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Expression of Prolactin Receptors in Human Osteosarcoma Cells

N. Bataille-Simoneau, K. Gerland, D. Chappard, M. F. Basle, and L. Mercier[1]

Laboratoire d'Histologie-Embryologie (LHEA), UFR Médecine,
1 rue Haute de Reculée, 49045 Angers Cedex, France

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The expression of prolactin receptor (PRL-R) mRNA was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) combined with Southern analysis in total RNA extracts from two human osteosarcoma cell lines (MG-63 and Saos-2). The level of PRL-R transcript was significantly enhanced in cells cultured in the presence of 1,25-$(OH)_2$ vitaminD_3 (10^{-7}M) and to a lesser extent in the presence of dexamethasone (10^{-6}M). This first demonstration of PRL-R gene expression in osteoblast-like cells supports the hypothesis of a direct action of prolactin in bone cells, which is further strongly suggested by the stimulatory effect of 1,25-$(OH)_2$ vitaminD_3 and dexamethasone on PRL-R mRNA level in these cells.

Prolactin (PRL) is one of the most versatile polypeptide hormones of pituitary origin in terms of biological actions (1, 2). This is consistent with the wide distribution of PRL-R mRNA reported in various tissues from several species at different development stages (3, 4, 5, 6, 7, 8). However, the biological significance of the wide distribution of PRL-R mRNA and the precise role of PRL in many given tissues remain to be determined.

Lactation in humans and animals is known to induce a maternal bone-resorptive state dependent on vitamin D, and it has been suggested that PRL might induce the release of a bone resorptive agent (9, 10, 11, 12, 13). In addition, the fact that some hyperprolactinemic women develop osteopenia (type 1 osteoporosis associated with a relatively greater loss of trabecular bone mineral compared to cortical bone mineral) in the presence of regular menses led to the speculation that PRL itself might directly influence bone metabolism (14, 15, 16, 17).

To unambiguously demonstrate a role for prolactin in bone cells, it was necessary to explore PRL-R gene expression in these cells. The present study demonstrates the presence of PRL-R mRNA in osteosarcoma cells with osteoblastic properties. This observation together with the stimulatory effect of osteotropic factors [1,25-$(OH)_2$ vitaminD_3 and dexamethasone] on steady-state PRL-R mRNA levels supports the hypothesis that PRL plays an important role in the regulation of bone cell function.

MATERIALS AND METHODS

Cell culture. MG-63 and Saos-2 cells, a gift from Dr. R. Bataille (INSERM U211, Nantes, France) were routinely grown in DMEM medium containing 5% FCS and 4 mmol/L glutamine. Except otherwise indicated, the medium was changed to 0.5% FCS (further referred as starvation medium or serum-free medium) for 8 to 24 hours and cells were treated with 1,25-$(OH)_2$ vitaminD_3 (10^{-7}M) or dexamethasone (10^{-6}M) for 16 hours before extraction of total RNA. 1,25-$(OH)_2$ vitaminD_3 was a gift of F. Hoffmann-La Roche SA (Basel, Switzerland) and dexamethasone was purchased from Sigma (St Louis, MO).

PCR amplification of reverse transcribed mRNA. Total cellular RNA was extracted as previously described (18).

[1] To whom correspondence should be addressed. Fax: (33) 41 73 58 88. E-mail: lmercier@club.med.univ-angers.fr.

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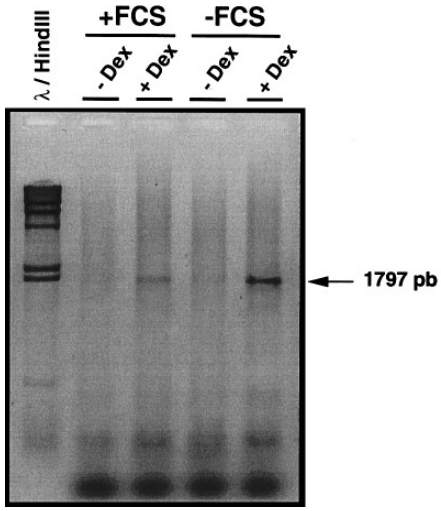

FIG. 1. Amplification of hPRL-R mRNA by RT-PCR in MG-63 cells. Ethidium bromide staining of PCR products separated on a 1% agarose gel. Cells were cultivated in medium supplemented or not with 5% fetal calf serum (±FCS) and in the absence or presence of 10^{-6}M dexamethasone (±DEX) for 16h at 37°C.

Expression of hPRL-R and hTFR mRNA was determined by RT-PCR and Southern blot analysis. First strand cDNA was synthesized from 1 μg of total RNA by incubation for 50 min at 42°C with 200U of M-MLV reverse transcriptase (Promega Corp, Madison, WI) after oligodT priming (0.25 μg). 5 μl (1/6 of the reaction mixture) were submitted to PCR for amplification of hPRL-R and hTFR sequences. The reaction mixture (50 μl) contained 50 pmol of each primer for hPRL-R (10 pmol each for hTFR primers), 0.2 mM dNTPs, 50 mM KCl, 2mM $MgCl_2$ and 1U Taq DNA polymerase. Amplification was performed using a Thermojet Thermal Cycler (Eurogentec, Liège, Belgium) with the following profiles (i) 94°C for 5 min × 1 cycle ; (ii) 94°C for 1 min, 65°C for 2 min, 72°C for 2 min × 30 cycles ; (iii) 72°C for 10 min × 1 cycle. Amplification products were resolved on 1% agarose gels. For hPRL-R, the specificity of the reaction was confirmed by Southern transfer onto Hybond N^+ membranes (Amersham) and hybridization with a ^{32}P labeled hPRL-R cDNA (a gift of P.A. Kelly, INSERM U344, Paris). Probes were labeled by random priming with the use of a Megaprime Labeling Kit and [α^{32}P dCTP] (3000Ci/mmol, Amersham). Hybridization was as previously described (19). Negative control PCR reactions were carried out on non-reverse transcribed mRNA. The primers for hPRL-R amplification were synthesized by Eurogentec (Liège, Belgium): 5′ AAATGTGGCATCTGCAAC-CGTTTTCAC 3′ (PRL-R sens) and 5′ GCACTTGCTTGATGTTGCAGTGAAGTT 3′ (PRL-R antisens). To ensure equal starting quantities of cDNA for the experiments and to allow quantification of the PCR products, the reverse transcribed mRNA samples were also amplified using oligonucleotides primers specific to the human transferrin receptor (hTFR) included in the 5′ AmpliFinder RACE kit (Clontech laboratories, Palo Alto, CA).

Northern blotting. Total RNAs was fractionated by electrophoresis on 1% formaldehyde agarose gels (30 μg per lane) and transferred onto Hybond N^+ membrane (Amersham). The 338 bp hTFR amplicon was recovered from agarose gels by using a Nucleotrap gel extraction kit (Macherey-Nagel, Düren, Germany) and ^{32}P labeled as described above. Hybridization and washing were carried out as previously described (20). Relative hTFR mRNA levels were normalized for loading variability by comparison with GAPDH mRNA levels in the same membrane. The hTFR probe was stripped off with boiling 0.5% SDS poored on the membrane and allowed to cool to room temperature prior to rehybridization with a ^{32}P labeled GAPDH cDNA probe (21).

RESULTS

The level of hPRL-R mRNA was assessed by PCR with primers which span most of the coding region of the hPRL-R cDNA (1797bp), after reverse transcription with oligodT. Figure 1 represents the results of a RT-PCR reaction performed with RNA extracted from MG-63 cells. It shows that in basal condition, hPRL-R mRNA level is extremely low, with no detectable signal after ethidium bromide staining of the amplification products resolved on 1% agarose gels. However, addition of 10^{-6}M dexamethasone markedly increased hPRL-R mRNA expres-

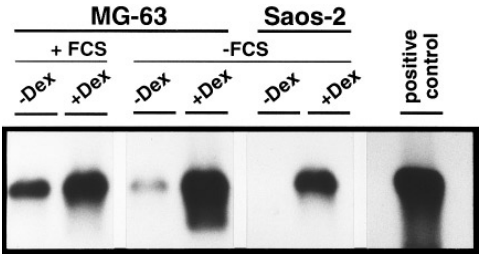

FIG. 2. Effect of dexamethasone on hPRL-R mRNA expression in MG-63 and Saos-2 cells. Cells were cultivated in medium supplemented or not with 5% fetal calf serum (±FCS) and in the absence or presence of 10^{-6}M dexamethasone (±DEX) for 16h at 37°C. Total RNA were reverse-transcribed and amplified by PCR for hPRL-R mRNA as described under Materials and Methods. PCR products were separated on a 1% agarose gel, transferred to nylon membrane, and hybridized with a [^{32}P]-labelled hPRL-R cDNA probe. Amplification product of the hPRL-R cDNA was run on the same gel and used as a positive control.

sion, as attested by the presence of a band of the correct size in treated samples. It should also be noted, that the signal was much more intense with dexamethasone in starvation medium.

As shown in figure 2, Southern hybridization of the amplification products with ^{32}P labeled hPRL-R cDNA and washing at high stringency, further confirmed that hPRL-R gene was expressed in the two human osteosarcoma cell lines studied, namely MG-63 and Saos-2. The increase in sensitivity gained with the use of a ^{32}P labeled probe revealed that hPRL-R mRNA was not only expressed in dexamethasone treated but also in untreated cells. The size of the amplification products was identical to that of a control PCR reaction performed simultaneously on a hPRL-R cDNA plasmid with the same primers. It also shows that MG-63 express higher levels of hPRL-R mRNA than Saos-2. Again, the effect of dexamethasone was more pronounced in starvation medium. The effect of serum is attested by the dramatic decrease in the level of hPRL-R mRNA observed in MG-63 cells cultured in serum-free medium. The same was also true for Saos-2 (data not shown). The stimulatory effect observed when cells were treated with 10^{-6}M dexamethasone in MG-63 cells and in Saos-2 cells, irrespective of FCS concentration, strongly suggests that in addition to serum, glucocorticoids are potent modulators of hPRL-R gene expression.

To determine whether hPRL-R gene expression was also induced by other important osteotropic factors, cells were cultured in the presence of 1,25-$(OH)_2$ vitaminD_3. Figure 3 shows that 1,25-$(OH)_2$ vitaminD_3 was an even more potent inducer of hPRL-R gene expression. In this experiment, hTFR cDNA co-amplified along hPRL-R cDNA was used as an internal control to account for sample-to-sample variations in RT and PCR reactions. Primers used in this case were derived from sequences located at the 5′ end of the 4.7 kb hTFR mRNA. This means that differences in the amount of hTFR cDNA amplicon reflect differences in the efficiency of the RT reaction. After normalization with respect to this internal control, hPRL-R mRNA levels in separate samples were directly compared (Figure 3, panel B). That hTFR mRNA is constituvely expressed in these cells was also verified by comparison with steady-state levels of GAPDH mRNA by northern blotting (Figure 4).

DISCUSSION

Prolactin exerts its action by binding to its receptor, a member of the GH/PRL/cytokine receptor subfamily (22). A long form of the hPRL-R has been identified that contains 598 amino acids in its mature form, whereas both long and short PRL-R forms have been identified in other species (23). While clinical studies have suggested a possible role for prolactin in

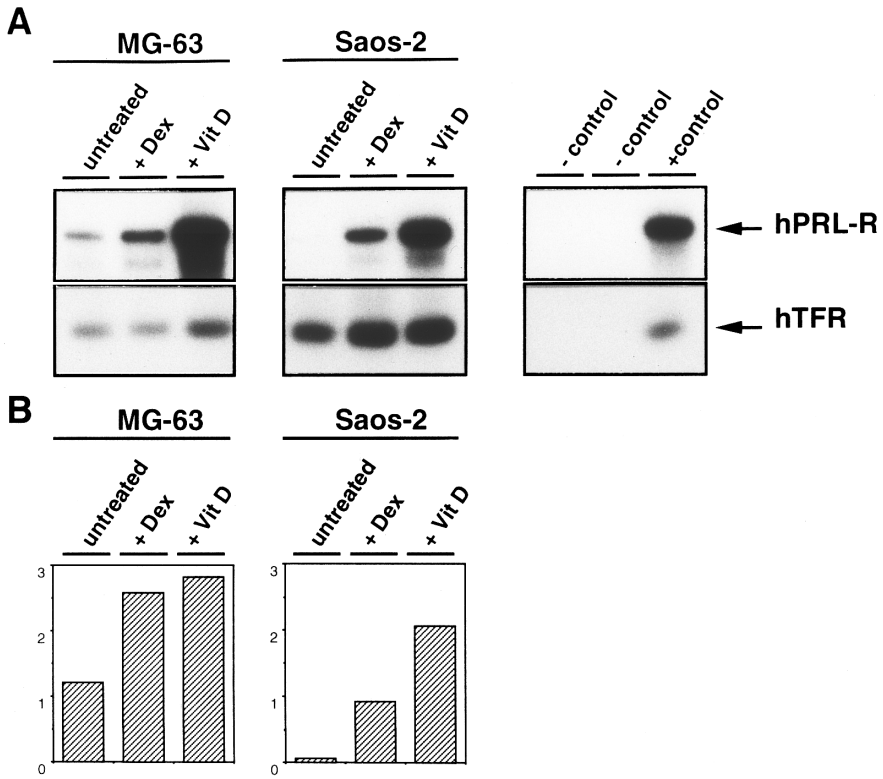

FIG. 3. Effect of dexamethasone and 1,25-(OH)2 vitamin D3 on hPRL-R mRNA expression in MG-63 and Saos-2 cells. (A) PCR products from cells cultivated in medium depleted in FCS (untreated) and containing 10^{-6}M dexamethasone (+Dex) or 10^{-7} M 1,25-(OH)2 vitamin D3 (+Vit D) were separated on a 1% agarose gel, transferred to nylon membrane, and hybridized with a [^{32}P]-labelled hPRL-R cDNA probe. Amplification products from non-reverse-transcribed MG-63 and Saos-2 mRNAs were used as negative controls. PCR products of the hPRL-R or hTFR cDNA were used as positive controls. (B) The intensities of autoradiographic signals for hPRL-R and hTFR were quantified and are shown as the ratio of hPRL-R versus hTFR.

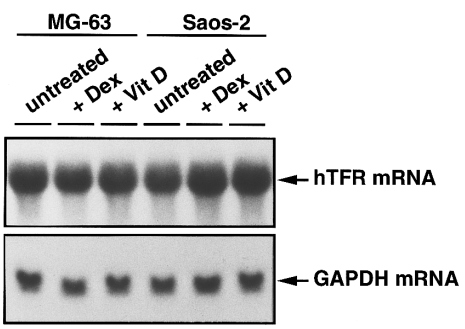

FIG. 4. Northern blot analysis of hTFR mRNA and GAPDH mRNA from MG-63 and Saos-2 under various culture conditions. 20 μg of total RNA from cells cultivated in medium depleted in FCS (untreated), containing 10^{-6}M dexamethasone(+Dex) or 10^{-7} M 1,25-(OH)2 vitamin D3 (+Vit D), was loaded in each lane. The blot was successively hybridized with a [^{32}P]-labelled hTFR cDNA probe and GAPDH cDNA probe.

bone, a direct demonstration of hPRL-R gene expression in bone cells was lacking and the putative role for hPRL in bone remained speculative. We report here the first demonstration that hPRL-R mRNA is expressed in osteosarcoma cells. Moreover, the data unambigously show that the steady-state level of this mRNA is strongly influenced by the presence of serum and osteotropic factors such as 1,25-$(OH)_2$ vitaminD_3 and dexamethasone which are systemic regulators of bone homeostasis and pathology (24). Due to the extremely low level of hPRL-R mRNA reported in most tissues, RT-PCR was the only way to explore osteoblastic cells for the presence of hPRL-R gene expression. It could then be argued that the presence of the mRNA does not necessarily reflects the level of expression of the protein of a target gene. However arguments in favour of the presence of hPRL-R in bone cells have recently been obtained in hPRL-R knock-out mice in which a disorganization of bone tissue in homozygous-/- animals was observed (P.A. Kelly, personal communication).

Serum, an incompletely defined mixture containing many factors that affect cell growth, glucocorticoids and 1,25-$(OH)_2$ vitaminD_3 are known to exert effects at the transcriptional level. Transcriptional control elements responding to these factors have been identified in many genes and respectively named SRE, GRE and VDRE. In the present state of our work, we can only be speculative about an up-regulation of hPRL-R gene transcription by serum, dexamethasone and 1,25-$(OH)_2$ vitaminD_3. A semi-quantitative study was also undertaken in order to assess the effect of increasing concentrations of dexamethasone on hPRL-R mRNA levels in MG-63, the most responsive of the two cell lines used in this study. Because of the low level of expression of hPRL-R mRNA, all assays were carried out in triplicate and analyzed by Southern transfer and hybridization with a ^{32}P labeled hPRL-R cDNA probe. This was essential for assays run with low dexamethasone concentrations for which we have to face a crucial sensitivity problem. Still a dose-dependent increase in hPRL-R mRNA relative to hTFR mRNA constituvely transcribed in these cells was observed (data not shown). Primers used for hTFR cDNA amplification were derived from the 5′ end of this long mRNA (4.7 kb) and make this control one of the most confident to evaluate the absolute efficiency of the reverse transcription reaction. However it does not mean that if the reverse transcription of the 4.7 kb mRNA is not complete this is also true for mRNA of lower size, such as the long form of hPRL-R mRNA. If this were the case, the hTFR mRNA would be underestimated resulting in an artifactual increase of hPRL-R mRNA relative to hTFR mRNA. Since differences observed in starvation medium between control and treated samples in this study are highly significant, dexamethasone and 1,25-$(OH)_2$ vitaminD_3 can be viewed as potent inducers of hPRL-R gene expression in osteosarcoma cells.

A multiple and differential control of the rat PRL-R was very recently reported (25, 26). This is consistent with the numerous functions of prolactin in different tissues. Such an observation suggested that the molecular basis of a tissue-specific regulation in diverse prolactin target cells lies in a differential promoter usage in PRL-R gene expression. Analysis of the three putative promoter sequences of the rat PRL-R gene has revealed consensus sequences for the binding of several transcription factors, which may be important for basal as well as hormonal control of promoter activity. Nevertheless, there was no evidence for the presence of GRE nor VDRE in these sequences (25, 26). In a next step it would be worth exploring PRL-R promoters sequences under various stimulatory conditions ($\pm$ FCS, $\pm$ dexamethasone, $\pm$ 1,25-$(OH)_2$ vitaminD_3) by methods such as *in-vitro* and *in-vivo* footprinting in osteosarcoma cells.

In conclusion, the present study provides the first evidence for the expression of hPRL-R mRNA in osteosarcoma cells. Moreover, this expression is strongly influenced by the presence of serum and osteotropic factors such as 1,25-$(OH)_2$ vitaminD_3 and dexamethasone. These data are consistent with a role for prolactin in bone which is also supported by the disorganization of

bone tissue observed in hPRL-R knock-out mice. While the link between binding, signaling pathways and the genes that become activated is still a challenge, our data implicate prolactin as a new regulator of bone cells function, together with GH and the IL-6/LIF/OSM family of cytokines.

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