promoted cell growth.

Key words: adipocyte, adipogenesis, differentiation, growth hormone, peroxisome proliferator–activated receptor γ.

Adipose tissue is one of the main targets of growth hormone (GH). GH has been proposed to regulate lipolysis and lipogenesis in adipocytes, as well as the differentiation of preadipocytes into adipocytes (*1*). Conversely, recent studies have shown that adipose tissue regulates the secretion of GH through two signals, free fatty acids and leptin (*2*). Thus, the relationship between the GH regulatory system and adipose function still requires clarification from both biological and clinical points of view.

GH was first shown to promote the differentiation of 3T3-F442A preadipocytes in culture (*3*). Studies employing other preadipocyte lines also revealed a stimulatory effect by GH on the differentiation into adipocytes (*4–7*). On the other hand, it is generally accepted that GH has anti-lipogenic activity, apparently antagonizing the actions of insulin in cultured adipocytes (*8*). GH exhibited activity to inhibit differentiation in primary preadipocyte cultures (*9–11*). A study with 3T3-L1 cells failed to show any effect of GH on differentiation into adipocytes (*12*). Genetic ablation in the GH regulatory system generally induces mild obesity in both humans (*13*) and laboratory animals (*14*). In this regard, an interesting observation (*15*) was made in children with GH deficiency, who are moderately obese. They had increased mean adipocyte volumes but reduced number of fat cells, as compared with healthy children, which tended to normalize upon GH administration. Thus, GH may have two paradoxical effects: increasing the number of adipose cells, but preventing their hypertrophy.

A major action of GH is to promote the secretion from the target cells of insulin-like growth factor (IGF)–1, which in turn acts on a variety of tissues to stimulate proportional body growth (*16*). Earlier study showed that IGF-1 was not able to substitute for GH in its adipogenic action, whereas GH increased the sensitivity of cells to IGF-1 (*17–19*). However, GH was found to stimulate the expression of IGF-1 in Ob1771 preadipocytes (*20*), and the secreted IGF-1 was proposed to induce the differentiation of this cell line in an autocrine/paracrine fashion (*21*). Another study (*12*) suggested that IGF-1 was an essential regulator of 3T3-L1 preadipocyte differentiation, while GH had no stimulatory effect.

Thus, there are many notions about the roles of GH in adipocyte differentiation. This is possibly because different preadipocyte cell lines and culture systems were used for the studies (*22*). It is likely that the cell lines as well as primary preadipocytes were in different stages of differentiation, which resulted in different responses to GH. To unify these notions, it is essential to show both effects of GH in a single experimental system. In this study, we have investigated the role of GH in the differentiation of 3T3-L1 preadipocytes, using a serum-free culture. GH indeed exhibited dual, apparently opposing effects on the differentiation.

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Abbreviations: aP2, adipose protein 2/fatty acid–binding protein; C/EBP, CCAAT/enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GH, growth hormone; GPDH, glycerol-3-phosphate dehydrogenase; IGF, insulin-like growth factor; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator–activated receptor; RT, reverse transcription.

That is, the differentiation was promoted when GH was applied in the induction phase of differentiation, but apparently suppressed when GH was added during the lipid-accumulation phase. Furthermore, we found that IGF-1 could not substitute for GH.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), in 5% CO_2 at 37°C. They were passaged every 3 days, and all the experiments were done between passages 3 and 10. For differentiation experiments, 3×10^4 trypsinized cells were seeded onto a 35-mm dish, and cultured in DMEM/10% FBS. After 3 days (day 0), confluent cells were treated for 2 days with a hormone cocktail containing 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/ml insulin (*23*), in a serum-free culture. The medium consisted of DMEM containing 5 μg/ml transferrin, 2 nM triiodothyronine, 30 ng/ml epidermal growth factor, 1 μM biotin, and 200 μg/ml fetuin (Sigma) (adapted from Ref. *24*). The cocktail was then removed, and the cells were cultured in the same medium supplemented with 5 μg/ml insulin. The medium was changed on day 5. On day 8, the cells were stained or processed for the determination of differentiation markers. As required, 50 ng/ml recombinant human GH (Sigma) was added for appropriate periods (see "RESULTS"). Unless otherwise stated, experiments were done in triplicate, and the data are presented as the mean ± SD. Statistical significance was analyzed by Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and N.S., not significant.

Analysis of Oil Deposits—Cells were fixed with 10% formalin in PBS and stained with 0.3% Oil Red O. Oil droplets in living cells were stained with Nile Red (*25*), and determined for the intensity of staining by monitoring the fluorescence in an imaging analyzer, FLA3000 (Fuji). The background fluorescence of un-differentiated cells was subtracted from the values of all samples. Triglyceride was determined using a Triglyceride E-Test Wako kit (Wako Pure Chemicals). Cell lysates were prepared as described below and incubated with 1 mg/ml *Pseudomonas* sp. lipoprotein lipase (Wako Pure Chemicals) for 1 h at room temperature. The samples were centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatants were used to determine the triglyceride content, according to the manufacturer's protocol.

Glycerol-3-phosphate Dehydrogenase (GPDH) Assay—GPDH activity was measured as described previously (*3*), with slight modifications. Cells were washed with PBS and lysed with 0.3 ml of lysis buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% Triton-X100. The lysates were centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatants were stored at –80°C until use. The reaction mixture contained, in a total volume of 1 ml, 50 mM triethanolamine-HCl (pH 7.5), 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1 mM NADH, 0.5 mM dihydroxyacetone phosphate, and 10 μl of cell lysate. GPDH activity was determined by following at 37°C the decrease in absorbance at 340 nm due to the oxidation of NADH. One unit of enzyme activity was defined as the amount of protein required for the use of 1 μmol NADH per min, dependent on dihydroxyacetone phosphate. The amount of protein was measured with a protein assay kit (BioRad).

Analysis of Adipocyte Marker mRNAs—Total RNA was prepared with RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. For reverse transcription (RT)-PCR, total RNA (1 μg) was mixed with a downstream primer mixture (10 pmol each), and the volume was adjusted to 10 μl with water. The solution was heated for 2 min at 90°C and quickly chilled on ice. RT was carried out for 1 h at 43°C with Moloney murine leukemia virus reverse transcriptase (Gibco BRL), as recommended by the manufacturer. PCR was performed with 2 μl of the RT product as a template, 10 pmol each of the upstream and downstream primers, and recombinant Taq DNA polymerase (TaKaRa). The reaction products were separated with a 2% agarose gel, the band images recorded in FLA3000, and their intensities analyzed using the software Image Gauge (Fuji). For each gene, the number of PCR cycles was adjusted based on the results of preliminary experiments, so that comparison of the band intensities was made in the exponential amplification phase. The concentrations of RNA samples were normalized, based on the intensity of the signal for a ribosomal protein, 36B4, a control unaffected by the differentiation. For Northern blotting, 25 μg/lane of total RNA was separated with an agarose/formaldehyde gel, and transferred onto a nylon membrane (Hybond-N+; Amersham Pharmacia). All later procedures were as described by the manufacturer.

Other Determinations—To estimate the extent of adipocyte differentiation, cells were stained with Nile Red, then with 20 μg/ml Hoechst 33342. The cells were then washed with PBS and observed under a fluorescence microscope. The ratio of differentiation was defined as the number of differentiated (Nile Red-stainable) cells divided by the total number of cells (Hoechst signals), which was determined using the software NIH Image.

For cell proliferation assays, confluent cells were further maintained for a day in the serum-free medium containing 5 μg/ml insulin, and then induced for differentiation with the hormone cocktail, as described above. Cells received 0.25 μCi/dish of [^{3}H]thymidine, 12 h after induction. After 12 h of radiolabeling, the cells were trypsinized and deposited on glass fiber filters using a cell harvester. After the filters were dried, the radioactivity was determined in a liquid scintillation counter.

To assess the adipogenic activity of GH intrinsically contained in FBS, DMEM containing 3% FBS was mixed with a 1/2,000 volume of an antiserum to bovine GH (Fitzgerald) or a preimmune serum, and kept for 1 h at room temperature. The treated medium was sterilized with a 0.22-μm membrane filter and used for the differentiation experiment.

RESULTS

Effect of GH in the Serum-Free Culture—To investigate the role of GH in adipocyte differentiation, we examined the effect of GH in a serum-free culture of 3T3-L1 preadipocytes. When the cells were differentiated in a medium containing 50 ng/ml GH, a larger number of cells became round-shaped on day 8 than when the cells were cultured without the hormone [Fig. 1B, compare upper panels of GH(–)(–) and (+)(+)]. However, GH increased only marginally the intensity of lipid staining with Nile Red [Fig. 1B,

lower panels of GH(–)(–) and (+)(+)], as well as Oil Red-O (data not shown). The lipid droplets that accumulated in the cells treated with GH were smaller than those in the untreated cells. Hence, we suspected that GH promoted differentiation of preadipocytes into adipocytes, but that it suppressed the maturation (lipid accumulation) of adipocytes once they had differentiated. Accordingly, we examined separately the effects of GH during and after the treatment with dexamethasone/3-isobutyl-1-methylxanthine/insulin (cocktail treatment), because the differentiation is triggered during the cocktail treatment, whereas the adipocyte maturation occurs after it (*23*).

We set four combinations of the timing of GH addition to the serum-free culture of 3T3-L1 preadipocytes (Fig. 1A). GH(+)(–) yielded a much larger number of cells with a round morphology and well-developed oil droplets than GH(–)(–) (Fig. 1B). In GH(+)(+), a considerable number of cells became rounded, but they had significantly smaller oil droplets and weaker Nile Red staining than GH(+)(–) cells. Cells of GH(–)(+) exhibited even poorer differentiation than GH(–)(–) cells with respect to both cell morphology and Nile Red staining. The specific activity of GPDH, a marker enzyme of adipocytes, was significantly increased by adding GH during the cocktail treatment, but unchanged by GH after the treatment (Fig. 1C). GH added during the cocktail treatment also increased lipid accumulation, as judged by both Nile Red staining and triglyceride content. GH added after the treatment, however, significantly reduced lipid accumulation. Thus, GH promoted adipocyte differentiation when it was added to the medium during the cocktail treat-

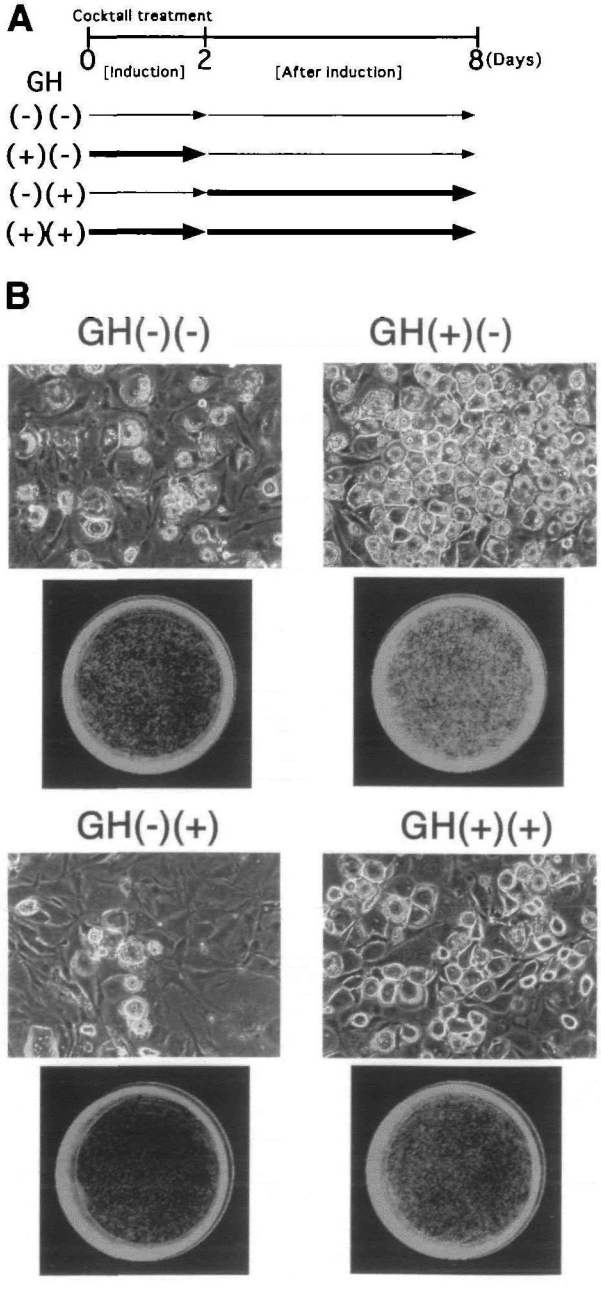

Fig. 1. **Effect of GH on the differentiation of 3T3-L1 preadipocytes in serum-free culture.** (A) Combinations of the timing of GH addition. In GH(–)(–), GH was absent throughout the experiment. In GH(+)(–) and GH(–)(+), GH was added only during and after the cocktail treatment, respectively, whereas GH was added all the time in GH(+)(+). Other conditions were as described in "EXPERIMENTAL PROCEDURES." (B) Morphology upon phase-contrast microscopy (upper panel) and Nile Red staining (lower panel) of the cells treated and cultured under the four sets of conditions. Stained cells appear gray in the pictures. (C) GPDH-specific activity (top), intensity of Nile Red staining (middle), and triglyceride content (bottom) of the cells treated and cultured as above. Data were analyzed and are presented as described in "EXPERIMENTAL PROCEDURES."

ment, but apparently suppressed it by lowering lipid accumulation when added after the treatment.

Effect of GH in the Presence of FBS—Most experiments of adipocyte differentiation have been performed in a medium containing 10% FBS, where 4–10 ng/ml GH is present intrinsically (*12, 26*). Hence, we were interested in whether GH also promoted adipocyte differentiation in the presence of FBS. When the cells were differentiated in the presence of 10% FBS, with or without GH throughout the experiment, Oil Red-O staining was slightly weaker when GH was added (Fig. 2A). This suppression was more prominent at lower concentrations of insulin. The suppressive effect of GH on both GPDH-specific activity and content of triglyceride was observed at all concentrations of FBS from 1 to 10% (data not shown).

As described above, in the serum-free culture, the apparent suppression of adipocyte differentiation by GH was caused by its presence after the cocktail treatment. Accordingly, we examined whether it was also the case in the serum-supplemented culture (Fig. 2B). In the medium containing 10% FBS, neither GPDH-specific activity nor triglyceride content was affected significantly when GH was added to the medium during the cocktail treatment. On the other hand, both parameters were decreased when GH was added after the treatment. Thus, it was also after the cocktail treatment that GH exerted an anti-adipogenic effect in the presence of FBS. A difference from the result with serum-free culture was that GPDH-specific activity was also decreased by GH added after the cocktail treatment (compare Figs. 1C and 2B).

GH Is One of the Intrinsic Adipogenic Factors in FBS—We speculated that GH did not stimulate adipocyte differentiation in the presence of 10% FBS, due to the presence of saturating concentrations of intrinsic differentiation-promoting factors, including GH itself. We indeed found that the stimulatory effect of GH appeared as the concentration of FBS was decreased (data not shown). On the other hand, the intrinsic GH was probably insufficient to suppress the adipocyte maturation. Accordingly, we examined the dose-dependence of the two actions of GH in a serum-free culture. The differentiation-promoting effect was monitored by adding different concentrations of GH only during the cocktail treatment. With regard to GPDH-specific activity, significant enhancement was observed by 2 ng/ml GH, and rose gradually at higher concentrations (over 20 ng/ml), with an apparent EC_{50} of around 10 ng/ml (Fig. 3A). Similar results were obtained for Nile Red staining and cell morphology (data not shown). To examine the inhibitory effect, differentiation was first induced for 2 days in the presence of 50 ng/ml GH, and then the culture was continued with medium containing various concentrations of GH. Nile Red staining decreased as the concentration of GH increased (Fig. 3B), though, as expected, the suppressive effect of GH was only marginal on GPDH-specific activity (data not shown). The inhibition was apparent from 10 ng/ml, and the IC_{50} was estimated to be 10–20 ng/ml. Thus, although the half-maximal concentrations differed little

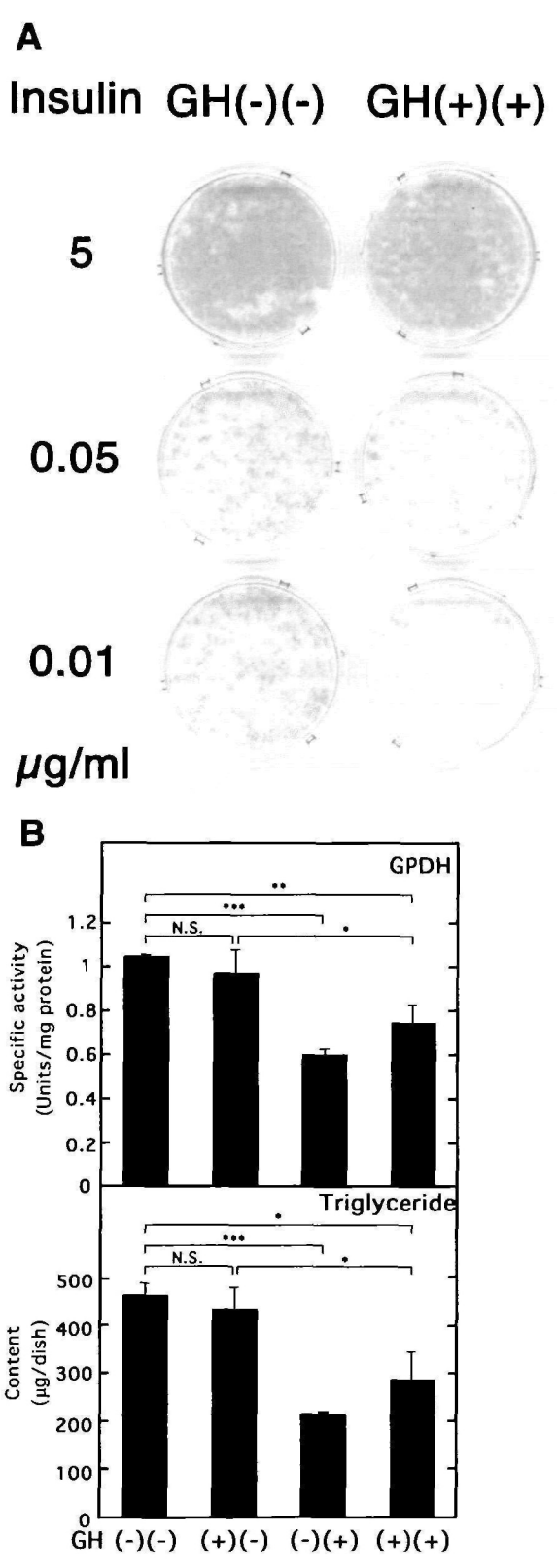

Fig. 2. **Effect of GH in the presence of FBS.** (A) Oil Red-O staining of 3T3-L1 cells that differentiated in the presence of different concentrations of insulin. Confluent cells were induced for 2 days to differentiate and cultured for 6 more days, in DMEM supplemented with 10% FBS instead of fetuin. GH was added or not, throughout the experiment. Insulin was added at the concentrations indicated. (B) GPDH-specific activity (top) and triglyceride content (bottom) of the cells that differentiated in the presence of FBS. Cells were treated and cultured under the four sets of conditions as described in Fig. 1A, except that 10% FBS was added to the medium instead of fetuin.

between the two effects, the stimulatory effect increased sharply at lower concentrations of GH. These results would partly explain why, in the presence of 10% FBS, the promoting effect is hardly observable, whereas the inhibitory effect is significant.

Based on the above results, we reasoned that part of the differentiation-promoting effect of FBS would be due to GH. We tested whether neutralization of GH with an anti-GH antiserum decreased the adipogenic activity of FBS. Differentiation was induced by adding 3% FBS during the cocktail treatment, in the presence of preimmune or anti-GH rabbit serum, followed by culture in the absence of the antiserum (Fig. 4). When anti-GH was added, the accumulation of triglyceride subsided, and the difference became apparent on day 5. On day 6, the antiserum had lowered triglyceride content by one third. A similar effect of the antiserum was observed on GPDH-specific activity, though slightly weaker than that on triglyceride content. These results suggest that part of the differentiation-promoting effect of FBS is indeed due to GH. In this experiment, we used a lower concentration of FBS and evaluated the differentiation at earlier points than usual to make the effect of the antiserum clearer. On day 8, the effect was less clear, because the differentiation was saturated.

Effect of GH on the Expression of Adipocyte Markers—We next investigated whether the stimulation of differentiation by GH involved the induction of representative adipocyte markers. We first followed by RT-PCR the time-course of mRNA expression in GH(+)(–) and GH(–)(–), with samples prepared from the cultures at days 0, 2, 4, and 8 (Fig. 5A). Peroxisome proliferator-activated receptor (PPAR) δ and lipoprotein lipase (LPL), both early markers of adipogenesis, were expressed at detectable levels from the beginning of the experiment, and increased at day 2 or 4. The expression of these two markers was not significantly different between GH(–)(–) and GH(+)(–), though the LPL signal was slightly stronger in GH(+)(–) on day 8. Although PPARγ1 mRNA was increased during adipocyte differentiation, it was expressed at a low level from day 0, reflecting its ubiquitous nature. Its expression was increased in GH(+)(–) on day 8. The expression of PPARγ2, adipose protein 2/fatty acid-binding protein (aP2), and adipsin, all middle to late markers, started relatively late, being consistent with published results (*27*). Their expression was promoted by the addition of GH during the cocktail treatment. The stimulatory effect first appeared on 2, and became prominent on day 8.

We also studied the mRNA expression of CCAAT/enhancer-binding protein (C/EBP) α and β by Northern blotting, because RT-PCR was unsuccessful for these markers due to their abnormally high G/C contents. C/EBPα is a key regulator of adipocyte differentiation, acting in the middle to late periods. Its mRNA expression was increased by GH on day 2, though in later stages the expression level was not significantly different between GH(–)(–) and GH(+)(–) (Fig. 5B). Another key regulator of adipogenesis, C/EBPβ, acts in the initial phase, when GH must be

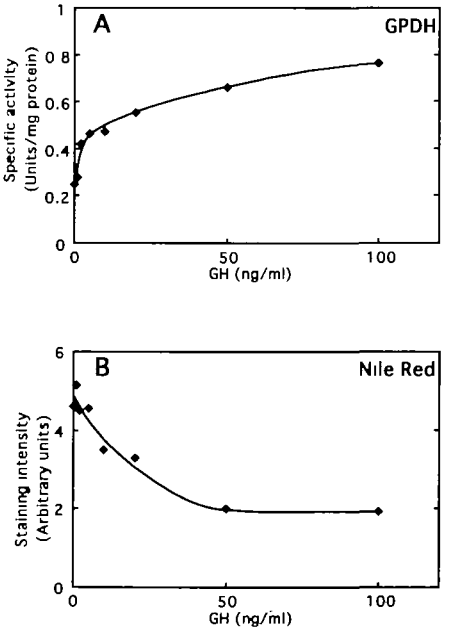

Fig. 3. **Dose-dependence of the differentiation-promoting and suppressing effects of GH.** (A) The promoting effect assessed based on GPDH-specific activity. Confluent cells were induced to differentiate in the serum-free medium by adding different concentrations of GH only during the cocktail treatment. After the treatment, cells were cultured for 6 more days without GH, and GPDH activity was measured. (B) The suppressive effect estimated by Nile Red staining. Confluent cells were induced to differentiate in the serum-free medium with 50 ng/ml GH for 2 days. After the induction, cells were cultured with the medium containing various concentrations of GH. On day 8, cells were stained with Nile Red and fluorescence intensity was determined. For both experiments, values are averages of duplicate samples. Similar results were obtained in two independent series of experiments.

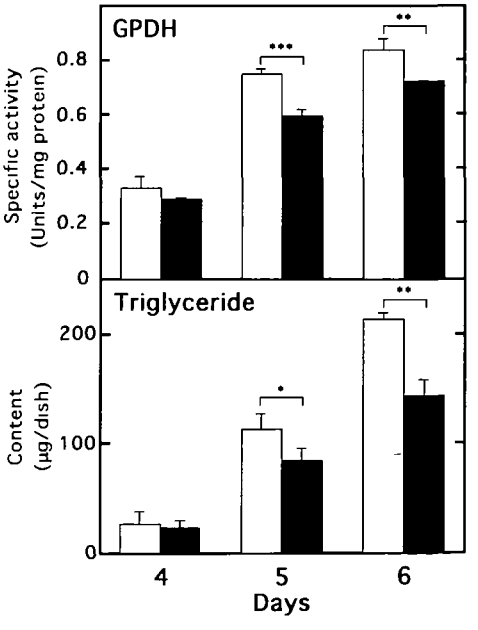

Fig. 4. **Neutralization of intrinsic GH in FBS with an anti-GH antiserum.** DMEM containing 3% FBS was pretreated with preimmune (open bar) or anti-GH (shaded bar) rabbit serum, as described in "EXPERIMENTAL PROCEDURES." Cells were induced to differentiate in the treated media, and cultured with the untreated medium (DMEM containing 3% FBS) for the periods indicated. GPDH-specific activity (top) and the content of triglyceride (bottom) were measured on the days indicated.

present for the stimulation of adipocyte differentiation. Expression of C/EBPβ mRNA was indeed increased from day 0 to 2, and decreased from day 4 to 8, but it was not affected by GH. Consistency between Northern blotting and RT-PCR was verified by confirming that the results for aP2 were similar between the two procedures. Hence, the promoting effect of GH became prominent relatively late in the course of differentiation with respect to the mRNA expression of adipocyte markers. This change was accompanied by the enhancement of the expression of PPARγ and C/EBPα.

On the other hand, we did not observe by RT-PCR a significant difference in the expression of the markers when GH was added after the cocktail treatment (data not shown). This result was consistent with the finding that GPDH-specific activity was largely unaffected by addition of GH after the treatment in the serum-free culture (see Fig. 1C).

IGF-1 Can Not Substitute for GH in the Stimulation of Adipocyte Differentiation—As one of the major mechanisms of GH action is triggering of IGF-1 secretion, we tested whether the differentiation-promoting effect of GH on the 3T3-L1 in the serum-free culture is mediated by IGF-1. The experiment was performed according to the schedule depicted in Fig. 1A, with 100 ng/ml IGF-1 instead of insulin. This concentration is three times the K_d value for the IGF-1 receptor in 3T3-L1 (32 ng/ml, 4.7 nM), and at this concentration more than 80% of the receptor is expected to be occupied (*12*). Both GPDH-specific activity and the triglyceride content were increased by the addition of GH during the cocktail treatment (Fig. 6), though the effects were less prominent than those in the presence of insulin (see Fig. 1C). Strong inhibition of lipid accumulation by GH after the treatment was also observed. Interestingly, GPDH-specific activity was also reduced significantly by GH added after the treatment, in contrast to the results with the medium containing insulin (compare Figs. 6 and 1C). We also observed that IGF-1 did not promote the differentiation of 3T3-L1 when added during the cocktail treatment in the presence of 5 μg/ml insulin (data not shown), under which conditions GH exerted a stimulatory effect. These results indicate that IGF-1 cannot replicate either the adipogenic or the anti-adipogenic action of GH.

Stimulation of Differentiation by GH Is Not Simply Due to an Increase in Cell Number—GH promotes adipocyte differentiation when added to the medium during the cocktail

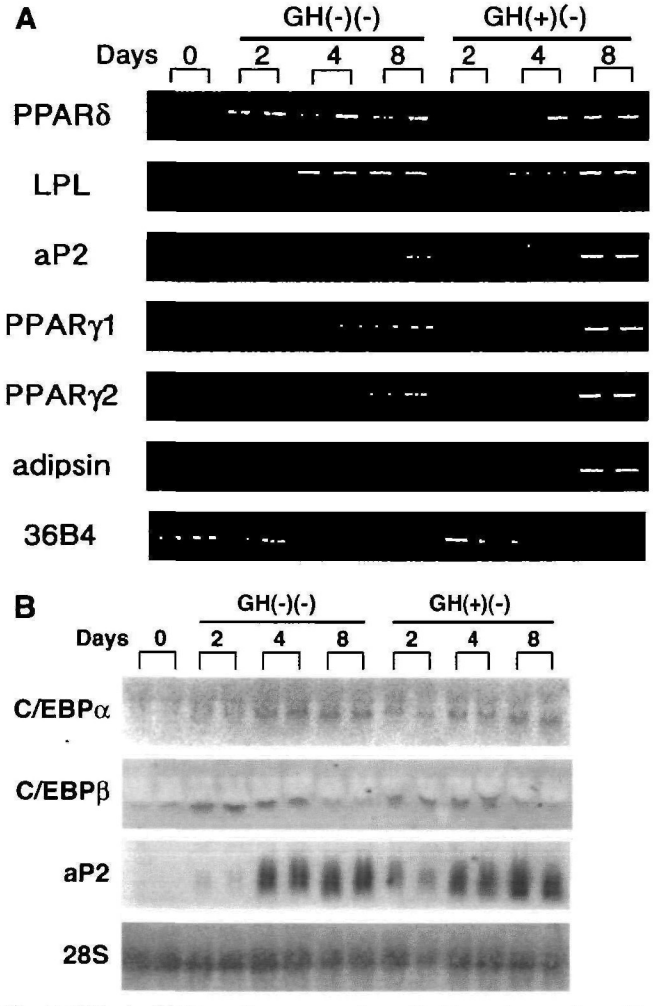

Fig. 5. **Effect of GH on the expression of adipocyte markers.** Total RNA was prepared on the days indicated from cells treated in the serum-free medium according to the GH(+)(–) or GH(–)(–) program. (A) Measurement of the mRNA levels of PPARδ, LPL, aP2, PPARγ1, PPARγ2, and adipsin by RT-PCR. The expression of 36B4 was also examined as a control that was not affected by the differentiation. (B) Estimation of the mRNA levels of C/EBPα and C/EBPβ by Northern blotting. Results for aP2 verified that comparable data were obtained by Northern blotting and RT-PCR. 28S ribosomal RNA represents the loading control.

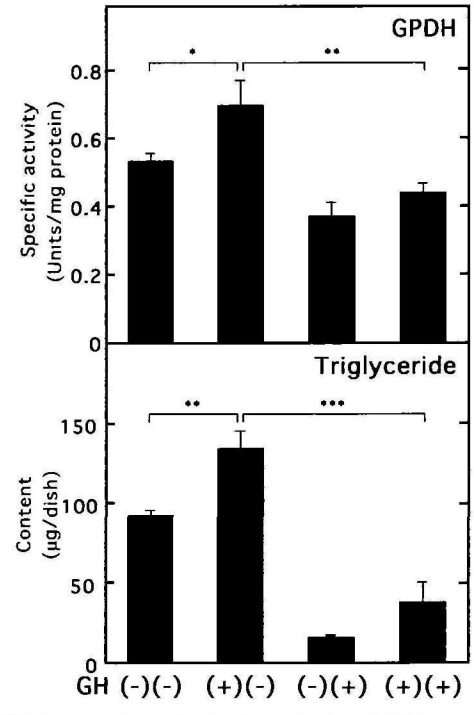

Fig. 6. **IGF-1 cannot replicate the effects of GH on adipocyte differentiation.** Confluent cells were induced to differentiate in the serum-free medium supplemented with 100 ng/ml recombinant human IGF-1 (Sigma) instead of insulin. GH was added according to the schedules depicted in Fig. 1A. GPDH specific activity (top) and triglyceride content (bottom) were measured on day 8. The results were reproduced in an additional experiment.

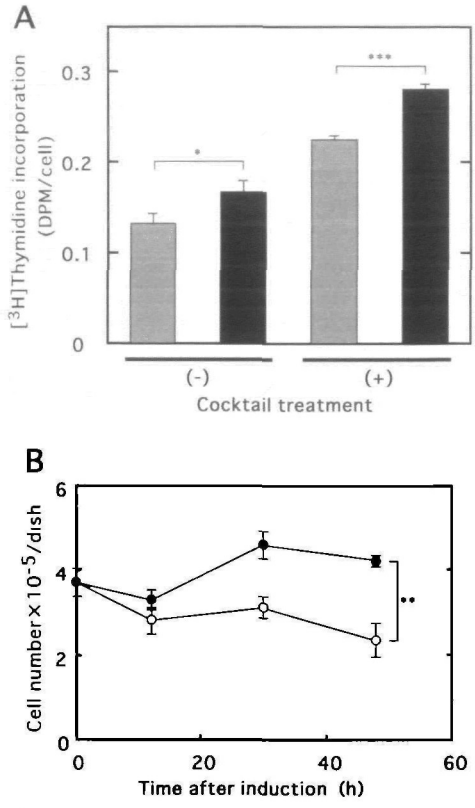

Fig. 7. **Effects of GH on the cell proliferation and total cell number of 3T3-L1.** (A) Stimulation of cell growth by GH. Cells cultured to confluence in the presence of 10% FBS were maintained in the serum-free medium for a day to assure that they were growth-arrested. The cells were then induced to differentiate by treatment with a hormone cocktail, with (closed bar) or without (striped bar) 50 ng/ml GH, and the incorporation of [^{3}H]thymidine was measured, as described in "EXPERIMENTAL PROCEDURES." Values are presented on the basis of cell number at the start of labeling, which was determined with another dish of cells cultured in parallel. (B) Change in cell number during the cocktail treatment with (solid circle) or without (open circle) GH. Cells were treated as in (A), and counted at the points indicated. The difference in cell number between 0 h and 48 h was significant for GH(–) ($p < 0.01$), but not for GH(+).

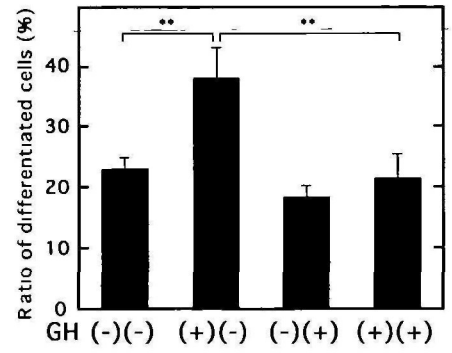

Fig. 8. **Increase in the ratio of differentiated cells by GH.** Cells were induced to differentiate and cultured in the serum-free medium, under the four sets of conditions described in Fig. 1A. On day 8, the oil droplets of the differentiated cells were stained with Nile Red and the nuclei of all cells were stained with Hoechst 33342. The ratio of differentiation was estimated as described in "EXPERIMENTAL PROCEDURES." For each dish, the averaged ratio of differentiation was measured with images of 10 randomly selected microscopic fields at low magnification. Values are averages of data on three dishes.

treatment, when clonal expansion occurs. Clonal expansion is defined as the division of confluent preadipocytes two or three times, before their entry into the course of differentiation (*28*). Accordingly, we examined whether the addition of GH during cocktail treatment promoted cell proliferation, by measuring the incorporation of [^{3}H]thymidine (Fig. 7A). The cocktail treatment promoted thymidine incorporation even in the absence of GH, confirming the occurrence of clonal expansion in the serum-free medium. GH further increased thymidine incorporation, indicating the stimulation of cell growth. The cell number did not change significantly in the presence of GH during the experiment, but it decreased in its absence (Fig. 7B). These results suggest that GH increased the total cell number by both stimulating cell proliferation and protecting the cells from death.

The above results raised the question of whether GH promoted the adipocyte differentiation *per se*, or only appeared to do so, by increasing the total number of cells per dish. To determine which is the case, we stained the differentiated cells with Nile Red and measured the ratio of Nile Red-positive cells to total cells following standard experimental procedures (Fig. 8). The ratio was significantly higher in GH(+)(–) than other samples. Hence, GH added during the cocktail treatment stimulated adipocyte differentiation by increasing the percentage of differentiated cells.

DISCUSSION

The many observations published on the role of GH in adipocyte differentiation indicate either a stimulatory effect or an anti-adipogenic action. This contradiction may be due to the use of different preadipocyte models and culture conditions. In this study, we examined the role of GH in the differentiation of 3T3-L1 preadipocytes, and for the first time observed that GH indeed has dual effects in a single experimental system. GH promoted adipocyte differentiation when added during treatment with a hormone cocktail, whereas it suppressed the accumulation of lipid when added after the treatment. An earlier experiment where no stimulatory effect of GH was observed (*12*) employed GH both during and after the cocktail treatment, and hence the two effects probably cancelled each other out.

An inhibitory but not stimulatory effect of GH on differentiation was observed in primary preadipocyte cultures (*9–11*). It was pointed out that primary preadipocytes may have been at a stage of differentiation beyond that of established preadipocyte models (*1, 22, 28, 29*). Provided that the primary preadipocytes were in a state corresponding to or ahead that of the cocktail-treated 3T3-L1 cells, it would not be surprising that only the suppressive effect of GH was observed in the primary cultures. The promotive effect of GH was hardly observable in the presence of 10% FBS, probably due to a saturating level of adipogenic activity at this concentration of FBS. Our immunological neutralization experiment showed that GH indeed contributes to this activity of FBS in 3T3-L1 preadipocytes (Fig. 4), being consistent with a previous study on 3T3-F442A cells (*30*). On

the other hand, the suppressive action of GH was obvious in the presence of FBS. This was apparently because the concentration of intrinsic GH in 10% FBS is not sufficient to cause significant suppression. In fact, we failed to show an increase in the adipogenic activity of FBS after the cocktail treatment by immunodepleting the intrinsic GH (data not shown).

GH promoted the mRNA expression of transcription factors, PPARγ and C/EBPα, when added during the cocktail treatment. GH did not affect the expression of C/EBPβ mRNA, even though the stimulatory effect of GH was apparent, only when it was added during the cocktail treatment. C/EBPβ is induced upon the cocktail treatment and positioned upstream in the cascade leading to PPARγ and C/EBPα (*31*). Thus, GH seems to induce PPARγ and C/EBPα, acting on the signaling step(s) beyond C/EBPβ gene transcription, and hence stimulate the expression of middle to late adipocyte markers.

GPDH-specific activity was not significantly decreased by GH after the cocktail treatment in the serum-free culture, despite an apparent suppression of differentiation. No significant change in the gene expression of adipocyte markers was observed either, under the same conditions. The latter result seems different from a report on primary preadipocytes, where the expression of representative adipocyte markers was decreased by GH treatment (*9*). The mechanisms of suppression by GH are possibly different in certain points between primary preadipocytes and 3T3-L1. There is a notion that GH may enhance the release of stored lipid (*32–34*). We followed the change in the content of lipids pre-stored in fully differentiated 3T3-L1 cells, by culturing the cells for an extended period, in the presence or absence of GH. No significant decrease in lipid storage was observed under either condition (data not shown). These results suggest that the inhibition of adipocyte maturation by GH was not due to suppression of the expression of adipocyte-specific genes or enhancement of lipolysis, but probably occurred through modification of the metabolic flow of the lipogenesis system. Our results indicate that the accumulation of lipids and expression of marker proteins (*e.g.* GPDH) may not occur in parallel during adipogenesis, under certain conditions. This should be noted in the quantitative assessment of adipocyte differentiation.

Both the differentiation-promoting and suppressing effects of GH were observable, even in the presence of nearly a saturating concentration of IGF-1. Therefore, we conclude that IGF-1 is not sufficient to substitute for GH in these actions, in our experimental system. It is noteworthy that in the differentiation of other preadipocyte lines, GH was not replaceable with IGF-1 (*7*), but the former increased the sensitivity to the latter (*17–19*). We also found that GH increased the percentage of differentiated cells. Hence, the differentiation-promoting effect of GH is not simply a result of promoting cell growth or improving cell viability, though it is possible that GH selectively increases the number of potent cells. The latter possibility seems rather remote, however, considering the recent finding (*35*) that clonal expansion is not a prerequisite for adipocyte differentiation. It was reported (*36*) that GH induced an antimitogenic state in 3T3-F442A preadipocytes, thereby leading to differentiation. 3T3-L1 and F442A are probably in different stages of differentiation, as suggested by the fact that the former requires cocktail treatment to differentiate, whereas the latter does not. Such a difference might explain the different modes of action of GH in these cell lines. For GH signaling, four main pathways have been elucidated to date, that is, those involving mitogen-activated protein (MAP) kinase, Janus kinase (JAK)-signal transducers and activators of transcription (STAT), protein kinase C, and insulin receptor substrates-1 and 2 (*16*). Studies are required to clarify how these signaling cascades contribute to each of the dual actions of GH on adipogenesis.

In GH deficiency, mild obesity is generally observed. GH-deficient children have increased mean adipocyte volumes but reduced number of fat cells as compared with healthy children, and GH replacement normalizes these changes (*15*). Our results may support the hypothesis (*29*) that GH expands the pool of adipocyte precursor cells, but prevents the cells from maturing further, thus resulting in a reduced fat mass. The physiological role of GH in adipose tissue development should be assessed by further study, in light of the functional interactions with other hormones and growth factors, as well as the short and long-term changes in their concentrations in plasma.

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