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Detection and regulation of leptin receptor mRNA in ovine mammary epithelial cells during pregnancy and lactation

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Abstract Adipocyte-epithelial cell interactions and their secretions are critical determinants of mammary gland development. In this present study, we examined the possible involvement of leptin and its receptors in the process of mammogenesis/lactogenesis. We demonstrated by reverse transcription and polymerase chain reaction analysis that long and short forms of leptin receptors were expressed in the ovine mammary gland during pregnancy and lactation. Furthermore, quantitative determinations, via ribonuclease protection assays, provided evidence that the level of leptin receptor expression was greatest during mid-pregnancy when active growth of the mammary gland is initiated. Location of the leptin receptors, as determined by in situ hybridization analysis, revealed that leptin receptor transcripts were expressed specifically in mammary epithelial cells. These data provide evidence that leptin, with its receptors, could be an important mediator in regulating mammary gland growth and development.

Key words: Leptin receptor; Mammary epithelial cell; Ovine

1. Introduction

Mammary gland development begins during fetal life, increases through puberty and terminates at the end of pregnancy. During all phases of development, the mammary epithelium is situated adjacent to stroma, which consists of a matrix of adipose and fibrous connective tissue called the 'mammary fat pad' [1]. The mammary fat pad was once considered as an inert matrix, but more recent evidence emphasizes the influence of adipocyte-epithelial interactions as being critical for mammary duct growth and morphogenesis [2–6]. Locally produced growth factors are believed to mediate the adipocyte-epithelial interactions and alter the actions of several steroid and peptide hormones on these tissues [7,8].

Leptin, a circulating hormone secreted by adipose tissue [9], is a cytokine that is capable of influencing the growth of various cell types [10,11]. Furthermore, leptin is expressed in parametrial adipose tissue and epithelial cells of mouse mammary glands [12]. Consequently, leptin may have both autocrine and paracrine actions in the mammary gland. We hypothesized that leptin plays an important physiological role in mammary epithelial cell growth by acting as a local factor which mediates adipocyte-epithelial cell interactions.

Leptin acts through single membrane receptors that have strong sequence homology to the class I cytokine receptor family [13]. Although multiple isoforms of leptin receptors have been described, they are generally categorized according to the length of their intracellular domains as long and short forms. In order to examine leptin's potential involvement as a local factor mediating the interaction between mammary gland adipocytes and epithelial cells, we used reverse transcription and polymerase chain reaction (RT-PCR) analysis to identify the expression of long and short form leptin receptor transcripts in ovine mammary glands. Furthermore, we used ribonuclease protection assay procedures to determine if leptin receptor mRNA was differentially expressed during pregnancy and lactation in mammary glands and we used in situ hybridization procedures to determine the cell types expressing the leptin receptor transcripts.

2. Material and methods

2.1. Tissue samples

Animal care and use procedures were approved by the French Ministry of Agriculture in adherence with French regulations for animal experimentation (guideline 19/04/1988). Mammary gland tissue was collected from three primiparous Préalpes du Sud ewes at each of the days 15, 50, 70, 106, 112 and 141 of pregnancy and days 3 and 48 of lactation.

2.2. RT-PCR analysis

Total tissue sample RNA was prepared by the guanidium isothiocyanate/phenol method, as described by Puissant and Houdebine [14]. The reverse transcription reaction was performed using 2 μg of total RNA, with 100 U Superscript II reverse transcriptase (Gibco BRL-Life Technologies) and 650 ng random primers in a final volume of 20 μl, for 50 min at 42°C. One μl of each reverse transcription reaction product was amplified by PCR in a final volume of 50 μl with 200 μM of each deoxynucleotide triphosphate (Pharmacia LKB Biotechnology, France), 45 pmol of each primer (Genset, Paris, France), 2.5 U Taq Polymerase (Oncor-Appligene, Illkirch, France) and 1×PCR buffer (Oncor-Appligene). Amplifications were performed using a Perkin-Elmer Thermocycler replicating for 30 cycles in the following sequence: denaturation at 94°C for 1 min, annealing at 55°C (long form ovine leptin receptor) or at 52°C (short form ovine leptin receptor) for 1 min, extension at 72°C for 1 min and a final extension step at 72°C for 10 min. To amplify the long and short forms of the ovine leptin receptors, human oligonucleotide primers were chosen according to the published human cDNA sequences [13,15]: antisense primer 5′-CAAAAGCACACCACTCTCTC-3′ and sense primer 5′-CCAGAAACGTTTCAGCATCT-3′ specific for the long form of the human leptin receptor or antisense primer 5′-TCAAAGAATGTCCGTTCT-3′ and sense primer 5′-GAGAAGTACCAGTTCAGT-3′ for the short form of the human leptin receptor. The expected sizes of the amplified fragments were respectively 609 bp (long form) and 279 bp (short form).

2.3. Analysis of the amplification products

Specificity of the amplified PCR products was confirmed by South-

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ern blot analysis using ovine and human leptin receptor cDNA probes, which specifically recognized the long and the short forms of ovine leptin receptor. PCR products were separated on a 2% agarose gel and blotted onto a nylon membrane. The blot was hybridized with α-^{32}P-labelled probes (3×10^6 cpm/ml; Amersham, Les Ulis, France). The ovine leptin receptor probe consisted of 365 bp [16] and the human leptin receptor probe consisted of 156 bp (nucleotides 2448–2604) nucleotides [13], which recognized the long and short forms of the ovine leptin receptors, respectively.

Amplification products were extracted from agarose gel using a DNA purification kit (Quiagen, France) and cloned into the pGEM-T vector, according to the manufacturer's instructions (Promega, Lyon, France). The cDNA inserts were then sequenced by automated DNA sequencing (Applied Bio Systems, Foster City, CA, USA).

2.4. Ribonuclease protection assay and in situ hybridization

Ribonuclease protection assay and in situ hybridization procedures were performed as described [17] using antisense and sense RNA probes. These riboprobes were transcribed using T7 or T3 RNA polymerase (Gibco BRL-Life Technologies, Cergy Pontoise, France) and labelled with [α-^{32}P]UTP for ribonuclease protection assay (800 Ci/mmol; Amersham, Les Ulis, France) or [α-^{35}S]UTP for in situ hybridization (1000 Ci/mmol; Amersham). Antisense riboprobes were produced using linearized recombinant Bluescript SK (−) transcription vector (Stratagene, Ozyme, Montigny-le-Bretonneux, France) containing a 484 bp cDNA fragment of the ovine leptin receptor [16]. This antisense riboprobe hybridizes with long and short forms of the ovine leptin receptor. Recombinant pGEM-T transcription vector containing a cDNA fragment (365 bp) specific for the long form of the ovine leptin receptor [16] was also linearized and radiolabelled. Labelled sense probes were used as negative controls for in situ hybridization. Bands appearing on the autoradiographs were scanned with a STORM-860 machine (Molecular Dynamics, Bondoufle, France) and quantified with ImageQuaNT software (IQNT-130; Molecular Dynamics). To control for RNA loading differences, ovine leptin receptor mRNA expression was standardized to ovine glyceraldehyde-3-phosphate dehydrogenase hybridization signal and the data are presented as corrected values.

3. Results

3.1. Detection of long and short forms of the leptin receptor in ovine mammary gland

Expression of the long (Fig. 1A) and short forms (Fig. 1B) of the ovine leptin receptor was detected in tissues from each stage of pregnancy and lactation that was examined, as determined by Southern blot analysis of RT-PCR products hybridized with leptin receptor probe corresponding to the long and short form. Subsequent sequencing of the 609 bp fragment revealed 100% identity to the published sequence of the long form of the ovine leptin receptor [13]. Moreover, the sequencing of the 279 bp fragment revealed 83% identity to the short form of the human leptin receptor [15].

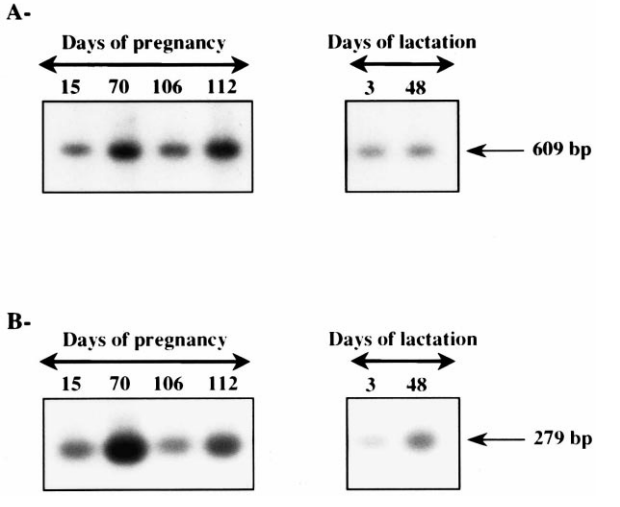

Fig. 1. Detection of the long (A) and short (B) forms of ovine leptin receptor by Southern blot analysis of RT-PCR products in ovine mammary gland during pregnancy and lactation.

3.2. Temporal expression of leptin receptor mRNA in ovine mammary glands during pregnancy and lactation

Expression of leptin receptor mRNA by mammary gland tissue collected from three ewes each on days 15, 50, 70, 112 and 141 of pregnancy (P) and days 3 and 48 of lactation (L) was quantified via a ribonuclease protection assay utilizing the 484 bp probe which recognizes the long and short forms of the ovine leptin receptor. The results are illustrated in Fig. 2 and are presented relative to the expression of ovine glyceraldehyde-3-phosphate dehydrogenase mRNA. Expression of ovine leptin receptors was greater during mid-pregnancy (days 70 and 106) than at any other time tissues were sampled.

In an effort to more precisely define the leptin receptor form (short vs. long) that changed during pregnancy and lactation, we hybridized total RNA from each sample with the 365 bp probe that was specific for the long form of the ovine leptin receptor. No hybridization signal was detected (data not shown), indicating that the short form of the ovine leptin receptor was predominantly expressed. Moreover, these data provide evidence that the short form of the ovine leptin receptor was differentially expressed during pregnancy and lactation.

3.3. Cellular localization of leptin receptor mRNA in ovine mammary gland

In order to determine which cells in the mammary gland expressed the leptin receptor gene, in situ hybridization was performed using the 484 bp cRNA probe that recognized both the long and short forms of the ovine leptin receptor. In cryostat sections of mammary gland collected across the various stages of pregnancy and lactation, we observed leptin receptor mRNA expression localized within the alveolar epithelial cells (Fig. 3). Moreover, in situ hybridization analysis provided a confirmation of the temporal variation in leptin receptor mRNA expression throughout pregnancy and lactation as observed via the ribonuclease protection assay analysis. Indeed, leptin receptor mRNA expression was greatest at day 70 of pregnancy (A,B), decreased to day 112 of pregnancy (C,D) and remained detectable during lactation (G and H = day 3 of lactation). Control tissue sections treated with the sense probe (E,F) exhibited no specific signal, further demonstrating the specificity of the reaction.

4. Discussion

We report here the first evidence, obtained via the use of RT-PCR procedures, that long and short forms of leptin receptor are expressed in ovine mammary tissue during pregnancy and lactation. However, when we utilized ribonuclease protection assay techniques, which are less sensitive, to quantify the relative abundance of the long and short forms of the leptin receptors, only the short form of the leptin receptor was detected. These observations are consistent with the reports of Tartaglia [13], Mercer [18], Ghilardi [19] and Hoggard [20]

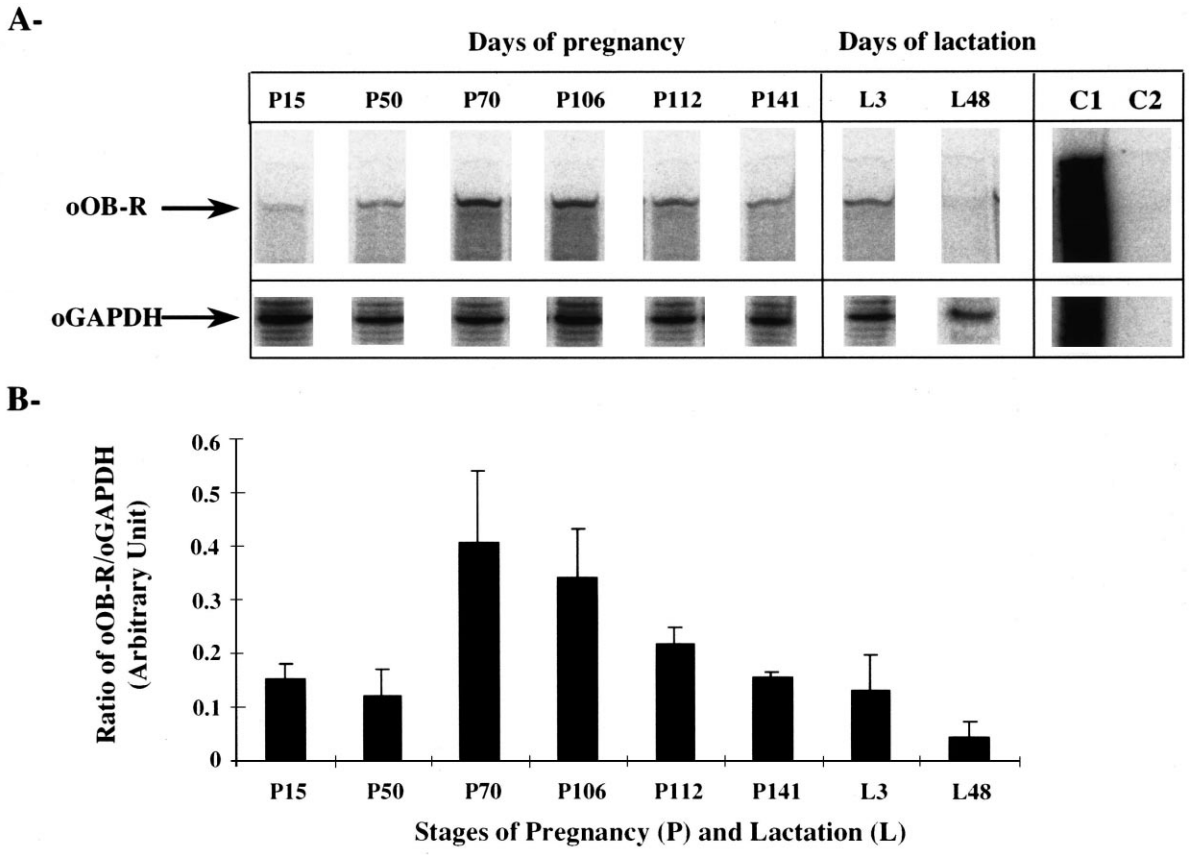

Fig. 2. (A) Detection, by ribonuclease protection assay, of ovine leptin receptor (OB-R) mRNA in ewe mammary tissue at various stages of pregnancy and lactation. The radiolabelled 484 bp leptin receptor fragment, used as the cRNA probe, was hybridized to 10 μg of mammary gland total RNA. As controls, the leptin receptor cRNA probe was hybridized with yeast tRNA and then treated with RNase (C2) or not (C1). (B) Determination, from STORM scanning of the ribonuclease protection assay gels, of the ovine leptin receptor mRNA to ovine glyceraldehyde-3-phosphate dehydrogenase (oGAPDH) mRNA.

where further evidence can be found that the long form of the leptin receptor was expressed at low levels in peripheral tissues and at high levels in the brain.

We also report here that transcripts of the short forms of the leptin receptor, as determined via ribonuclease protection assay, was in greatest abundance in the ovine mammary gland during mid-pregnancy (days 70–106) and lowest at the end of pregnancy and during lactation. Furthermore, in situ hybridization analysis of these tissues reinforced this differential pattern of leptin receptor transcript expression and moreover localized the leptin receptor transcripts to the mammary alveolar epithelial cells. The abundant expression of the leptin receptor transcript within the mammary epithelial cells at day 70 of pregnancy coincides with the initiation of mammary epithelial cell proliferation which occurs, as growth of the mammary gland begins, during mid-pregnancy in the ewe [21]. Concomitant with this stage of pregnancy and mammary development, peripheral concentrations of oestradiol are increasing and may contribute to the variation in the expression of the leptin receptor transcripts [21].

In concert with the leptin receptor dynamics occurring in the alveolar epithelial cells, we suggest that coincident expression and regulation of leptin mRNA may also be occurring within the epithelial cells and parametrial adipose tissues, as was reported to occur in the mouse mammary gland [22]. In the present study, utilizing Northern blot procedures, we detected leptin transcripts in the adipose tissue of the ovine mammary gland (data not shown). This finding is crucial, as the stromal-epithelial interactions are fundamental for mammary gland growth and development. Indeed, each stage of mammary glandular development, from ductal branching through terminal end bud development, alveolar formation and milk production, is dependent on the presence of the mammary fat pad [2–6]. Our findings provide evidence to support the hypothesis that leptin might be a local growth factor, which acts as a functional link between adipocytes and epithelial cells of the mammary gland. By acting as an autocrine or paracrine factor in the epithelial cell, leptin may play an important role in the modulation of the mammary gland growth. This hypothesis is further supported by several reports which indicate that leptin is a cytokine that is capable of influencing the growth or apoptosis of various cell types [10,11].

Adipocyte-epithelial interactions are critical in determining the pattern of ductal growth and morphogenesis in the mammary gland via the effect of local growth factors. These local factors are implicated as paracrine mediators of several steroids and peptides [7,8]. The ovarian steroids, oestrogen and progesterone, play an essential role in the development of the mammary gland during pregnancy. By in vivo and in vitro studies, it was demonstrated that the mammogenic effects of oestrogen occur indirectly via epithelial proliferation and morphogenesis and require the cooperative action of mammary stromal fibroblasts and adipocytes [23–25]. Moreover, it has

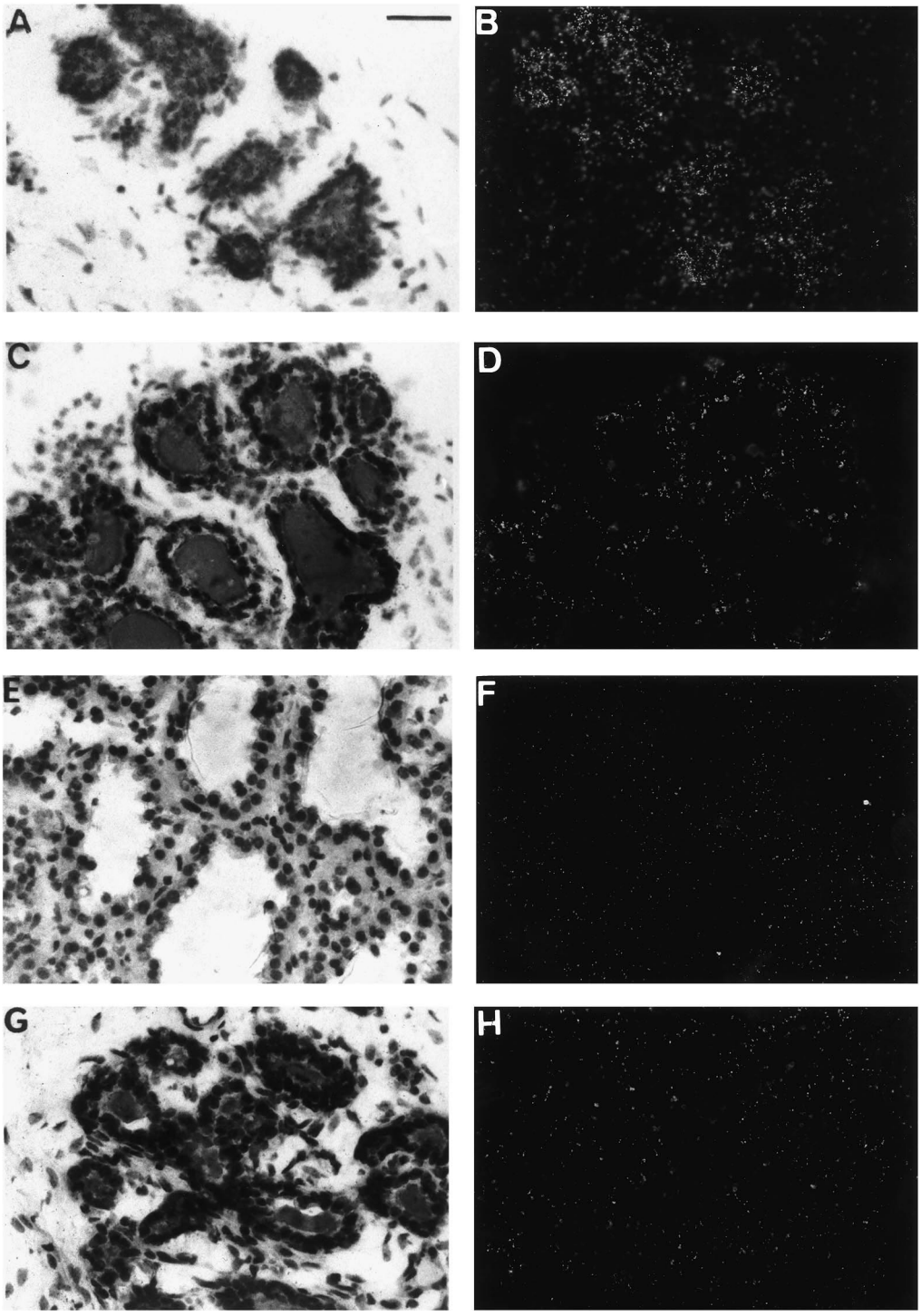

Fig. 3. In situ hybridization analysis of ovine leptin receptor mRNA expression in the ovine mammary gland at day 70 (A,B) and 112 (C,D) of pregnancy and day 3 of lactation (E,F). A section of the mammary gland at day 70 of pregnancy (G,H) was hybridized with the sense probe and used as a control of specificity. The right and left column correspond to bright-field and epi-illuminated photomicrographs. Bar = 39 μm.

been reported that oestrogen receptors are present on mammary fibroblasts and adipocytes [26,27]. Several reports have described that leptin expression is induced by oestradiol in adipocyte [28,29].

In conclusion, leptin is ideally suited as a potentially important downstream mediator of steroid action, acting locally to regulate mammary gland growth and development via adipocyte-epithelial interactions. The data we present here provide evidence that indeed leptin is produced by the mammary gland fat pad and receptors for leptin exist on mammary alveoli epithelial cells. Furthermore, the expression of the leptin receptor gene increases during mid-pregnancy, coincident with the initiation of mammary development and rising peripheral concentrations of oestradiol.

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