Leptin gene in rabbit: cloning and expression in mammary epithelial cells during pregnancy and lactation

Emmanuelle Koch,1,2* Cathy Hue-Beauvais,1,2 Laurent Galio,1 Gili Solomon,2 Arieh Gertler,2 Françoise Révillon,3 Valérie Lhotellier,3 Etienne Aujean,1 Eve Devinoy,1 and Madia Charlier1

1INRA, UR1196, Génomique et Physiologie de la Lactation, Jouy-en-Josas, France; 2Institute of Biochemistry Food Science and Nutrition, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel; and 3Laboratoire d’Oncologie Moléculaire Humaine, Centre Oscar Lambret, Lille, Cedex, France

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Leptin is encoded by the ob gene, which is composed of three exons in most species (18, 21, 22, 45), except in the rabbit where only two exons have been described (43). Transcripts are strongly expressed in adipose tissue and increase in diet-induced obesity (16). However, other studies have demonstrated that leptin mRNA are also detectable in several peripheral tissues such as the mammary gland (7, 10, 37–39). Leptin has been reported to be produced both in mammary epithelial cells prior to parturition in humans (39), sheep (10), rodents (7) and cattle (8), and in myoepithelial cells (10) after parturition.

Our objective was to gain a comprehensive view of leptin expression in the mammary gland throughout pregnancy and lactation in rabbits. Such data are of importance because maternally produced leptin is secreted into milk (7) and is taken up by the newborn during suckling (11). Thus leptin may enter the circulation of the newborn as already described in rat (31, 33) and contribute to reorganization of the central neural pathways. Indeed, leptin has strong neurotrophic properties that may underlie some developmental changes during the perinatal period (1, 24, 30). The study of induced metabolic syndrome in the rabbit is a pertinent model because of this animal’s predisposition to accumulate visceral fat when subjected to a high-lipid diet. We therefore chose this species as our model. However, as no information about the presence and expression of leptin in rabbit mammary tissue has been published to date, our aim was to determine whether the leptin gene structure in this species differed from that seen in other species.

MATERIALS AND METHODS

Animals and Experimental Design

All animal studies were carried out in compliance with French regulations on animal experimentation and with the authorization of the French Ministry of Agriculture (Animal Health and Protection Directorate, accreditation number 78-119).

We housed 18 female rabbits (New Zealand White, 1077-INRA) individually in an indoor facility under controlled light conditions (8 h light/16 h darkness except from the week before mating when they were under 16 h light/8 h darkness) and temperature (18°C). The rabbits were killed during early (day 3, three animals), mid-(days 9–14, five animals), and late pregnancy (days 25–29, three animals) and during lactation on days 3 (three animals) and 16 (three animals). After animals were euthanized, the left lower mammary gland from each animal was excised and dissected. Mammary epithelial tissue and surrounding mammary adipose tissue were either processed or snap-frozen and stored for later RNA isolation, immunohistochemistry, and in situ hybridization (ISH).
Isolation of RNA and Reverse Transcription

Total RNA was isolated from the mammary epithelial tissue and from the surrounding adipose tissue by Trizol extraction according to manufacturer’s protocol (Invitrogen). RNA integrity (RIN: RNA integrity number) was assessed with an Agilent Bioanalyzer (average RIN of 9.1 for our samples) with a cut-off value of 8. RNA was kept at −80°C until further analyses.

Cloning of Rabbit Leptin cDNA From Mammary Adipose Tissue

We reverse transcribed 5 μg of total RNA in a total volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM deoxy-NTP, 0.5 μg oligo-dT, 0.1 μg hexamer primers (Roche), and 1 U Superscript II (Invitrogen). The reaction temperatures were 42°C for 1 h and 70°C for 15 min.

Regions with high interspecies similarities between human and mouse leptin sequences were used to design primers to amplify larger regions than those already described in the rabbit (45). The oligonucleotide primers for leptin used were as follows, forward primers: RL1: 5'-GGGCCAGAAACATCCTGGG-3' and RL3: 5'-GGAGGAGGTCCCGGCGCCAGC-3' and reverse primer: RL2: 5'-GGGAGGAGCAGCCCCCAGGGGC-3'. Amplification reactions (50 μl) were performed using 5 μl of the reverse transcription (RT) products, 10× PCR Master Mix (5 μl, MP Biomedicals), 200 μM of each primer, and 2.5 U of Taq DNA polymerase (MP Biomedicals). Thermal cycling proceeded with 30 cycles at 95°C for 1 min, either 60°C (primers RL1 and RL2) or 65°C (primers RL3 and RL2) for 1 min and 72°C for 1 min, with a final step of 10 min at 72°C. The purified 554 bp (primers RL1 and RL2) or 582 bp (primers RL3 and RL2) PCR products were subcloned into a pGEM-T Easy vector (Promega) according to manufacturer’s instructions. The presence of the inserts in purified recombinant plasmids was determined by PCR, and five clones were sequenced (MWG). The sequences of cloned fragments were aligned using the National Center for Biotechnology Information’s public domain database. The nucleotide sequence data described in this paper were submitted to GenBank and assigned the accession numbers JX262378 (554 bp DNA fragment) and KC461185 (582 bp DNA fragment). The 554 bp cDNA fragment was used to prepare recombinant leptin, while the 582 bp constitutes the full-length rabbit leptin cDNA.

Preparation of Recombinant Rabbit Leptin

Preparation of a rabbit leptin prokaryotic expression plasmid. The 554 bp cDNA fragment of rabbit leptin cloned into the pBluescript vector, prepared previously, served as the starting material. To facilitate its transfer to the prokaryotic pMon3401 vector, PCR was performed to introduce new NcoI and HinIII restriction sites at the 5' and 3'-ends respectively, along with an additional Met-Ala encoding sequence (Met being cleaved by bacteria), thus enabling subsequent subcloning of the rabbit leptin cDNA into the pMon3401 vector linearized with NcoI and HinIII. The PCR product was digested with NcoI and HindIII restriction enzymes and purified by electrophoresis in 1% agarose gel and a GeneClean II purification kit (BIO 101, Carlsbad, CA). The rabbit leptin cDNA was then ligated to the pMon3401 expression vector. Escherichia coli MON105 competent cells were transformed with the new expression plasmid and plated on LB-agar plates containing 75 μg/ml spectinomycin for plasmid selection. Four E. coli colonies were isolated and confirmed to contain the rabbit leptin cDNA by digestion with NcoI/HindIII restriction enzymes. All colonies were positive and expressed rabbit leptin, and one of them (clone 1) was sequenced and used for large-scale rabbit leptin preparation.

Expression, refolding, and purification of rabbit leptin. Recombinant rabbit leptin with an extra Ala at the NH₂ terminus was expressed in a 2.5 l culture. After an optical density of 0.9 was attained, nalidixic acid was added to reach a final concentration of 50 μg/ml and was then grown for an additional 4 h. Inclusion bodies (IBs) were then prepared as described previously (40) and frozen. Subsequently, IBs obtained from 2.5 l of bacterial culture were solubilized in 250 ml 4.5 M urea and 40 mM Tris base containing 0.1 mM cysteine. The pH of the solution was adjusted to 11.3 with NaOH. After 2 h of stirring at 4°C, we added three volumes of 0.67 M Arg to a final concentration of 0.5 M and stirred for an additional 0.5 h. The solution was then dialyzed against 10 l of 10 mM Tris-HCl, pH 10, and, with five external solution exchanges. NaCl was added to a final concentration of 150 mM, and the protein solution was applied at maximum flow rate (400–500 ml/h) to a Q-Sepharose column (30 ml bead volume), pre-equilibrated with 10 mM Tris-HCl, pH 10, and containing 150 mM NaCl. The flow-through fraction, which contained the rabbit leptin, was collected, concentrated sixfold, and applied in 10 consecutive 18 ml portions to a preparative Superdex 75 column (2.6 × 60 cm) pre-equilibrated with 10 mM Tris-HCl, pH 9, and containing 150 mM NaCl. Fractions containing the monomeric protein as determined by analytical gel filtration were pooled, dialyzed against NaHCO₃ to ensure a 4:1 (wt/wt) protein-to-salt ratio, and lyophilized.

Preparation of a rabbit leptin prokaryotic expression plasmid.

The primers and the TaqMan Minor Groove Binder (MGB) fluorogenic probe used for leptin had the following sequences: sense primer 5'-CAGCACTGGTCGCTCTCCGCT-3', antisense primer 5'-GGTTGACTTCATCCTGGGCGC-3', and probe 5'-CAGACAGAGGGTGTCCTGCT-3'.

Quantitative RT-PCR of Rabbit Leptin mRNA From Mammary Epithelial and Adipose Tissues

The proliferation rate of leptin-sensitive BaF3 1442-Cl 4 cells transfected with the long form of human leptin receptor was used to estimate the agonistic activity of rabbit leptin, by the MTT method as previously described (35).

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1997), where the C_T variable is defined as the cycle number at which the fluorescent signal generated by cleavage of the dual-labeled probe is first detectable. This method is based on the use of a calibrator sample (i.e., 1× sample), which permits quantification in unknown samples. The mammary gland at day 16 of lactation (L16) was chosen as the calibrator sample (i.e., target expression = 1) since preliminary data showed a high expression level of leptin.

The relative target expression was given by the formula: 2^(-ΔΔCT), where ΔΔCT = ΔCT_target sample - ΔCT_calibrator sample; with ΔCT = C_T_target - C_T_bp.

All data were evaluated by the Mann-Whitney U-test. P values of <0.05 were considered as statistically significant.

### Immunohistological Analyses of Mammary Tissue

For immunohistochemistry, mammary tissue samples were fixed in 4% paraformaldehyde for 10 min at 4°C. The samples were then cryoprotected in 40% sucrose, embedded in TissueTek (Sakura, Torrance, CA), and kept in liquid nitrogen before being stored at −80°C. We mounted 5 μm sections on slides.

Leptin, cytokeratin 8, and smooth muscle actin were localized by immunohistochemical analyses using the following dilutions of primary antibodies: goat anti-rat leptin (1:100, AbDSerotec), mouse anti-cytokeratin 8 (1:100, Covance), and mouse anti-smooth muscle actin (1:300) (Sigma). Briefly, 5 μm thick frozen sections were treated in 50 mM ammonium chloride for 30 min followed by permeabilization in 2% BSA, 0.05% saponin, and 0.05% sodium azide in PBS 1× for 1 h. The tissue sections were then incubated overnight at 4°C with primary antibodies diluted in the same buffer. After being washed, antibody binding was visualized after incubation for 45 min with fluorescence-labeled secondary antibodies (anti-goat TRITC-conjugated, 1:300; anti-mouse FITC-conjugated, 1:300; Jackson Immunoresearch) in PBS 1×, 4.6-Diamidino-2-phenylindole was diluted to 1:500 in PBS 1× and applied for 3 min at room temperature as a nuclear counterstain. The secondary antibody control slide was treated as the others, with the omission of the goat anti-rat leptin antibody. As a control, the primary goat anti-rat leptin antibody was incubated for 1 h at room temperature with recombinant rabbit leptin in an excess quantity (3-fold), before treatment.

The slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed with a Zeiss microscope coupled with ApoTome technology at the MIMA2 facilities (INRA, F78350 Jouy-en-Josas, France).

### ISH

LNA-modified DNA probes were synthesized by Eurogentec. The sequences of the LNA-modified oligonucleotides (LNA-modified bases shown in upper case) were: leptin probe, 5′-gCccTatCcgcTccTgtGttCc-3′; nonspecific antisense probe, 5′-gGaaAcAggAcggGatAggGc-3′.

We digoxigenin (DIG)-labeled 100 pmol of leptin and antisense control LNA-probes using the 3′-terminal transferase labeling kit (Roche) for 30 min at 37°C. The ISH protocol was performed as described (17). Briefly, 7 μm thick frozen sections of mammary tissue were postfixed for 10 min in 4% PFA in PBS (pH 7.5), acetylated for 10 min (100.4 mM triethanolamine, 21 mM HCl, 26.5 mM anhydride acetic acid), and finally digested with 5 μg/ml proteinase K (Roche Diagnostics) for 5 min. The sections were incubated for 30 min at room temperature in prehybridization buffer (50% formamide, 5× SSC, 5× Denhardt’s, 200 μg/ml yeast RNA, 500 μg/ml salmon sperm DNA, and 20 μg/ml Roche blocking reagent) and then for 1 h at 55°C in hybridization buffer (the same as the prehybridization buffer plus 0.25% CHAPS, 0.1% Tween 20) containing the probes. The sections were then washed three times for 15 min in 0.2× SSC at 60°C followed by serial incubations for 10 min in buffer A (100 mM Tris pH 7.5, 150 mM NaCl), for 30 min in blocking solution (buffer A supplemented with 10% fetal calf serum and 0.05% Tween 20), and for 1 h in the anti-DIG antibody (anti-DIG-PA Fab fragments, Roche) diluted to 1/2,000 in blocking solution. After three washes in buffer A, the pH of the sections was equilibrated at pH 9.5 for 10 min in buffer B (100 mM Tris pH 9, 100 mM NaCl, 50 mM MgCl2). Revelation was achieved with a solution stabilized with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyolphosphate p-toluidine (Eurobio) for 15 h. The slides were finally mounted in Diamount mounting medium (Diapath, Microm/Microtech).

### RESULTS

#### Cloning and Sequencing of Rabbit Leptin cDNA

The DNA fragment we cloned from rabbit mammary adipose tissue by RT-PCR using RL1 and RL2 primers designed in leptin mRNA conserved regions was 554 nucleotides in length. It contained a 501 bp open reading frame (ORF) encoding a putative polypeptide of 167 amino acids. The amino acid sequence was similar to the leptin sequences of other species. The NH2 terminus displayed features consistent with a signal peptide of 21 residues, as defined by SignalP program analysis (http://www.cbs.dtu.dk/services/SignalP/). A predicted cleavage site could therefore be expected between amino acid positions 21 and 22, giving rise to a mature protein with a predicted molecular weight of 16 kDa and a theoretical isoelectric point 6.31. The cloned PCR fragment also contained a 29-nucleotide 5′-untranslated region (UTR) and a 23-nucleotide 3′-UTR.

The cloned sequences were aligned with the rabbit leptin mRNA sequence whose coding region has already been described (43) and with the rabbit leptin gene (Ensembl). In the cloned sequences, there were seven nucleotide differences from the two sequences already described, corresponding to codons 8, 12, 15, 16, 118, 163, and 164 of the preprotein. Three of them were silent substitutions (12, 118, and 163) whereas the other three, in the signal peptide, resulted in substitutions of R to Q, C to Y, and L to P, respectively, and one in the mature protein resulted in D to S. These differences probably represented natural polymorphisms among different strains of rabbits. The sequence we report here extended the coding sequence to 5′- and 3′-regions. Both 3′- and 5′-sequences presented 100% similarities with the rabbit genome sequence. When the leptin cDNA sequence we obtained was compared with other vertebrate leptin mRNA sequences, we observed that it was closer to human than to mouse sequences (83.8 vs. 78.7%).

Genome alignment between the rabbit and other mammalian species revealed that a conserved region was located further upstream of the above ORF and included a TATA box and a putative first exon. We therefore designed a primer (RL3) in the first putative exon and were able to amplify the anticipated 582 bp fragment whose nucleotide sequence was identical to that of the 554 bp cDNA fragment except that it contained 28 additional nucleotides upstream. Our data led to the conclusion that in the rabbit the leptin gene contains three exons, which is consistent with data presented in other species.

#### Preparation and Characterization of Rabbit Leptin

Preliminary experiments in four E. coli clones designed to express rabbit leptin indicated strong expression in most of them (Fig. 1A). Clone 1 was selected for large-scale expression. After refolding and dialysis, rabbit leptin was purified by...
Sterile 0.2 mM solutions for at least 14 days at pH 6 or 8; no leptin in solution was tested during storage at 4 and 37°C as value of 6.8 pM for human leptin (36). The stability of rabbit leptin was tested using a BaF3 bioassay. Both rabbit and human ovine leptins exhibited almost identical activity with their respective EC50 values of 6.5 pM, close to the previously reported value of 6.8 pM for human leptin (36). The biological activity of the purified rabbit leptin was tested using a BaF3 bioassay. The sample marked MLM is mouse leptin (5 μg/lane). B: SDS-PAGE (15%) of purified rabbit prolactin run after lyophilization in absence (lanes 1–2 from the left) or presence (lanes 4–5 from the left) of β-mercaptoethanol (ME) at 2 concentrations: lanes 1 and 4–5 μg, lanes 2 and 5–10 μg. Lane 3, molecular weight markers (see above); C: gel-filtration analysis of the purified rabbit leptin on analytical Superdex 75 column pre-equilibrated with TN buffer (25 mM Tris·HCl, 150 mM NaCl, pH 8). The main peak with retention time of 15.57 min corresponds to monomer and the preceding small shoulder to dimer. To estimate the molecular mass the column was calibrated with BSA (66 kDa), rat ciliary neurotrophic factor (CNTF) (22 kDa) and human leptin (16 kDa). D: effect of rabbit and human leptin on proliferation of BaF3 cells stably transfected with the long form of human leptin receptor (hLEPR). A representative experiment (out of 3 performed) is shown. The results are presented as means ± SE, but the SE values are in most cases so small that they could not be seen on the graph. For other details, see text.

Expression of Leptin mRNA in Rabbit Mammary Gland During Pregnancy and Lactation

During pregnancy, leptin mRNA in mammary epithelial tissue was weakly expressed but detectable at all stages. By the third day of lactation, we observed an increase in leptin mRNA expression levels compared with pregnancy samples (leptin/TBP ratios of 3.22 vs. 0.72, respectively; P = 0.05). Leptin mRNA expression continued to increase during lactation; indeed, at day 16 of lactation, the ratio leptin/TBP reached 12.61 (P = 0.05) (Fig. 2). Compared with the mammary-surrounding consecutive anion-exchange and gel-filtration chromatography. The fractions containing pure monomer were pooled and dialyzed against NaHCO3 at pH 8 and a 4:1 (wt/wt) protein-salt ratio and then lyophilized. The yield was 300 mg from 2.5 l of bacterial culture. The purity and homogeneity of the purified leptin were documented by three separate methods. SDS-PAGE yielded only one band of ~16 kDa under both reducing and nonreducing conditions (Fig. 1B), and reverse-phase chromatography also yielded a single peak (not shown). As expected, in the absence of a reducing agent, mobility was slightly higher, indicating a globular structure. The band appeared slightly below the 15 kDa protein marker despite the predicted 16 kDa molecular mass. Since bands of the same size had also been observed in human and ovine leptins (not shown), this discrepancy was likely related to the slower mobility of the marker. Gel filtration at pH 8 under native conditions yielded a single monomeric peak consisting of >95% monomer, corresponding to a molecular mass of ~15–16 kDa (Fig. 1C). Specific extinction coefficients at 280 nm for a 0.1% solution, assuming an extra Ala at the NH2 terminus, were calculated according to the method already described (29) and yielded a value of 0.200. The biological activity of the purified rabbit leptin was tested using a BaF3 bioassay. Both rabbit and human ovine leptins exhibited almost identical activity with their respective EC50 values of ~11.5 pg per well (Fig. 1D) or 6.5 pM, close to the previously reported value of 6.8 pM for human leptin (36). The stability of rabbit leptin in solution was tested during storage at 4 and 37°C as sterile 0.2 mM solutions for at least 14 days at pH 6 or 8; no changes were observed in their monomeric content, and their activity in the BaF3 bioassay was retained.

Fig. 1. A: miniexpression of 4 clones of rabbit leptin. The bacteria were cultured in 30 ml of TB medium at 37°C, and the expression was induced by nalidixic acid. After 4 h the culture was centrifuged and frozen. Next day the pellets were digested with lysozyme and DNase and sonicated. The supernatant was saved, and the pellet was sonicated again and suspended in 1 ml distilled deionized water. Aliquots (corresponding to 16 μl) were separated by 15% SDS-PAGE. Lanes marked with + were induced by nalidixic acid, and lanes marked with − were the noninduced cultures. The molecular mass markers from the bottom up (lane 4) are (in kDa): 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250. The sample marked MLM is mouse leptin (5 μg/lane). B: SDS-PAGE (15%) of purified rabbit prolactin run after lyophilization in absence (lanes 1–2 from the left) or presence (lanes 4–5 from the left) of β-mercaptoethanol (ME) at 2 concentrations: lanes 1 and 4–5 μg, lanes 2 and 5–10 μg. Lane 3, molecular weight markers (see above); C: gel-filtration analysis of the purified rabbit leptin on analytical Superdex 75 column pre-equilibrated with TN buffer (25 mM Tris·HCl, 150 mM NaCl, pH 8). The main peak with retention time of 15.57 min corresponds to monomer and the preceding small shoulder to dimer. To estimate the molecular mass the column was calibrated with BSA (66 kDa), rat ciliary neurotrophic factor (CNTF) (22 kDa) and human leptin (16 kDa). D: effect of rabbit and human leptin on proliferation of BaF3 cells stably transfected with the long form of human leptin receptor (hLEPR). A representative experiment (out of 3 performed) is shown. The results are presented as means ± SE, but the SE values are in most cases so small that they could not be seen on the graph. For other details, see text.

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Fig. 2. Quantification of leptin mRNA in mammary gland throughout pregnancy and lactation determined by reverse transcription and real-time polymerase chain reaction assay. Leptin and TBP levels were measured from adipose tissue (AT) and mammary tissue collected from rabbits at 3, 9–14, and 25–29 days of pregnancy (P3, P9–P14, P25–P29, respectively) and at 3 (L3) and 16 days (L16) of lactation. Leptin/TBP mRNA ratios were calculated. Significant differences (at least P < 0.05) among the stages are indicated with different letters.
adipose tissue, leptin transcripts in the mammary gland were expressed at lower levels until early lactation but at equivalent levels by midlactation (L16).

Temporal Expression of Leptin in Rabbit Mammary Gland

The mammary gland is a complex tissue in which the acini develop from pregnancy to lactation. To verify whether leptin is produced by acini, mammary glands were collected at various stages of pregnancy and lactation, and cryosections were analyzed by indirect immunofluorescence using an anti-leptin antibody. All acini were labeled in mammary gland sections (Fig. 3).

Even though we have not used a quantitative method, we observed that during the first half of pregnancy the leptin signal was detectable, although weak [pregnancy day 3 (P3) to pregnancy day 14 (P14)]. This signal appeared stronger toward the end of pregnancy (P25). A strong labeling could be observed on L3 and L16. At all stages of pregnancy and lactation, no signal was detected when a secondary antibody was added alone (L16/H11002). Moreover, no specific signal was observed when anti-leptin antibody was preadsorbed with leptin (L16/H11001), further demonstrating the specificity of the immunolabeling technique.

Tissue Localization of Leptin mRNA in Rabbit Mammary Gland

To determine whether the leptin detected above in the alveolar compartment was produced locally, ISHs were performed with a DIG-labeled oligonucleotide LNA probe on rabbit mammary gland sections at selected physiological stages (Fig. 4; P9, P25, L3, and L16). The signal was mainly observed in the alveolar compartment at all stages, even though some extremely weak signals could be detected in some stromal cells. Leptin probe specificity was assessed at all stages using an antisense oligonucleotide LNA probe. A representative image is shown in Fig. 4 (P25 antisense). In the same way, the specificity at all stages of the alkaline phosphatase-coupled anti-DIG antibody was controlled in experiments run in the absence of a DIG-labeled oligonucleotide LNA probe. A representative image is depicted in Fig. 4 (P25 Ac−). These results show that leptin transcripts were expressed in alveolar cells, thus demonstrating a local production of this protein.

Cellular Localization of Leptin in Rabbit Mammary Gland

To determine which cells in the mammary alveoli express leptin, we performed a double staining analysis using antibodies to leptin and CK8 or leptin and smooth muscle actin. These latter antibodies are specific to the epithelial and myoepithelial cells surrounding mammary alveoli, respectively. These experiments were performed on P16 when leptin had reached its peak of expression in the mammary gland, as already shown in Fig. 4.

When observed at a low magnification, all luminal alveolar cells expressed leptin (Fig. 5A). These cells, strongly stained with CK8 antibody as well, are luminal epithelial cells. At a higher magnification, a signal (Fig. 5B1) was also localized in

Fig. 3. Immunolocalization of leptin with a TRITC-conjugated antibody in rabbit mammary gland from pregnancy (P3, P9, P14, P25) and lactation (L3 and L16). Negative control (L16−) and specific control (L16+) are also shown. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The scale bar represents 20 μm.

Fig. 4. Expression of leptin mRNA detected by in situ hybridization on rabbit mammary gland sections at different stages of pregnancy and lactation. Leptin mRNA expression was detected by in situ hybridization with a digoxigenin (DIG)-labeled oligo LNA probe onto mammary gland sections at different stages of pregnancy (P9 and P25) and lactation (L3 and L16). We performed 2 negative controls on 25-day pregnancy sections either with an antisense LNA probe (P25 antisense) or in experiments run in the absence of a DIG-labeled oligonucleotide LNA probe (P25 Ac−). Scale bars, 200 μm.
the cytoplasm of some cells, stained with smooth muscle actin antibody (Fig. 5B2) and which could easily be recognized as myoepithelial cells from both their elongated shape and elongated nuclei (Fig. 5B3).

**DISCUSSION**

During this study, full-length leptin cDNA was cloned from rabbit adipose tissue and shown to encode a predicted polypeptide of 167 amino acids with a theoretical value of 18.24 kDa and an isoelectric point of 7.62. Consistent with the other known leptin sequences, it contained a predicted 21-amino acid signal peptide. The respective prepared recombinant rabbit leptin exhibited biological activity similar to that seen in other mammalian species, thus verifying the correctness of the predicted amino acid sequence. Alignment of the nucleotide sequence of rabbit leptin with human and mouse sequences revealed a greater similarity with human than with mouse. This was confirmed from examination of the amino acid sequences, 80.2 vs. 79.8%.

We also report evidence that rabbit mammary tissue expresses leptin mRNA and protein during pregnancy and lactation. These findings are in agreement with the synthesis of leptin previously described in humans (39), mice (7), sheep (10), and cattle (8). We further show that leptin gene expression in rabbit mammary glands is strongly dependent on the stage of pregnancy or lactation. However, our data contrasted with some previous findings in different animal models. In mouse and ovine models, leptin expression in the mammary gland throughout lactation was significantly lower than during pregnancy (7, 10). Possible interspecies differences could be related to species-specific endocrine profiles and subsequent hormonal regulation.

Using an immunohistochemical approach, we were thus able to clearly establish that leptin is present in the rabbit mammary gland. Moreover, the expression pattern of the protein mirrored that of mRNA assessed by real-time PCR.

Using ISH with specific oligo-LNA probes, we demonstrated that leptin is synthesized by mammary secretory tissue during both pregnancy and lactation. During the early stages of pregnancy, stromal tissue containing adipocytes is predominant in the mammary gland. Because we focused our studies on the epithelium, we chose to use cryosections to minimize leptin signals due to adipocytes. Even though they represented a major source of leptin, mammary adipocytes were therefore scarcely labeled in our ISH experiments. By contrast, after full cell differentiation during lactogenesis, adipose tissue had...
clearly regressed in the mammary gland so that local leptin mRNA synthesis was not due to that cell population.

Specific mammary epithelial and myoepithelial cell markers (CK8 and α-smooth muscle actin, respectively) were used to discriminate between the luminal epithelial and basal myoepithelial production of leptin during the period of its strongest expression. At midlactation, leptin was clearly present in the cytoplasm of luminal epithelial cells and detected in a few basal myoepithelial cells. Although our experiments clearly showed that mRNA was present in luminal cells, ISH experiments did not show if basal cells, which exhibit typical myoepithelial phenotype, also expressed this transcript. It is nonetheless likely that the local synthesis of leptin mRNA also occurs in myoepithelial cells during lactation as already described in sheep (10). However, in contrast to sheep, in the rabbit, leptin is not produced exclusively by myoepithelial cells during lactation. Our findings are in line with those describing leptin expression in a human breast epithelial cell line (27). Leptin production by mammary epithelial cells is of considerable interest because leptin receptors have also been observed in mammary epithelial cells in several species (7, 8, 23, 25, 38) including rabbit (preliminary results). These data strongly support the hypothesis that leptin may constitute a major local regulator of mammary gland development. By acting as an autocrine and/or paracrine factor, leptin may contribute to the modulation of mammary growth and differentiation. Moreover, leptin gene expression by the rabbit mammary gland may be related to leptin secretion into colostrum and milk, as has already been observed in humans (9, 11, 13, 20, 26, 39), rats (11), cows (32), pigs (14), and mice (7). The presence of leptin in breast milk raises important questions as to the possible physiological role of this cytokine in newborns and particularly its effect in the metabolic imprinting process.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


