ORIGINAL

Progesterone dose-dependently modulates hepatocyte growth factor production in 3T3-L1 mouse preadipocytes

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Abstract. It is well documented that estrogen is predominant inducer of hepatocyte growth factor (HGF) in a variety of cell types. However, the effect of progesterone (P) remains to be elusive. Thus, in the present study, we examined the effect of P and combined effect of P and 17 β -estradiol (E2) on HGF expression and production in 3T3-L1 fibroblastic preadipocytes and mature adipocytes, as a model of stromal cells. Northern blot analysis showed that *hgf* mRNA expressed in preadipocytes was notably higher than that of mature adipocytes, and increased by treatment of preadipocytes with E2 or 10 nM P, but not with 1,000 nM P. The E2-induced *hgf* mRNA expression was enhanced by 10 nM P, but suppressed by 1,000 nM P. Western blot analysis revealed that biological active forms of HGF protein was found in the preadipocyte culture medium, while the lesser amount of HGF precursor protein was detected in the mature adipocyte culture medium. The amounts of HGF were changed dependently on the *hgf* mRNA expression levels. These results indicate that HGF production is intricately regulated by E2 and P at the transcriptional levels in 3T3-L1 cells, and may explain the changes in the HGF production during the mammary gland development, especially decrease in HGF expression during pregnancy when P concentration is high.

Key words: Adipocyte, Estrogen, HGF, Mammary gland, Progesterone

HEPATOCYTE GROWTH FACTOR (HGF), also known as a scatter factor, is a heparin-binding glycoprotein mainly derived from stroma, and acts on a variety of epithelial cells expressing c-Met proto-oncogene, a HGF receptor, to induce cell proliferation, motility, differentiation and morphogenesis such as formation of branching tubules [1-4]. In the mouse mammary gland, *hgf* mRNA is detected in a thin layer of mesenchymal cells adjacent to the ductal epithelium, while the transcript for the c-Met receptor is localized within the basal epithelial cell layer of the ductal epithelium [5]. Moreover, mouse mammary fibroblast produces HGF [6], which induces branching morphogenesis of mammary epithelial cells [6, 7]. HGF is therefore thought to play pivotal roles in the mammary gland development as a mediator of epithelial-stromal interactions [5-9].

Interestingly, *hgf* mRNA expression in the mammary gland varies during the cycle of morphologic and functional change associated with the process of puberty, pregnancy, lactation and involution in postnatal life. That is, *hgf* mRNA expression is high after the puberty, decreased during pregnancy, almost vanished during lactation, and re-appeared at involution [5, 7].

It is well known that the processes of mammary gland development are highly controlled by ovarian steroids such as estrogen and progesterone (P), and that these hormonal levels are altered dramatically during the process of puberty, estrus cycle, pregnancy, lactation and involution [8-11]. In addition, expression and production of HGF in mammary fibroblasts, ovary cells, and macrophages are enhanced in response to estrogen [8, 12-14], suggesting that estrogen is predominant inducer of HGF in a variety of cell types. However, the effects of P on expression and production of HGF remain elusive [14], whereas both progesterone receptor and estrogen receptor are expressed in mammary stroma [15].

The mammary stroma is comprised of not only fibroblasts including fibroblastic preadipocytes, but

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also a variety of cells including adipocytes and endothelial cells [16]. Although endothelial cell is a target of HGF action [17], adipocytes as well as fibroblasts are shown to produce HGF [6, 18, 19]. To investigate the regulation by ovarian steroids of HGF production in fibroblastic preadipocytes and adipocyte, in the present study we examined the effect of 17 β -estradiol (E2) and/or P in 3T3-L1 pre- and mature adipocytes as a model of stroma cells.

Materials and Methods

Materials

17 β -estradiol (E2), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (Dex), and phenol red-free Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich Fine Chemical Co. (St. Louis, MO, USA). Progesterone (P) was bought from Nacalai Tesque Inc. (Kyoto, Japan). Fetal calf serum (FCS) was from Trace Scientific Ltd. (Melbourne, Australia) and a part of FCS were treated with mixture of NotA charcoal (J.T Baker Chemical Co., Phillipsburg, NJ, USA) and dextran T-70 (GE Healthcare, Little Chalfont, Bucks, UK) (10:1 in weight), to remove endogenous sex steroids. Troglitazone was kindly provided by Daiichi Sankyo Co. (Tokyo, Japan).

Cell culture and treatments

Mouse 3T3-L1 cells were cultured in phenol red-free DMEM supplemented with 10% FCS at 37°C under 5% CO₂. After reaching 80% confluency, the cells were cultured in DMEM containing 5% charcoal-dextran treated FCS for 2 days and used as preadipocytes. Fully confluent 3T3-L1 cells were also cultured sequentially in DMEM containing 10% FCS, 0.5 mM IBMX, 1 μ M Dex for 2 days, DMEM containing 10% FCS, 10 μ M troglitazone and 1 μ g/mL insulin for 4 days, and DMEM containing 5% charcoal-dextran treated FCS, 10 μ M troglitazone and 1 μ g/mL insulin for 2 days. The latter cells were used as mature adipocytes. Pre- and mature adipocytes were treated with various concentrations of E2, P or combination of both (originally dissolved in 70% ethanol) for indicated time periods.

RNA extraction and Northern blotting

Total RNA was isolated from cultured 3T3-L1 pre- and mature adipocytes with RNAiso (Takara Bio Inc., Siga, Japan) according to the manufacture's instructions. For Northern blot analysis, total RNA (40

μ g) was resolved on 1% agarose-formaldehyde gel, transferred onto a nylon membrane (Hybond-N⁺; GE Healthcare) and cross-linked under UV light (70,000 mJ/cm²). Both prehybridization and hybridization were performed at 65°C for 2 h and overnight, respectively, in a buffer containing 7% SDS, 0.5 M Church's phosphate buffer (pH 7.2), 1 mM EDTA and 0.4 mg/mL salmon sperm DNA. After prehybridization, the membrane was sequentially hybridized with a cDNA probe encoding mouse *hgf* cDNA (L25131; +2,803 to NM_010427; +2,514) or mouse *gapdh* cDNA (NM_008084, +537 ~ +1,027) as the internal control for loading. The probe was labeled with [α -³²P]-dCTP using the Megaprime DNA labeling systems (GE Healthcare) according to the instructions provided. After hybridization, the membrane was stringently washed for 20 min twice with 2x SSC containing 0.1% SDS, and once with 0.1 x SSC containing 0.1% SDS at 65°C before exposure onto a X-ray film (RX-U; Fuji Photo Film Co., Ltd, Tokyo, Japan). Intensities of bands were analyzed densitometrically using NIH Image program (<http://rsb.info.nih.gov/nih-image/>).

Western blot analysis on heparin binding proteins

Pre- and mature adipocytes 2 days after replacement of the 5% charcoal-dextran treated FCS were cultured further for 48 h with fresh DMEM containing 3% charcoal-dextran treated FCS with E2, P or both. The conditioned culture medium (CM, 10 mL) was then collected and incubated with 50 μ L of heparin-agarose (50% slurry, Sigma) overnight at 4°C to concentrate heparin-binding proteins. After washing three times with 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, heparin binding proteins were recovered in 50 μ L of SDS sample buffer [50mM Tris-HCl (pH 7.5), 8% SDS, 15% glycerol, 12% 2-mercaptoethanol and 6% bromophenol blue] by boiling for 3 min.

The proteins were separated by SDS-PAGE (10% gel) under reducing conditions and proteins were electroblotted onto PVDF membrane (ImmobilonTM, Millipore, Bedford, MA, USA). The membrane was blocked overnight at 4°C in Ez Block (ATTO, Tokyo, Japan), followed by incubation with anti-mouse HGF antibody (R&D systems Inc., Minneapolis, MN, USA) diluted 2,000-fold with the Can Get Signal solution 1 (Toyobo Co., Osaka, Japan) for 4 h at 4°C. The membrane was washed 8 times with 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.1% Tween 20, and incubated with horseradish peroxidase-conjugated

anti-goat IgG antibody (1:4,000, Jackson Immuno Research Laboratories, West Grove, PA, USA) for 2 h at 4°C. Visualization was performed using enhanced chemiluminescence detection system (Millipore) according to the manufactures' instruction. Intensities of immunoreactive bands were analyzed densitometrically using NIH Image program.

Statistical analysis

Results are expressed as means $\pm$ standard error of the mean (SEM) of independent experiments. Statistical analyses were performed using either Student's *t*-test or ANOVA and Bonferroni *post-hoc* test, with $p < 0.05$ being considered as statistically significant.

Results

Northern blot analysis was performed with mouse 3T3-L1 pre- and mature adipocytes cultured in phenol red- and steroid-free medium. As shown in Fig. 1A, three transcripts (6.3 kb, 3.1 kb, 2.3 kb) of *hgf* mRNA were detected in both preadipocytes and mature adipocytes. Of these, full length 6.3 kb mRNA and alternative spliced 3.1 kb mRNA encode for HGF precursor protein [20-22]. Interestingly, the amounts of each transcript in preadipocytes were significantly higher than those of mature adipocytes (Fig. 1B). To examine the HGF production from pre- and mature adipocytes, Western blot analysis was performed with heparin-binding fraction of

Fig. 1 Expression and production of HGF in 3T3-L1 cells before and after adipogenic differentiation

(A, B) Northern blot analyses of *hgf* mRNA in 3T3-L1 pre- and mature adipocytes were performed. A: Representative autoradiographs of *hgf* mRNA and that of *gapdh*. B: Quantitative changes in the expression of respective *hgf* mRNA splicing variants (6.3kb, 3.1kb, 2.3kb) normalized with *gapdh* expression. (C, D) Western blot analyses of HGF proteins in the heparin binding fractions of the culture medium were performed. C: Representative blot showing three major bands corresponding to pro-HGF (95 kDa), α chain (62kDa) and β chain (33kDa) of biologically active HGF proteins. D: Quantitative changes in the production of respective HGF proteins. Data are expressed as means $\pm$ SEM of three independent preparation. Statistical differences between pre- and mature adipocytes are shown as * $p < 0.05$; ** $p < 0.01$.

respective CM. As shown in Fig. 1C, three bands corresponding to HGF precursor (pro-HGF, 95 kDa), α -chain (62 kDa) and β -chain (33 kDa) of active form of HGF were clearly detected in the preadipocyte CM. In contrast, in the CM of mature adipocytes, α - and β - chains of HGF were hardly detected, while HGF precursor was apparently present. Similar to the changes in HGF transcript, the amounts of HGF proteins in the preadipocyte CM were more abundant than those of mature adipocytes (Fig. 1D).

Effects of E2 and P on *hgf* mRNA expression in 3T3-L1 pre- and mature adipocytes were examined. Treatment of preadipocytes with 10 nM E2 increased expression of *hgf* mRNA, especially 6.3kb splicing variant in a time-dependent manner and the increase was lasted for at least 24 h (Fig. 2A and 2B). However, E2 treatment of mature adipocytes did not cause any effect on the expression (Fig. 2A and 2C). When treated with increasing concentrations of E2 for 6 h, preadipocyte increased expression of *hgf* mRNA dose-dependently (Fig. 2D and 2E).

Treatment of preadipocytes, but not mature adipocytes, with 10 nM P, increased expression of *hgf* mRNA, especially 6.3kb splicing variant in a time-dependent manner and the increase was lasted for 24 h as observed in the cells treated with E2 (Fig. 3A, 3B, and 3C). Preadipocyte treated with 1 and 10 nM P increased expression of *hgf* mRNA, but the cells treated with 100 and 1,000 nM P did not affect *hgf* mRNA expression (Fig. 3D and 3E).

Next, preadipocytes were treated simultaneously with E2 and P. As shown in Fig. 4A and 4B, the cells treated with 10 nM E2 and 10 nM P increased 6.3 kb *hgf* mRNA more than that of E2 or P alone. However, the cells treated with 10 nM E2 and 1,000 nM P did not influence the expression of *hgf* mRNA (Fig. 4C and 4D).

To examine the effects of E2 and P on HGF production in 3T3-L1 preadipocytes, Western blot analysis was performed as in Fig. 1C. Treatment with either 10 nM E2 or 10 nM P alone increased the amounts of HGF proteins as a whole, which consist of HGF precursor, α - and β - chains of active form of HGF, compared with those of cells without hormonal stimulation (Fig. 5A and 5B). Moreover, treatment simultaneously with 10 nM E2 and 10 nM P enhanced HGF production in the CM, although such enhancement was not observed in the CM of cells treated with 10 nM E2 and 1,000 nM P (Fig. 5A and 5B).

Discussion

It has been shown that human, rat and mouse fibroblasts including that isolated from mouse mammary gland and 3T3-L1 adipocytes and human isolated adipocytes express and produce HGF [6, 18, 19]. By direct comparison of 3T3-L1 pre- and mature adipocytes, it was found that three transcripts of *hgf* mRNA were abundantly expressed in fibroblastic preadipocytes, and their expression were significantly reduced after the adipogenic differentiation (Fig. 1), suggesting at least in 3T3-L1 cells HGF is produced by fibroblastic preadipocytes rather than adipocytes.

In accordance with levels of the mRNA expression, three protein bands corresponding to HGF precursor (95 kDa), and α -chain (62 kDa) and β -chain (33 kDa) of active form of HGF were richly present in the CM of 3T3-L1 preadipocytes, whereas significantly lesser amounts, mainly HGF precursor, were present in that of mature adipocytes (Fig. 1). Since we found the CM from mature adipocytes failed to induce any morphological changes of mammary epithelial cells, while the CM from preadipocytes induced mammary epithelial tubular formation *in vitro* (Yamaji, *et al.*, manuscript in preparation) like recombinant HGF [23], it is suggested sufficient amounts of biologically active HGF is only present in CM of preadipocytes. Similarly, in the CM of mammary fibroblasts, 60 kDa and 33 kDa proteins are detected and identified as active HGF [24]. On the other hand, it is reported in the CM of 3T3-L1 adipocytes, only single 82KDa protein immunoreactive to HGF antibody, presumably identical to HGF precursor, is detected [18]. Although the CM of adipocytes containing 82 kDa is reported to increase growth of breast cancer cell line [18], it might be explained that cleavage of HGF precursor occurred on the surface of cancer cells to form active HGF [25, 26]. Our results, together with the previous findings, suggest that the cleavage activity of HGF precursor might also be different between before and after adipogenic differentiation.

Treatment of preadipocytes with E2 increased HGF expression and production (Figs. 2 and 5), in line with the previous reports in mammary fibroblasts, ovary cells, and macrophages [8, 12-14], possibly through a direct interaction of the E2-estrogen receptor complex with *cis*-acting estrogen responsive elements present in the promoter region of the *hgf* gene [13, 27]. It is worth to note that only the longest transcript encoding for full

Fig. 2 17 β -estradiol (E2) enhances *hgf* mRNA expression in preadipocytes, but not in mature adipocytes, in a dose-dependent manner. 3T3-L1 pre- and mature adipocytes were stimulated by 10 nM E2 for 1, 3, 6, 9 and 24 h (**A–C**) and preadipocytes were incubated by increasing concentrations of E2 for 6 h (**D, E**). Expression of *hgf* mRNA was determined by Northern blot analysis and normalized with that of *gapdh*. **A** and **D**: Representative autoradiographs. **B, C**, and **E**: Quantitative changes in the expression of respective *hgf* splicing variants (6.3 kb, 3.1 kb, 2.3 kb) in preadipocyte (**B** and **E**, $n = 4$) and mature adipocyte (**C**, $n = 3$). Data are expressed as relative values to control without E2 treatment (time 0 or dose 0). Statistical differences are shown as * $p < 0.05$; ** $p < 0.01$ (vs. control).

Fig. 3 Progesterone (P) influences *hgf* mRNA expression in preadipocytes, but not in mature adipocytes, in a dose-dependent manner. 3T3-L1 pre- and mature adipocytes were stimulated by 10 nM P for 1, 3, 6, 9 and 24 h (A–C) and preadipocytes were incubated by increasing concentrations of P for 6 h (D, E). Expression of *hgf* mRNA was determined by Northern blot analysis and normalized with that of *gapdh*. A and D: Representative autoradiographs. B, C, and E: Quantitative changes in the expression of respective *hgf* splicing variants (6.3kb, 3.1kb, 2.3kb) in preadipocyte (B and E, $n = 4$) and mature adipocyte (C, $n = 3$). Data are expressed as relative values to control without P treatment (time 0 or dose 0). Statistical differences are shown as * $p < 0.05$; ** $p < 0.01$ (vs. control); # $p < 0.05$ (vs. P 10 nM).

Fig. 4 Effects of high and low doses of progesterone (P) on 17 β -estradiol (E2)-induced HGF mRNA expression in preadipocytes 3T3-L1 preadipocytes were stimulated by 10 nM E2, 10 nM P, and both for 6 h (**A**, **B**), and by 10 nM E2 with either 10 nM or 1,000 nM of P (**C**, **D**). Expression of *hgf* mRNA was determined by Northern blot analysis and normalized with that of *gapdh*. Shown in **A** and **C**: Representative autoradiographs of four respective experiments. **B** and **D**: Quantitative changes in the expression of *hgf* splicing variants (6.3kb, 3.1kb, 2.3kb). Data are expressed as relative values to control without E2 and P treatments. Statistical differences between indicated columns are shown as * $p < 0.05$; ** $p < 0.01$.

Fig. 5 Effects of 17 β -estradiol (E2) and progesterone (P) on HGF production from preadipocytes 3T3-L1 preadipocytes were stimulated by 10 nM E2, 10 nM P, and 10 nM E2 with either 10 nM or 1,000 nM of P for 48 h. Western Blot analysis of HGF proteins in the heparin binding fractions of the culture medium were performed. **A**: Representative blot. **B**: Quantitative changes in the expression of respective HGF proteins. Data are expressed as relative values to control without E2 and P treatments. Statistical differences are shown as * $p < 0.05$; ** $p < 0.01$ (vs. control); # $p < 0.05$ (vs. E2 10 nM plus P 10 nM).

length HGF protein [20-22] was induced and the E2 effect was limited to preadipocytes.

In the current study mature adipocytes were cultured with troglitazone, a peroxisome proliferator-activated receptor γ (PPAR γ) ligand. It was thus predicted that transcription factors such as PPAR γ and CCAAT/enhancer-binding protein β (C/EBP β) increased in the process of adipogenesis bind to the respective *cis*-acting elements in the HGF promoter to enhance *HGF* mRNA expression [28, 29]. However, as negative regulators for HGF transcription such as chicken ovalbumin upstream promoter-transcription factor (COUP-TF) can suppress the stimulatory functions of both estrogen receptor and PPAR in the presence of their own ligands [30], certain negative regulator(s) might be induced after the differentiation, resulting in competing estrogen response. In addition, as cortisol (1,000 nM) is shown to decrease HGF production in osteoblast-like cells [31], treatment with Dex, a synthetic glucocorticoid, for first two days to induce adipogenic differentiation in this study might cause a suppression of HGF expression directly through glucocorticoid response element (GRE) in the promoter region of *hgf* gene [27].

Treatment of preadipocytes, but not mature adipocytes, with P at less than 10 nM increased HGF expression and production, and potentiated the estrogen effect (Figs. 3-5). In contrast, the treatment with P at more than 100 nM had no effect on HGF mRNA induction, and at 1,000 nM abolished the estrogen effect. There is no report that showing the presence of progesterone response element (PRE) in the HGF gene. However, as PRE and GRE share the same consensus sequence and GREs from several genes can also mediate response to

progesterone [32, 33], therefore P effects might occur through interaction of P-progesterone receptor complex with GRE, but the complete mechanism behind the dual effects of P remains to be elucidated.

HGF expression is high in the inactive stage of mammary gland, decreased during pregnancy, almost vanished during lactation, and re-appeared at involution [5, 7]. During estrus cycle, circulating P concentrations vary in a range of 3~48 nM, but rise over 100 nM after pregnancy and decrease just before parturition [11]. Based on the current findings, the changes in HGF expression in the mammary gland development might be attributed to physiological changes in the circulating E2 and P concentrations that directly affect HGF expression in fibroblasts.

In summary, in the present study, we have first demonstrated that HGF production in mouse 3T3-L1 preadipocytes is regulated intricately at transcription by ovarian steroids, especially progesterone. We have also shown that HGF mRNA expression and protein production are suppressed after preadipocyte differentiate into mature adipocyte. The latter might partly explain our findings that diet-induced obesity disrupts ductal development in the mammary glands of non-pregnant mice [34].

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