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Removal of serum factors by charcoal treatment promotes adipogenesis via a MAPK-dependent pathway

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Abstract

In vitro differentiation of the progenitor cells or preadipocytes into adipocytes is usually achieved by adding an adipogenic mixture (isobutylmethylxanthine, dexamethasone, and insulin, IDI) to medium supplemented with fetal bovine serum (FBS). To study the effects of steroid hormones *in vitro*, endogenous hormones, growth factors and cytokines are removed by charcoal stripping of serum. However, the effects of charcoal-stripped serum (CS-FBS) *per se* on adipogenesis have been ignored. Here, we showed that alkaline phosphate activity and nodule formation of osteoprogenitor KS483 cells were lower in CS-FBS than in FBS. Concurrently, abundant amounts of adipocytes were only observed in KS483 cells cultured with CS-FBS, irrespective of the brands of serum used. Inhibition of the p42/44 MAPK pathway by its specific inhibitor PD98059 increased adipogenesis of KS483 cells with FBS, whereas activation of this signalling pathway by EGF blocked adipogenesis of these cells with CS-FBS. Furthermore, the p42/44 MAPK phosphorylation of KS483 cells cultured with CS-FBS was decreased compared with FBS. We concluded that charcoal-stripping of serum removed stimulators of the MAPK signalling pathway and in turn led to downregulation of osteogenesis and upregulation of adipogenesis. Interestingly, the adipogenic mixture IDI stimulated adipogenesis of KS483 cells cultured with CS-FBS, but not with FBS. Furthermore, differential effects of genistein on adipogenesis were observed in KS483 cells cultured with FBS or CS-FBS in combination with IDI. Our results showed that charcoal stripping of serum affected the commitment of KS483 cells and therefore differentially regulated adipogenesis influenced by IDI alone and in combination with genistein. (Mol Cell Biochem **268**: 159–167, 2005)

Key words: adipocytes, osteoblasts, genistein, estrogen, PPARγ, serum

Introduction

Adipogenesis *in vitro* has been mainly studied in two types of cell lines: multipotent stem cell lines and preadipocyte cell lines [1–3]. The differentiation of these cells into adipocytes *in vitro* is often performed in the medium supplemented with fetal bovine serum (FBS) by adding so called adipogenic factors like the thiazolidinediones or the mixture of isobutylmethylxanthine, dexamethasone, and insulin (IDI) and/or indomethacin [4–6]. Serum in most cell cultures is essential and several studies showed that serum *per se* is the most powerful factor influencing cellular proliferation and differentiation *in vitro*. For example, to eliminate endogenous steroid hormones, growth factors and cytokines in the culture medium, charcoal-stripping of the serum is often used [7–9]. However, the performance of the cells cultured in medium containing charcoal-stripped fetal bovine serum (CS-FBS) may differ from that of the cells in medium containing FBS.

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It has been reported that growth inhibition was observed in human CFU-megakaryocytes [10], and in MCF-7 breast cancer cells [7] when these cells were cultured with CS-FBS instead of FBS. Moreover, preosteoblastic MC3T3-E1 cells had a limited growth and did not increase alkaline phosphatase (ALP) activity when FBS was replaced by CS-FBS [9]. Despite of enormous differences in the composition between FBS and CS-FBS, both types of sera have been equally used in adipogenesis *in vitro* [11, 12]. It is unknown, however, whether charcoal stripping of serum affects adipogenesis *in vitro*.

Adipogenesis is controlled by several transcriptional regulators including the CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor-γ (PPARγ) [13–15]. Among these transcriptional factors, PPARγ2, the adipocyte-specific isoform, is a critical dominant regulator of this process [14, 16, 17]. Ligands like thiazolidinediones and indomethacin can bind to and activate PPARγ, resulting in adipogenesis [18, 19]. Furthermore, it has been shown that PPARγ [14, 16] contains a consensus mitogen-activated protein kinases (MAPK) site in the A/B domain [20–22]. Phosphorylation of this site by p44/42 MAPKs decreases PPARγ activity, leading to a downregulation of adipogenesis. In constrast, PD98059, an inhibitor of p44/42 MAPK pathway, increased PPARγ activity leading to an upregulation of adipogenesis [20, 23].

As mentioned above, PPARγ activity can be regulated by two ways: ligand binding or phosphorylation of this receptor, which in turn influences adipogenesis *in vitro*. We have recently shown that the tyrosine kinase inhibitor genistein is a ligand of PPARγ and an inhibitor of p42/44 MAPK activity which dually activated PPARγ, resulting in adipogenesis in osteoprogenitor KS483 cells [24]. In contrast, several studies from different groups showed that genistein at the same concentration range as we used inhibited adipogenesis in preadipocyte 3T3-L1 cells [25, 26]. One of the striking differences between these two culture systems is adding IDI or not. In 3T3-L1 cell cultures, IDI was used to stimulate adipogenesis, whereas IDI was not used in KS483 cell cultures. In this study, we tested whether IDI affects genistein-induced adipogenesis in KS483 cells.

KS483 cells, cloned from mouse calvaria, have been shown to differentiate into only osteoblasts when cultured in the medium containing FBS [27–29]. Surprisingly, we observed that these cells could differentiate into both osteoblasts and adipocytes once FBS was replaced by CS-FBS [24, 30]. These differential responses of KS483 cells in FBS and CS-FBS promoted us to study whether the responses are purely limited to specific brands of serum or due to the charcoal stripping of the serum.

Here, we showed that charcoal stripping of sera promoted adipogenesis. The possible mechanisms of this adipogenesis have been further explored. In addition, we showed that IDI only increased adipogenesis of KS483 cells cultured with CS-FBS, but not with FBS. Moreover, the differential effects of genistein on adipogenesis have been observed when KS483 cells were cultured with FBS, CS-FBS, and in combination with IDI.

Materials and methods

Cell culture

KS483 cells were cultured in phenol red free α-minimum essential medium (α-MEM) supplemented with 10% FBS (Gibco BrL Life Technologies, GIB) and penicillin/streptomycin as maintenance medium at 37 °C in a humidified atmosphere of 5% CO_2 in air. For the experiments, cells were plated at a density of 15,000 cells/cm^2 cultured in the medium with a supplement of ascorbic acid (50 μg/mL) from day 4 on and β-glycerophosphate (5 mM) from day 11 on after plating. Cell culture medium was changed every 3 to 4 days.

Mouse bone marrow cells were isolated from the marrow of tibias and femurs of 6- to 8-week-old female mice. All cultures were carried out in phenol red free α-MEM supplemented with 15% FBS or CS-FBS, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate and 10^{-8} M dexamethasone as well as penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were plated at a density of 6×10^5 cells/cm^2 in a 12-well-plate and cultured for 21 days. Cell culture medium was changed every 3 to 4 days.

Sera

Various brands of FBS used in this study were from following companies: PAA Laboratories GmbH, Catalogue Number A11-043; Boehringer ingelheim Bioproducts partnership, Catalogue Number 14-602E; Gibco BrL Life Technologies, Catalogue Number 10270-098; Greiner, Catalogue Number 758094, and HYCLONE, Catalogue Number CH30160. Stripping of sera was prepared in our lab based on a published protocol [9]. Briefly, Dextran-coated charcoal was prepared by adding 2.5 g of charcoal (Sigma) into the solution that contains 0.25 g of dextran (T500, Sigma) and 1000 mL of Tris-HCL (0.1 M, pH 8.0). After stirring overnight at 4 °C, this suspension was centrifuged at 1000 *g* and the supernatant was discarded. The dextran-coated charcoal pellets was mixed with 250 mL heat inactivated sera and incubated at 45 °C for 45 min. Charcoal was then removed by centrifugation and the sera were sterilized by filtering. In addition, one brand of commercially available CS-FBS (Biological industries Co. Israel) was used. The estrogen concentrations before and after charcoal stripping have been measured (Table 1), which suggest

Table 1. Parameters in sera measured before and after charcoal treatment

	PA	Spa	BO	Sbo	GI	Sgi	GR	Sgr	IB
Estradiol (pmol/L)	163.8	<40	117,1	<40	121	<40	105.2	<40	<40
T3 (nmol/L)	1.6	0,5	2,1	<0.5	1,2	<0.5	2.2	<0.5	<0.5
T4 (nmol/L)	136	25	167	36	168	29	183	42	142
25-OH-VIT.D (nmol/L)	27	26	36	39	48	44	28	23	33
1,25-OH2-D (pmol/L)	80	35	76	42	116	24	115	68	100
total cholesterol (mmol/L)	0.34	0.23	0.34	0.25	0.27	0.25	0.34	0.31	0.29

that charcoal stripping of serum is efficient to remove steroid hormones. The concentration of indomethacin and genistein used in this study was 50 μM and 30 μM, respectively. The IDI mixture (including 1.6 μM human insulin, 0.25 μM dexamethason and 0.5 mM 1-methyl-3-isobutylxanthine) was added when osteoprogenitor cells became confluent.

Assays for ALP and DNA

Cellular ALP activity and the content of DNA from intact KS483 cells in the same well were measured. Cells were washed with phosphate buffered saline (PBS), then frozen immediately and kept at -20 °C until use. A solution of 1*SSC with 0.01%SDS was added to the cells which were then sonificated. ALP activity was kinetically determined at a wavelength of 405 nm by a colorimetric assay using *p*-nitrophenylphosphate as a substrate at pH 10.5. DNA was measured by the method of an enhancement of fluorescence using Hoechst 33258 (Sigma) binding to DNA [31].

Expression of PPARγ2, aP2 and LPL

The method of RNA isolation, competitive and semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and the description of primers of PPARγ2, aP2 and LPL have been described previously [30].

Staining

Nodules and adipocyte before any staining were objectively counted under a light microscope based on their distinct morphology. Adipocytes were further confirmed by *Oil Red O* staining, which is specific for the lipid droplets in the adipocytes. Oil Red O (4.2 g) was dissolved in 1200 mL of isopropanol and then diluted with distilled water at a ratio of 4:3. Cell monolayers were rinsed with PBS and fixed with 4% formalin for 10 min. After washing with water, cells were stained for 1 h by immersion with Oil Red O solution. The staining was stopped by rinsing with water.

Double staining for ALP and Oil-Red-O

After Oil-red-O staining, cells was washed with water and then stained for ALP. These cells were immersed in 0.5 mL ALP staining medium, which contained fast blue and naphthol AS-MX phosphate (Sigma). The staining was stopped by washing with PBS after colouring for 10 min.

Immunoblot analysis of p42/44 MAPKs

KS483 cells were seeded in 6-well plates and cultured in α-MEM contining 10% FBS. After four days of cell culture, the medium was changed and these cells were cultured in serum free α-MEM overnight and then exposed to FBS, CS-FBS (serum from GIBCO) for 80 min. Epidermal growth factor (EGF) stimulation was carried out by adding 50 ng/mL EGF for 15 min. Cells were washed twice in PBS and then collected. Proteins of KS483 cells were fractionated by SDS-PAGE with a 12.5% acrylamide separation gel, and the separated proteins were transferred to nitrocellulose membranes (pore size 0.45 μm; Schleicher and Schuell, code 401196). After blocking the membranes with the mixture of 2% milk powder, 1% BSA and 0.1% gelatin, proteins were probed with the p42/p44 MAPK phospho-specfic antibody (Sigma). After overnight incubation with the first antibody at 4 °C, the second antibody anti-mouse IgG-peroxidase conjugates was used. The results were visualized by ECL detection system.

Transfection of cultured cells and reporter assays

The luciferase reporter construct contained three copies of PPRE cloned in front of a minmal TK promoter [24]. KS483 cells were seeded into 24-well plates. After 24 h, these cells were transfected using a lipid-based FuGENE 6 transfection reagent (Roche, Basel, Germany). For each triplicate of sample, 100 ng luciferase reporter and 500 ng β-galactosidase expression vector were applied. Then after 16 h, the medium was changed into experimental medium. After 48 h exposure of KS483 cells to the experimental medium, KS483 cells were

washed twice with PBS, lysed in PBS containing 1% Triton X-100 and sonificated. Luferase activity was measured and expressed as fold induction ±S.E.M. The transfection efficiency was corrected by β-galactosidase activity.

Statistics

Data are presented as means ±S.E.M. Differences between groups were accepted at $P < 0.05$, which were assessed by one-way ANOVA or related test using software Instat.

Results

KS483 cells cultured with the medium containing CS-FBS differentiated into both osteoblasts and adipocytes, as indicated by ALP staining for osteoblasts and Oil-Red-O staining for adipocytes (Fig. 1A). These cells were also able to increase ALP activity in a time-dependent way and form mineralised nodules with both FBS and CS-FBS [29, 30]. In this study, the effects of CS-FBS on osteogenesis of KS483 cells have been further studied using different brands of sera. As shown in Fig. 1B, typical osteogenic parameters, ALP activity and nodule formation, varied with different brands of sera. For each brand of the serum, however, both ALP activity and nodule formation were decreased with CS-FBS when compared with FBS (Fig. 1B and C).

Adipogenesis of KS483 cells was confirmed by Oil-Red-O staining of adipocytes and the expression of typical adipocyte marker PPARγ2, aP2 and LPL. Adipocytes appeared after seven days of culture, reached a peak value at day 14, and decreased thereafter. Expression of PPARγ2 and aP2 was only detected when adipogenesis appeared in our culture system. The number of adipocytes during differentiation was correlated with the changes in mRNA expression of PPARγ2, aP2 and LPL [24, 30]. To test whether adipogenesis promoted by CS-FBS is limited to a specific brand of serum or not, KS483 cells were cultured for 14 days in the medium supplemented with various brands of FBS or CS-FBS. As shown in Fig. 2, adipocytes were not found in KS483 cells cultured with two brands of FBS, GIBCO, and BOEHRINGER during the whole period of cell culture. Only a few adipocytes were observed when other two brands of sera, GREINER and PAA, were used. In contrast, abundant amounts of adipocytes were observed in all CS-FBS. In our lab, KS483 cells are routinely cultured in FBS from either GIBCO or HYCLONE that does not promotes adipogenesis. To further confirm that CS-FBS-promoted adipogenesis in KS483 cells, we used a commercially available CS-FBS and found that adipocytes appeared in these cultures (Fig. 2). To show that adipogenesis promoted by CS-FBS is not restricted to KS483 cells, we also used primary mouse mesenchymal

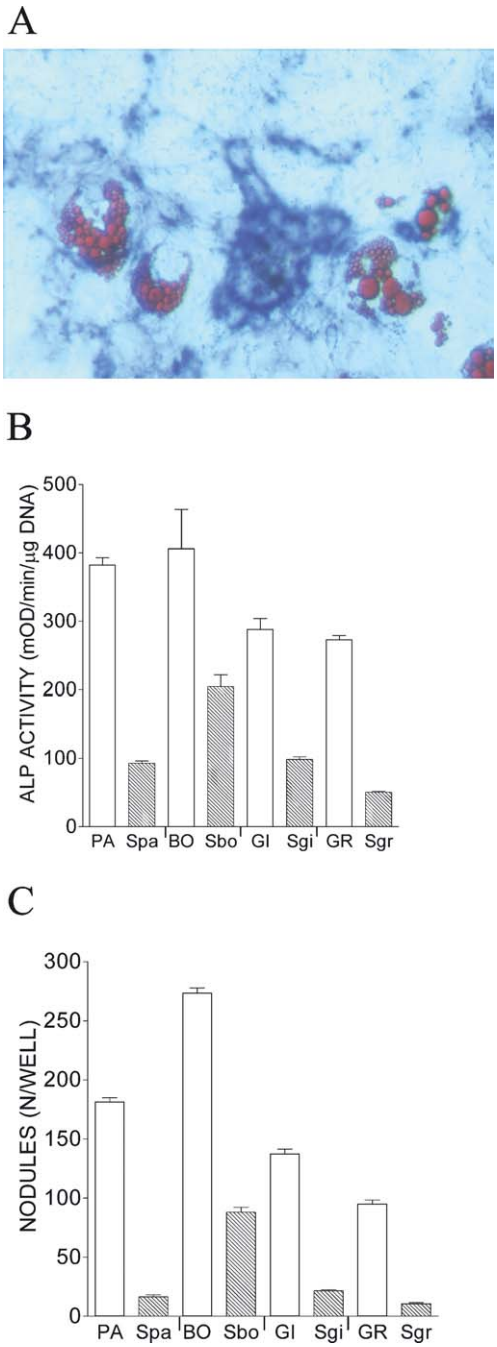

Fig. 1. Effects of charcoal stripping of sera on osteogenesis. KS483 cells were cultured in 12-well plates in non-stripped- (FBS) and charcoal-stripped-sera (CS-FBS) for 14 days. These cells are able to differentiate into both ALP-positive osteoblasts (A, blue staining) and Oil-red O-positive adipocytes (A, red staining). Osteoblastic markers ALP activity (B) and nodule formation (C) were measured. Each value is the mean ± S.E. of the results from three different wells and is representative of results from three different experiments. PA, serum from PAA Laboratories; BO, serum from Boehringer ingelheim Bioproducts partnership; GI, serum from Gibco BrL Life Technologies; GR; serum from Greiner. Charcoal-stripped sera were indicated as S in front of the company name as mentioned above. Results for FBS were expressed by empty bars and CS-FBS by black bars.

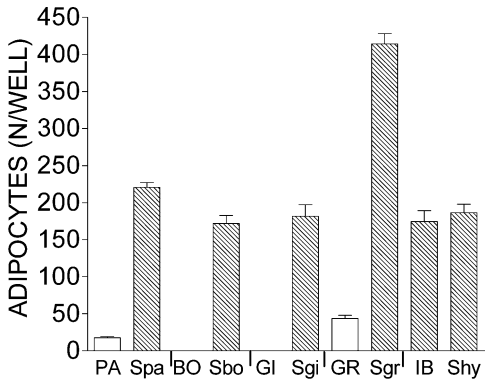

Fig. 2. Charcoal-stripped serum induces adipogenesis. KS483 cells were cultured in 12-well plates in the medium supplemented with FBS and CS-FBS for 14 days. Each value is the mean ± S.E. of the results from three different wells and is representative of results from three different experiments. PA, serum from PAA Laboratories; BO, serum from Boehringer ingelheim Bioproducts partnership; GI, serum from Gibco BrL Life Technologies; GR; serum from Greiner. Shy, the charcoal stripped serum from HYCLONE. Charcoal-stripped sera were indicated as S in front of the company name as mentioned above. In addition, IB is the commercially available stripped-serum. Results for FBS were expressed by empty bars and CS-FBS by black bars.

bone marrow cell cultures. Adipocytes were not found in cultures with FBS. In contrast, adipocytes (408 ± 63 adipocytes per well) were observed when these cells cultured with CS-FBS for 21 days. Clearly, these results demonstrated that CS-FBS has an effect on the commitment of KS483 cells, resulting in a decrease of osteogenesis and an increase of adipogenesis.

We further explored the possible mechanisms responsible for the promotion of adipogenesis by CS-FBS. Charcoal-stripping of the serum is a well-known method to remove steroid hormones [9]. For this reason, we first measured the concentrations of several hormones in the sera before and after charcoal-stripping. As shown in Table 1, charcoal stripping of sera efficiently decreased the levels of 17β-estradiol (E2), T3, and T4, indicating that the efficiency of our method is comparable to that described in the literature [9]. It has been shown previously that the level of IGF-I was decreased from 119.4 ng/mL to 27 ng/mL after charcoal treatment [9]. Since the method we used and the parameters measured were comparable to those described in the literature [7–9], it is likely that our charcoal treatment of sera eliminated not only endogenous steroid hormones but also growth factors and cytokines.

In the following experiments, the GIBCO serum, unless indicated, was used. We first tested whether CS-FBS (using sera from both GIBCO and HYCLONE) promoted adipogenesis is due to a removal of E2. To test this, we added E2 ranging from 10^{-15} to 10^{-5} M back to the CS-FBS and found a dose-dependent inhibition of adipogenesis starting at the concentration from 10^{-13} M, with a maximum inhibition at 10^{-9} M (238 ± 33 adipocytes per well in CS-FBS and 55 ± 12 adipocytes per well in the 10^{-9} M E2 treated group). However, E2 at all tested concentrations did not completely inhibit CS-FBS-promoted adipogenesis. Furthermore, when KS483 cells were cultured in FBS and exposed to the specific antiestrogenic compound ICI164,384 or ICI182,780 at concentrations between 10^{-9} and 10^{-5} M, adipocytes were hardly found in the cultures. We observed only a few adipocytes in some but not all culture wells. Based on these results, we concluded that removal of estrogen was not the exclusive factor responsible for CS-FBS-promoted adipogenesis.

It has been reported that an inhibition of gap junctional communication by 18α glycyrrhetinic acid induced adipogenesis in mouse preosteoblast MC3T3-E1 cells and human osteoblast cells [32]. In KS483 cells cultured with FBS, however, 18α glycyrrhetinic acid at all tested concentrations ranging from 2×10^{-4} to 10^{-9} M did not induced adipogenesis. Furthermore, no adipogenesis was observed when these cells were exposed to a specific p38 pathway inhibitor SB203580 at concentrations of 10^{-4} to 10^{-9} M. Taken together, these results indicate that CS-FBS-promoted adipogenesis was not due to an inhibition of either gap-junctional pathway or the p38 pathway.

PPARγ is a critical transcriptional factor for adipogenesis [14, 16, 17]. PPARγ agonists, ciglitazone or LY-171883 (10^{-6} M) induced adipogenesis (87 ± 10 or 104 ± 10 adipocytes per well, respectively) in KS483 cells that were cultured in FBS, suggesting that PPARγ plays an essential role in adipogenesis of these cells. Charcoal stripping of serum only removes factors present in the serum. Therefore, it is unlikely that adipogenesis promoted by CS-FBS was due to a ligand activation of PPARγ. Another possibility is that the A/B domain of PPARγ contains a consensus MAPK site and phosphorylation of this site inhibits adipogenesis [20–22]. We tested whether adipogenesis promoted by CS-FBS is due to an inhibition of p42 and p44 MAPK phosphorylation. When KS483 cells were cultured with FBS and exposed to the MAPK inhibitor PD98059 at concentrations between 1 and 25 μM, it dose-dependently induced adipogenesis. In contrast, growth factor EGF activated p44 and p42 MAPKs and in turn led to a dose-related inhibition of adipogenesis of KS483 cells cultured with CS-FBS (Fig. 3A and B). EGF at concentrations higher than 1 ng/mL could completely block adipogenesis of KS483 cells cultured with CS-FBS (Fig. 3). These results suggest that CS-FBS might inhibit p42/44 MAPKs, leading to an increase in adipogenesis. To show that CS-FBS inhibited p42/44 MAPKs, we did Western blot analysis and found that p42 and p44 MAPKs were lower in KS483 cells cultured with CS-FBS than those with FBS (Fig. 3C). Taken together, these data strongly suggest that adipogenesis promoted by CS-FBS might result from

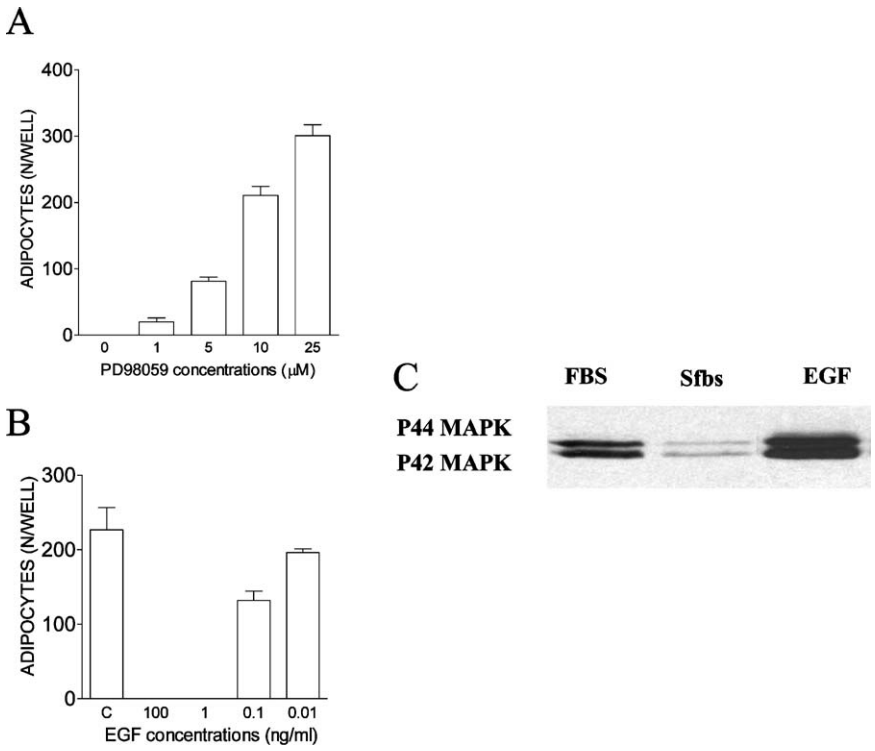

Fig. 3. Adipogenesis of KS483 cells was affected via p42/p44 MAPK pathway. KS483 cells were cultured in 12-well plates in the medium supplemented with FBS and exposed to PD98059 for 14 days. A dose-related increase in adipogenesis was found (A). In contrast, a dose-related inhibition of adipogenesis, with the complete inhibition above 1 ng/mL, was observed in KS483 cells cultured with CS-FBS and continuously exposed to EGF (B). The number of adipocytes was expressed as means ± S.E. from three different wells, which is representative of results from three different experiments. For western blot analysis (C), KS483 cells were cultured in 6-well plates in the α-MEM containing 10% FBS. After four days of cell culture, these cells were cultured in serum free α-MEM overnight and then exposed to FBS, CS-FBS or EGF. CS-FBS had reduced activation of p42/p44 MAPK activity, whereas EGF activated dually-phosphorylated form of p42 and p44 MAPKs.

a reduction of p42/44 MAPK phosphorylation. To further show that CS-FBS-promoted adipogenesis is due to an activation of PPARγ, we transfected KS483 cells with PPRE-luc. Compared to FBS, the PPRE-luc reporter activity of KS483 cells cultured with CS-FBS was increased to 1.5 ± 0.17. This increase is comparable to the reporter activity of other PPARγ agonists, ciglitazone (1.5 ± 0.11) and LY-171883 (1.7 ± 0.21). In summary, our results strongly suggest that CS-FBS downregulated p42/44 MAPKs, leading to an activation of PPARγ and adipogenesis.

It has been well established that IDI promotes adipogenesis in preadipocyte 3T3-L1 cells [1, 25]. There was no difference in IDI-enhanced adipogenesis of 3T3-L1 cells cultured with FBS and CS-FBS in our study (data not shown). To our surprise, the well-known adipogenic mixture IDI did not increase adipogenesis in KS483 cells cultured with FBS. In contrast, IDI increased the number of adipocytes at least five-fold in KS483 cells cultured with CS-FBS (Fig. 4). These results showed that serum *per se* differentially influenced IDI-stimulated adipogenesis of KS483 cells.

We further tested whether adipogenesis induced by agonists of PPARγ was influenced by the widely used basic culture medium, i.e. serum or serum in combination with IDI. Indomethacin, an agonist of PPARγ, has been shown to induce adipogenesis [18]. In KS483 cells, indomethacin potently induced adipogenesis in the medium supplement with either FBS or CS-FBS. Moreover, indomethacin-induced adipogenesis was potentiated by IDI when these cells cultured with either FBS or CS-FBS (Fig. 4A).

Genistein, another agonist of PPARγ, has been shown to affect biphasicly adipogenesis of KS483 cells cultured with CS-FBS [24]. In this study, we chose the concentration of genistein that stimulated adipogenesis. As shown in Fig. 4B, genistein increased adipogenesis of KS483 cells cultured with FBS or CS-FBS. Furthermore, IDI promoted genistein-induced adipogenesis of KS483 cells cultured with either FBS or CS-FBS. Interestingly, genistein only decreased IDI-stimulated adipogenesis of KS483 cells cultured with CS-FBS, which is consistent with those reported in 3T3-L1 cells [25, 26] and observed in our studies using 3T3-L1 cells cultured with

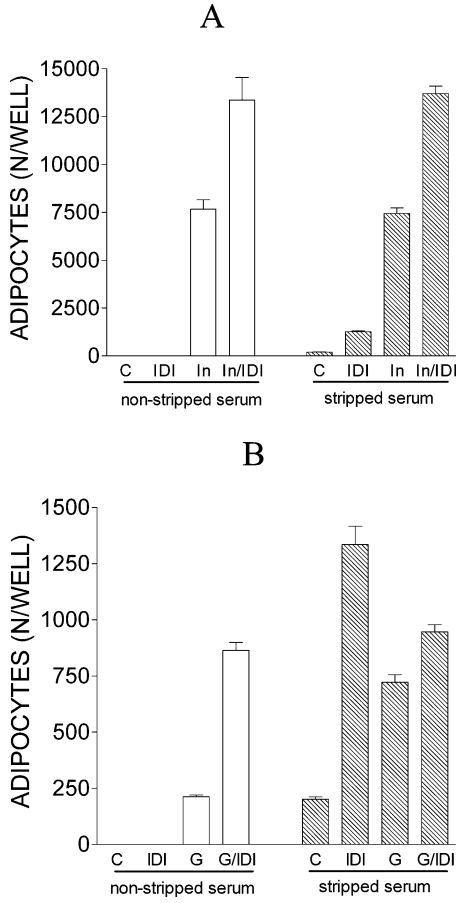

Fig. 4. Adipogenesis under different culture conditions. KS483 cells cultured in 12-well plates were exposed, after confluent, to the adipogenic mixture IDI, 50 μM indomethacin (In), indomethacin plus IDI (IDI/In), 30 μM genistein (G), or genistein plus IDI (G/IDI) in FBS or CS-FBS for 14 days. The number of adipocytes was quantified and expressed as mean $\pm$ S.E. of the results from three different wells. The results for FBS were expressed by empty bars and CS-FBS by black bars.

either FBS or CS-FBS in combination with IDI (data not shown).

Discussion

From the current study, three conclusions can be drawn. First, charcoal stripping of serum removes serum factors that activated p42/44 MAPKs, leading to a decrease in osteogenesis and an increase in adipogenesis of KS483 cells. Second, the well-known adipogenic mixture IDI only promoted adipogenesis of KS483 cells that were committed into the adipogenic linage. Third, genistein-induced adipogenesis was differentially affected in KS483 cells cultured with FBS and CS-FBS in combination with IDI. Clearly, adipogenesis *in vitro* is affected by the serum and the adipogenic mixture IDI.

It has been shown that charcoal-stripping of serum removes endogenous steroid hormones, growth factors and cytokines [7–9]. As shown in this study, removal of these active compounds resulted in a decrease in p42/44 MAPK phosphorylation. It has been well established that MAPK-dependent phosphorylation of Cbfa1 increases osteogenesis, whereas an inhibition of this phosphorylation decreases osteogenesis [33, 34]. Our observations that ALP activity of KS483 cells in all tested sera was lower with CS-FBS than with FBS suggest that the decrease in p42/44 MAPK activity in CS-FBS affects osteogenesis of KS483 cells. This is in line with data on MC3T3-E1 cells that also exhibited a decreased ALP activity when cultured with CS-FBS compared to FBS [9].

In the original paper of Yamashita *et al.*, the calvaria-derived clonal cell line KS483 was selected for its commitment towards the osteoblastic lineage [27, 28]. As expected, when KS483 cells were cultured with several brands of FBS, they only developed into fully differentiated osteoblasts [28, 29]. To our surprise, these cells, when cultured with CS-FBS even under so-called osteogenic conditions, concurrently differentiated into both osteoblasts and adipocytes. As shown in this study, the promotion of adipogenesis by CS-FBS is not limited to specific brands of sera from different companies nor to the cell line used because similar results were found in bone marrow mesenchymal cell cultures. Our results indicate that serum *per se* influences the commitment of osteoprogenitor cells *in vitro*.

We further studied the possible factors or mechanisms that are involved in adipogenesis promoted by CS-FBS. By adding various inhibitors, antagonists, and agonists to FBS or CS-FBS, we were unable to identify the specific factor(s) responsible for adipogenesis. For example, removal of E2 is unlikely the exclusively factor responsible for adipogenesis because E2 only partially inhibited adipogenesis in CS-FBS and antiestrogens did not induce adipogenesis in FBS. Furthermore, we were unable to show adipogenesis of KS483 cells induced by low-density lipoprotein (data not shown), which has been reported to promote adipogenic differentiation of marrow stromal cells [35]. Rabbit serum, which contains much higher content of fatty acids compared to fetal bovine serum, induces adipogenesis [36]. However, we demonstrated that KS483 cells cultured with FBS did not differentiate into adipocytes, which indicated that the level of potential ligands for PPARγ, e.g. fatty acids, is not high enough. Since charcoal-stripping only removed factors present in the serum, ligands for PPARγ like fatty acids are unlikely the factors responsible for adipogenesis of KS483 cells cultured with CS-FBS. It may be impossible to identify a single factor that is key to the promotion of adipogenesis of KS483 cells. Collectively, all the factors removed from charcoal stripping may contribute to the disturbance of essential pathways that are involved in adipogenesis.

Instead of identifying key factors responsible for adipogenesis, we identified p42/44 MAPK pathway as an essential signaling pathway for adipogenesis promoted by CS-FBS. This conclusion is in line with the consensus that p44/42 MAPK phosphorylation regulates PPARγ activity [20–22]. Our results that PD98059, an inhibitor of p42/44 MAPKs, induced adipogenesis and EGF, an activator of p42/44 MAPKs, inhibited adipogenesis support this conclusion. As estrogen and growth factors like PDGF, EGF, FGF, IGF-I regulate p42/44 MAPKs and inhibit adipogenesis [30, 37], it is likely that a combination of different factors, rather than just one factor, is responsible for regulation of this pathway, leading to the promotion of adipogenesis of KS483 cells cultured with CS-FBS. Furthermore, it has been shown that an inhibition of gap-junctional communication induces adipogenesis in human osteoblastic cells [32]. However, an inhibitor of gap-junctional communication, 18α-glycyrrhetinic acid, did not induce adipogenesis of KS483 cells cultured with FBS.

The adipogenic mixture IDI has been widely used in studies of adipocyte differentiation [1, 2, 25]. However, the results are not consistent [1]. Our results showed that IDI had differential effects on adipogenesis when KS483 cells cultured with FBS or CS-FBS. These differential effects result from serum *per se*, which influence the commitment of these cells. Moreover, our results suggest that IDI only increased adipogenesis once these cells are committed to the adipogenic linage and PPARγ is activated, i.e., when KS483 cells were cultured with CS-FBS or exposed to indomethacin, and genistein. This conclusion is further supported by the observations that IDI stimulated adipogenesis in bone marrow mesenchymal cells, bone marrow stromal cell lines, human trabecular bone derived cells and 3T3-L1 cells, which already had adipogenic potentials [5, 6, 12, 38].

Differential effects of genistein on adipogenesis in KS483 cells cultured with FBS and CS-FBS in combination of IDI resulted from the serum *per se* and the counteraction of genistein and IDI. When KS483 cells cultured with FBS plus IDI, IDI-enhanced adipogenesis can only be found in KS483 cells in which adipogenesis was induced, i.e. genistein-treated cells. When KS483 cells were cultured with CS-FBS, however, CS-FBS alone induced adipogenesis and IDI could further enhance adipogenesis. Compared to the IDI treated group, genistein decreased IDI-enhanced adipogenesis, which showed a counteraction between genistein and IDI. Several molecular mechanisms of this counteraction have been proposed. It has been suggested that genistein-inhibited adipogenesis is via a decrease in IDI-stimulated phosphotyrosine of Syk that influenced the expression of G protein α subunit (Gsα), an important protein for regulating differentiation of 3T3-L1 cells [25, 39]. It has also been shown that IDI-stimulated tyrosine phosphorylation of C/EBPβ [26]. Genistein blocked C/EBPβ activity, which in turn decreased the expression of two critical adipogenic transcription factors C/EBPα and PPARγ and led to an inhibition of adipogenesis [26].

In conclusion, our results showed that charcoal stripping of serum affected the commitment of osteoprogenitor cells, which resulted in differential effects on adipogenesis affected by IDI alone and in combination with genistein.

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