Insulin-Like Growth Factors 1 and 2 Regulate Expression of β-Casein *In Vitro* in Mouse Mammary Epithelial Cells

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We studied the role of insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) in functional differentiation of HC11 mouse mammary gland cells. It was found that both IGF-1 and IGF-2 activate the expression of milk protein β-casein in the presence of prolactin and hydrocortisone. It was found that β-casein expression is accompanied by cyclin D1 coexpression.

Key Words: *insulin-like growth factor-1 (IGF-1); insulin-like growth factor-2 (IGF-2); cell differentiation*

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) play the key role in normal embryogenesis, proliferation, survival and differentiation of cells in many human and animal tissues [1,7]. IGF-1 and IGF-2 share a 62% homology at the amino acid sequence level and bind to IGF-1 receptor (IGF-1R) which is a tyrosine kinase receptor [11]. Binding of IGF-1 and IGF-2 to IGF-1R leads to activation of the PI-3K/Akt and Raf-1/MEK/ERK signal transduction pathways [11].

In late 1990s, it was first demonstrated that IGF-1 and its receptor are involved in differentiation of the mammary gland. It was established that IGF-1 and IGF-1R are expressed in the epithelial and stromal cells in the mouse mammary gland, and are essential mediators of growth and branching of mammary gland ducts during puberty [5,6].

Our experimental *in vivo* study on transgenic mice carrying mutant IGF-1R gene under WAP promotor revealed markedly reduced expression of milk proteins caseins α, β, γ, δ and WAP, on the 2nd day of lactation [13]. In addition, we have shown that primary culture of mammary epithelial cells derived from pregnant mice carrying mutant IGF-1R under MMTV promoter, yields decreased levels of caseins and WAP during *in vitro* differentiation [13]. Thus, our preliminary data support the fact that IGF-1R is involved in functional differentiation of mammary gland, *i.e.* milk protein production.

To confirm our *in vivo* data and obtain new evidence for the assumption that IGF-1/IGF-1R- and IGF-2/IGF-1R-mediated signal pathways are essential for functional differentiation of mouse mammary epithelial cells, experiments were performed on HC11 cells (mouse mammary gland cells), a typical model for studies of the molecular mechanisms of mammary gland differentiation *in vitro*. We studied IGF-1- and IGF-2-dependent expression of β-casein, well-known and widely used marker of mouse mammary gland differentiation *in vitro,* in the presence of lactogenic hormones, prolactin, and hydrocortisone.

Expression of cyclin D1 in epithelial cells is necessary for functional differentiation of mouse mammary epithelial cells. It was shown that *Cyl-1*$^{-/-}$-mice lacking cyclin Dl protein grew slowly and remained proportionately smaller than normal mice. However, they were viable, reached sexual maturity and were able to produce live offspring, but they could not rear their progeny because of inability to produce milk [4]. Considering the fact that IGF-1/IGF-1R-signaling pathway is involved in the regulation of cyclin D1 synthesis, we studied the expression of this cyclin during IGF-1-dependent differentiation of HC11 cells to confirm the existence of a relationship between IGF-1-dependent of β-casein and expression of cyclin D1 *in vitro*.

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MATERIALS AND METHODS

We used HC11 mouse mammary epithelial cells, which was cloned from COMMA-1D mouse mammary epithelial cells derived from BALB/c mice [2]. The cells were grown until confluence in RPMI-1640 with 8% FCS in the presence of 5 μg/ml insulin (Sigma, I6634), 10 ng/ml epidermal growth factor, and 50 μg/ml gentamicin. The medium was then removed, the cells were washed in phosphate-buffered saline, and the medium was added. Control differentiating medium contained RPMI-1640 without FCS, 5 μg/ml insulin, 2 μg/ml hydrocortisone (Sigma, H0888), and 5 μg/ml prolactin (Sigma, L-6520). In the experimental differentiating medium, insulin was replaced with 20 ng/ml IGF-1 (Millipore, 01-208) or 28 ng/ml IGF-2 (Millipore, 01-142). Mediums containing only insulin, or IGF-1, or IGF-2, or hydrocortisone and prolactin together were used as additional controls. The cells were then cultured for 5 days. The medium was removed, cells washed with phosphate-buffered saline and lysed for 30 min at 4°C in lysing buffer (10 mM tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol, and 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, 5 mg/ml aprotinin). The lysates were centrifuged at 12,000*g* for 10 minutes at 4°C, the upper fraction was removed, and its protein concentration was measured.

For Western immunoblotting, 40 mg total protein was used. The samples were heated at 95°C for 5 minutes and immediately snap-cooled on ice. The samples were then centrifuged, loaded onto a 4-12% Bis-Tris denaturing gel, and electrophoresis was performed. Separated proteins were transferred to nitrocellulose membranes and blocked in phosphate-buffered saline containing 0.1% Tween-20 (FSB/0.1% Tween-20) with 5% milk. Then, the membranes were incubated with primary antibodies (anti-β-casein, Santa Cruz, sc-17969 and anti-cyclin D1, Cell Signaling, N 2926) diluted 1:500 in FSB/0.1% Tween 20 with 5% milk in a shaker at 4°C overnight. To determine the expression of β-actin (internal control), the membranes were incubated with antibodies against β-actin (Sigma, A5441) diluted 1:5000 in FSB/0.1% Tween 20 with 5% milk at room temperature for 1 h. After incubation with first antibodies, the membranes were washed in FSB/0.1% Tween-20 and incubated with second antibodies conjugated with HRP diluted 1:5000 in FSB/0.1% Tween 20 with 5% milk at room temperature for 1 h. To detect HRP-conjugated secondary antibodies, Enhanced chemiluminescence plus (Perkin Elmer Life science) was used. The images were captured with an UltraLum (Ultra-Lum, Inc., CA 91711) imaging system. The intensity of each band was quantified by densitometry using Scion Image software. The numerical values of β-casein and cyclin D1 expression levels are presented in graphs as the ratio of each protein expression value to the expression value of β-actin used as an internal control.

Statistical analysis was performed using GraphPad Prizm software. Statistical significance of differences was determined using Student's *t* test. The differences were considered significant at $p<0.05$.

RESULTS

β-Casein is one of the most common markers for *in vitro* functional differentiation of mouse mammary gland. In HC11 cell line, which is widely used to study the molecular mechanisms of mammary gland dif-

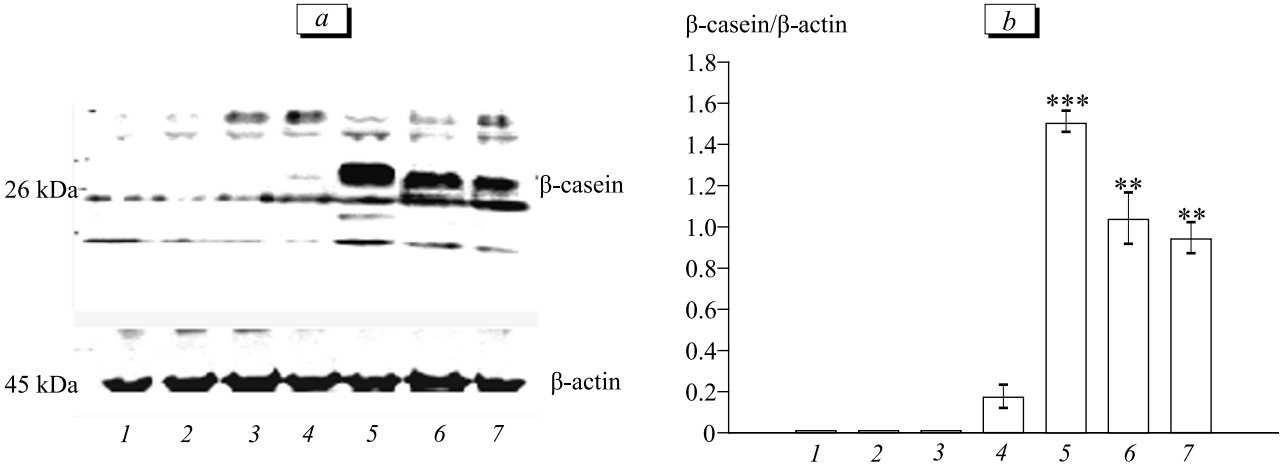

Fig. 1. β-Casein expression (*a*) and relative expression (*b*) in HC11 cells. *1*) in the presence of 5 μg/ml insulin; *2*) 20 ng/ml IGF-1; *3*) 28 ng/ml IGF-2; *4*) 5 μg/ml prolactin and 2 μg/ml hydrocortisone; *5*) insulin, prolactin and hydrocortisone; *6*) IGF-1, prolactin and hydrocortisone; *7*) IGF-2, prolactin, and hydrocortisone. β-Actin, internal control. *b*: β-casein/β-actin expression. $^{**}p<0.001$, $^{***}p<0.0001$ in comparison with *4*.

ferentiation *in vitro*, β-casein expression can be obtained when insulin, hydrocortisone, and prolactin are simultaneously present in the cultural medium [14]. Hydrocortisone in the presence of insulin promotes the formation and maintenance of rough endoplasmic reticulum playing a crucial role in casein synthesis [8-10]. Prolactin in the presence of insulin induces transcription from promoters of different casein genes including β-casein [15,16]. Insulin initiates DNA synthesis followed by mitosis in undifferentiated epithelial cells [12,16]. Since IGF/IGF-1R-mediated signaling plays a role in regulating cell division, namely promotes G1/S transition during the cell cycle [3,11], we used IGF-1 and IGF-2 instead of traditionally used insulin. Addition of insulin alone or IGF-1, or IGF-2 to the culture medium does not promote β-casein expression in HC11 cells (Fig. 1, *a*, *1-3*, *b*). Hydrocortisone and prolactin in the absence of insulin or IGF-1 or IGF-2 insignificantly activated β-casein expression (Fig. 1, *a 4*, *b*). In the contrary, the combination of insulin, hydrocortisone, and prolactin significantly activated β-casein expression in the control (Fig. 1, *a*, *5*, *b*). Simultaneous presence of IGF-1, hydrocortisone, and prolactin also notably increased β-casein expression (Fig. 1, *a*, *6*, *b*). β-Casein was also considerably expressed in the presence of IGF-2, prolactin, and hydrocortisone (Fig. 1, *a*, *7*, *b*). In all these cases, β-casein expression significantly differed from that in the presence of prolactin and hydrocortisone. Thus, both IGF-1 and IGF-2 are evidently capable to activate β-casein expression in the presence of lactogenic hormones. The used concentrations of IGF-1 and IGF-2 were 20 and 28 ng/ml, respectively. These concentrations are known to promote exclusively IGF-1R and IGF-1R-mediated signaling pathways. In this experiment, β-casein expression in the presence of insulin, hydrocortisone, and prolactin was taken as the control parameter. Despite the fact that IGF-1, IGF-2, and insulin genes are located in different chromosomes and their expression is regulated through several mechanisms, IGF-1 and IGF-2 stimulated β-casein expression *in vitro* in HC11 cells. These data suggest that IGF-1 and IGF-2 participate in the regulation of molecular mechanisms of functional differentiation of mammary gland, *i.e.* milk protein production.

It was found that cyclin D1 was overexpressed in proliferating cells and promoted cell cycle regulation at the G1-S transition [3,11]. In our experiments, HC11 cells were located in a dense monolayer and did not divide for more than 5 days. Lack of growth factors resulted in weak cyclin D1 expression (Fig. 2, *a*, *1*, *b*). Addition of prolactin and hydrocortisone to the medium did not increase cyclin D1 content (Fig. 2, *a*, *2*, *b*). At the same time, simultaneous presence of insulin, prolactin, and hydrocortisone strongly activated

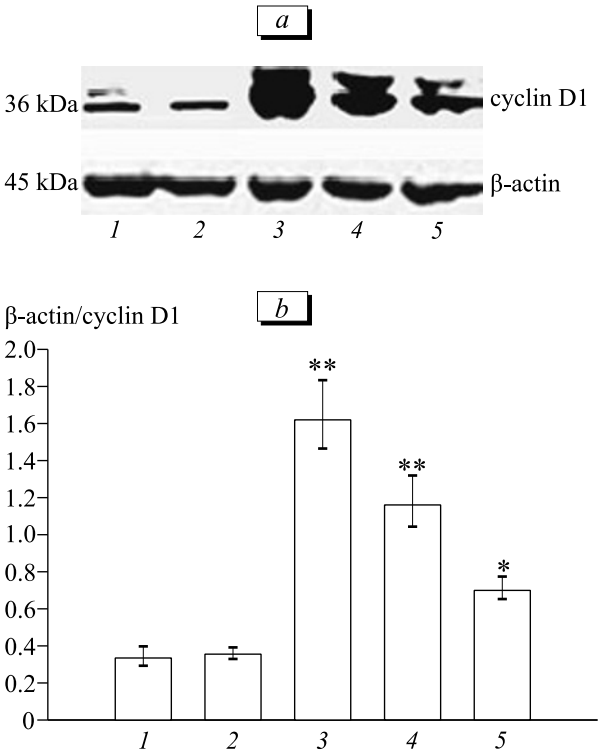

Fig. 2. Cyclin D1 expression (*a*) and relative expression (*b*) in HC11 cells. *1*) serum-free; *2*) in the presence of 5 μg/ml prolactin and 2 μg/ml hydrocortisone; *3*) 5 μg/ml insulin, prolactin, and hydrocortisone; *4*) 20 ng/ml IGF-1, prolactin, and hydrocortisone; *5*) 28 ng/ml IGF-2, prolactin and hydrocortisone. β-Actin, internal control. *b*: cyclin D1/β-actin expression. **p*<0.05, ***p*<0.001 in comparison with *2*.

cyclin D1 expression (Fig. 2, *a*, *3*, *b*). Replacement of insulin for IGF-1 or IGF-2 in the presence of prolactin and hydrocortisone also markedly increased cyclin D1 expression (Fig. 2, *a*, *4*, *5*, *b*). The expression of cyclin D1 in all these cases significantly differed from that in the medium without growth factors or in the presence of only hydrocortisone and prolactin. Thus, cyclin D1 expression in HC11 cells is linked to either the presence of insulin, or IGF-1, or IGF-2. Comparison of cyclin D1 and β-casein expression revealed coexpression of cyclin D1 and β-casein during functional differentiation of HC11 cells *in vitro* (Fig. 1, *a*, *5-7*; Fig. 2, *a*, *3-5*). Our studies showed that cyclin D1, contrary to popular opinion about its critical role in cell division, proved to be overexpressed in non-proliferating functionally differentiated HC11 epithelial cells of the mammary gland in the presence of IGF-1 or IGF-2, and its expression is associated with that of β-casein.

Thus, the results obtained confirm the data [13] of our previous *in vivo* experiments in mice and support the view that IGF-1 and IGF-2 are actually involved in functional differentiation of mammary gland and regulate β-casein expression. We have also revealed that β-casein expression is accompanied with IGF-1- and IGF-2-dependent cyclin D1 coexpression.

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