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Cloning of bovine RANTES mRNA and its expression and regulation in ovaries in the periovulatory period

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Abstract RANTES may be one of the chemoattractants involved in stimulating eosinophils and macrophages to migrate selectively into bovine dominant follicles and into developing corpora lutea. We sequenced a 736 bp fragment of the bovine RANTES mRNA encoding the complete protein and defined the ovarian source of RANTES mRNA. As demonstrated by competitive RT-PCR, follicle-derived macrophages showed a 100–1000 times higher RANTES mRNA level compared to unpurified granulosa cells or follicle-derived fibroblasts. By means of in situ hybridization, RANTES mRNA positive macrophages were located in the former thecal layer of the developing corpora lutea.

Key words: Bovine RANTES; Ovary; Macrophage; Eosinophil

1. Introduction

Leukocytes are attracted into the ovary and influence cyclically changing structures [1–3]. Recently, we reported that the eosinophils suddenly increase in number to roughly 90% of the leukocytes in the bovine corpus luteum of early development [4]. In sheep, the eosinophils are assumed to influence the neovascularization of the corpus luteum [5]. Macrophages appear to increase in number within the thecal layer of dominant follicles [6] and to contribute to periovulatory events such as dilation, permeability and the sprouting of microvessels, as well as to the degradation of the basal membrane [3,7].

There are only a few data available on the chemoattractants by which the various leukocyte subpopulations are drawn into specific ovarian areas at a precisely defined moment [8,9]. RANTES is a favorite candidate in the CC (β-) chemokine family for selectively recruiting both eosinophils and macrophages [10–12]. It is produced by a variety of cell types and can be induced in many tissues by specific stimuli [13,14]. RANTES certainly plays a role in reproduction. The concentration of leukocytes, and especially macrophages, also changes dramatically in the uterine epithelium during the estrous cycle. The instillation of RANTES into the uterine lumen of macrophage-colony stimulating factor (M-CSF) nullizygous mutant mice ($csfm^{op}/csfm^{op}$) resulted in a dramatically increased number of macrophages [15].

We investigated whether RANTES is upregulated in the bovine ovary around the periovulatory period in order to attract eosinophils and/or macrophages. FSH, LH and PGE_2 are potential candidates for triggering RANTES secretion, and they have been investigated in cultures of isolated ovarian cells. Whereas granulosa cells and fibroblasts are easily obtained from the bovine ovary, the small number of leukocytes separated from dominant follicles was insufficient for this purpose. Recently, we described an easy method of accessing macrophage-like cells in the bovine ovary [16]. In the present study, these macrophage-like cells have definitely been identified as true macrophages. We also determined the bovine RANTES mRNA sequence, since it was not available from data bases, and evaluated the RANTES mRNA expression and regulation in different cell types derived from dominant follicles and additional ovarian tissues by semiquantitative RT-PCR and in situ hybridization.

2. Materials and methods

2.1. Isolation of cells and cell cultures

Bovine ovaries with regressed corpora lutea were obtained from the local abattoir immediately after the death of the animal and staged as recently described [16]. Dominant follicles were punctured to release granulosa cells containing a low number of leukocytes. These leukocytes consisted mainly of macrophages. In order to obtain pure granulosa cells, the leukocytes were removed from the primary cell harvest (also described as unpurified granulosa cells) by incubation with an anti-bovine CD18 monoclonal antibody (VMDR, Pullman, WA, USA) followed by goat anti-mouse IgG-coated M-450 Dynabeads (DYNAL GmbH, Hamburg, Germany). The cells were cultured with DMEM/Ham's F12 (GIBCO BRL, Grand Island, NY, USA) with 5% FCS (unpurified granulosa cell cultures accumulate macrophage-like cells after few days [16]). These cells were phenotyped by flow cytometry, using anti-bovine CD18, CD14, CD45 and MHC-class II monoclonal antibodies (VMDR) followed by FITC-labeled goat anti-mouse $F(ab')_2$ immunoglobulins. The macrophage-like cells expressed all those antigens (data not shown) which are characteristic for peripheral blood monocytes and tissue macrophages. The cells were therefore classified as true macrophages. Fibroblasts were obtained from dominant follicles by cultivating small pieces of the wall in DMEM with 10% FCS. Immunocytological methods, using anti-CD18 and anti-vimentin (DAKO, Hamburg, Germany) monoclonal antibodies and detection of 3β-hydroxy-steroid dehydrogenase (3β-HSD) activity [17], revealed that the fibroblasts and purified granulosa cells represented pure fractions. Purified granulosa cells were $CD18^-$, $vimentin^+$ and showed 3β-HSD activity, whereas the fibroblasts expressed only vimentin (data not shown). 3×10^5 cells were seeded into six-well plates for 24 h. The medium was replaced with OPTI-MEM (GIBCO) for another 12 h to eliminate possible RANTES mRNA upregulation by FCS. Following that, the cells were incubated with OPTI-MEM, containing bovine FSH (100 ng/ml; Biogenesis, Poole, UK), LH (100 ng/ml; Biogenesis), lipopolysaccharide *E. coli* 055:B5 (LPS; 100 ng/ml; Sigma), prostaglandin E_2 (PGE_2, 500 U/ml; Sigma) and 10 ng/ml phorbol-myristate acetate (PMA, Sigma).

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2.2. RNA isolation, cDNA synthesis and cloning of bovine RANTES mRNA

Total RNA was isolated using the QIAGEN total RNA isolation kit (QIAGEN GmbH, Hilden, Germany). Genomic DNA was digested with 0.02 U DNase/μg RNA (Roche Molecular Biochemicals, Mannheim, Germany). 5 μg of RNA was employed to synthesize cDNA, using a first-strand cDNA synthesis kit from Amersham Pharmacia Biotech (Freiburg, Germany) in a reaction volume of 15 μl. The homologous RANTES primers (5′-aca ggt acc atg aag gtc tc-3′) and (5′-ctc atc tcc aaa gag ttg atg-3′) were designed by comparing human and guinea pig RANTES mRNA sequences [18]. With these primers, we identified the predicted 279 bp fragment in bovine small-intestine cDNA by RT-PCR. The bovine primers were designed on the basis of the determined sequence. The BoRants1 primer (5′-ata tgc ctc gga cac cac g-3′) and a $d(T)_{18}$ primer (Amersham Pharmacia Biotech) were used to amplify the 3′ untranslated region of bovine RANTES cDNA. The unknown 5′ sequence of RANTES mRNA was amplified by single-strand ligation to single-stranded cDNA as described by Stappert et al. [19]. Briefly, the technique is based on covalent linkage of an anchor oligonucleotide (5′-P-gta gga att cgg gtt gta ggg agg tcg aca ttg cc-3′) with T4 RNA ligase (MBI Fermentas GmbH, St. Leon-Rot, Germany) to first-strand cDNA treated with RNase H (Roche Molecular Biochemicals) and primed with the specific primer BoRantr2 (5′-aag ttc agg ttc aag gcg tc-3′). The anchored cDNA was then amplified by using an anchor-specific primer (5′-ggc aat gtc gac ctc cct aca ac-3′) and BoRantr2 for 20 cycles, followed by a nested PCR with a second anchor primer (5′-ctc cct aca acc cga att cct ac-3′) and BoRantr1 (5′-tcg cac cca ctt ctt ctc tg-3′). All fragments were cloned into a pGEM-T vector (Promega Corporation, Mannheim, Germany). The sequence was determined in both strands.

2.3. Semiquantitative competitive RT-PCR

To quantify bovine GAPDH and RANTES cDNA, a rapid one-step method was introduced to synthesize internal homologous competitors [20]. Amplification of bovine small intestine cDNA with the Borants1 (5′-ata tgc ctc gga cac cac g-3′) and a hybrid primer (5′-aag ttc agg ttc aag gcg tct cgc acc cac ttc ttc tct g-3′) resulted in a 191 bp bovine RANTES competitor. In order to quantify bovine GAPDH cDNA, a 476 bp GAPDH competitor was synthesized, using BoGapdhs1 (5′-tga agg gtg gcg cca aga gg) and a hybrid (5′-tgc cag ccc cag cat cga agc ctg ctt cac cac ctt ctt g-3′) primer. The competitors were prepared and stabilized at defined concentrations in the PCR tubes in accordance with the method of Köhler et al. [21].

Variations across different cDNA preparations were corrected. All samples were first adjusted to contain equal-input GAPDH cDNA concentrations in a semi-quantitative PCR, using BoGapdhs1 and BoGapdhr1 (5′-tgc cag ccc cag cat cga ag-3′) [22]. We then estimated RANTES cDNA in the adjusted samples. cDNA samples were titrated in RT-PCR tubes containing known copies of the RANTES competitor. Both the sample cDNA and the competitor were co-amplified, using the BoRants1 and BoRantr2 primers. With this approach, two products were generated. One was derived from the cDNA (218 bp) and the other, 27 bp smaller in size, from the RANTES competitor. Based on the length difference, the sample cDNA and competitor PCR products were resolved by gel electrophoresis. The sample cDNA and competitor were quantified by measuring the intensity of ethidium fluorescence with a cooled CCD 8 bit image sensor and the data analyzed by Phoretix 1 D plus software (Phoretix International, Newcastle-upon-Tyne, UK). The target copies equation was used to determine the ratio of sample cDNA copies/PCR to the number of competitor copies added, multiplied by the quotient of the cDNA signal divided by the competitor signal. Each 25 μl amplification reaction contained 2.5 μl 10× concentrated PCR buffer (15 mM $MgCl_2$), 0.3 U *Taq* DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), 100 μM dNTPs, 0.1 μM of each primer, 1 μl sample cDNA and the stabilized competitor in adjusted dilutions. The mRNA levels of cultures obtained from separate experiments ($n=4$) were presented as mean ± S.E.M. The Mann–Whitney test was used to determine the statistical significance of the difference between basal and stimulated cells.

2.4. In situ hybridization of cultured cells and luteal tissue

The 303 bp RANTES riboprobe was synthesized by RT-PCR using the BoRants2 and BoRantr2 primers, cloned into the pGEM-T vector, and linearized. The digoxigenin (DIG)-labelled antisense and sense strands were generated, using the DIG RNA-labelling kit (SP6/T7, Roche Molecular Biochemicals). Cells on chamber slides were fixed in 4% buffered formaldehyde and permeated with 0.1% Triton X-100 in PBS. The pieces of ovarian tissue were fixed in SERRA solution (12 parts 95% ethanol, 6 parts 35% formaldehyde and 1 part acetic acid) and embedded in paraffin wax. The section processing, hybridization (500 ng DIG-labelled riboprobe/ml), washing and staining were carried out in accordance with the protocol described by Braissant and Wahli [23].

3. Results

3.1. Cloning of bovine RANTES mRNA

RT-PCR from the bovine small intestine cDNA with homologous human RANTES primers resulted in a predicted 279 bp fragment which did not contain the start and stop codons of the coding region. The missing 5′ coding region and a 54 bp fragment of the 5′ untranslated region were amplified by the single-strand ligation to single-stranded cDNA technique. 3′ rapid amplification of cDNA end (3′ race) produced the sequence up to the stop codon, and an additional 409 bp fragment on the 3′ untranslated region. This region did not contain the poly-A tail, since the oligo-dT primer seems to bind to a poly-A-rich region approximately 360 bp further down from the stop codon than the whole human RANTES mRNA sequence. The nucleotide and predicted amino acid sequences of the coding region of the bovine RANTES cDNA are shown in Fig. 1. The partial bovine RANTES mRNA sequence is 736 bp long (EMBL, accession number AJ 007043) and contains a 276 bp open reading frame that encodes a 91 amino acid protein. Comparison at the amino acid level revealed 80.6%, 76.3%, 75.3% and 82.8% identity with the human, mouse, rat and guinea pig RANTES amino acid sequences, respectively.

3.2. Semiquantitative RANTES cDNA determination

Tissue cDNA samples were prepared from various regions of the ovary. Most segments, including the wall of dominant follicles and developing corpora lutea, were positive for

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                                                                  M  K
CGGAGCTGCAGAGGATCAGCACGCGGATCGCCCCAGCCTCTGCCCACAGCTACCATGAAG                60
                                                                BoRants2
 V  S  A  T  A  F  A  V  L  L  M  A  A  A  L  C  A  P  A  S
GTCTCTGCCACTGCCTTCGCTGTCCTCCTGATGGCGGCCGCCCTCTGCGCTCCTGCTTCT               120

 A  S  P  Y  A  S  D  T  T  P  C  C  F  A  Y  I  S  R  P  L
GCCTCCCCATATGCCTCGGACACCACGCCCTGCTGCTTTGCCTATATCTCCCGCCCGCTG               180
       BoRants1
 P  R  T  H  V  Q  E  Y  F  Y  T  S  S  K  C  S  M  A  A  V
CCCCGCACCCACGTCCAGGAGTATTTCTACACCAGCAGCAAGTGCTCCATGGCAGCAGTT               240
 V  F  I  T  R  K  K  R  Q  V  C  A  N  P  E  K  K  W  V  R
GTCTTTATCACCAGGAAGAAGCGCCAGGTGTGCGCCAACCCAGAGAAGAAGTGGGTGCGA               300
                                                    BoRantr1
 E  Y  I  N  A  L  E  L  S  •
GAGTACATCAACGCTTTGGAGTTGAGCTAGGGTGGAGGACGCCTTGAACCTGAACTTGCG               360
                                BoRantr2

CCAACTTTGCTTCTCGCTCTTGTCCTAAGCAGCTTGGGAGGCTCCCCCGCAATGCCCTCC               420

TCCCACCCTCTCCTGGGAAGGCACAGATTCCACCCCGCGCAGCAGCAGCTGCTGTGAAAG               480

ACCCTCAGTGCTCCTGGGGCTCTGCCCTTGTGCACAGGAGGTCTCTAAGCTCCGAGCTCC               540

TGAGCCCCTGCCCACCCACCGCTCTGCAGTCAGAAAGGATGCCGGCGTCTCTGGAGGGGA               600

AGGAGGGCAGGAGACTGGGCTCCTCTGGTCATGGCCCGTGACCAGAGCCCCCATCTCGGT               660

CCCTCACTGGGGAGGGCTGCACTGGCAAATAAGAAGAAATCAGCTGTTCATTAAAATTCT               720

CCAAGCGATTGCAAAA    736
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Fig. 1. Nucleotide sequence and predicted amino acid sequence of bovine RANTES. Primer sequences are underlined.

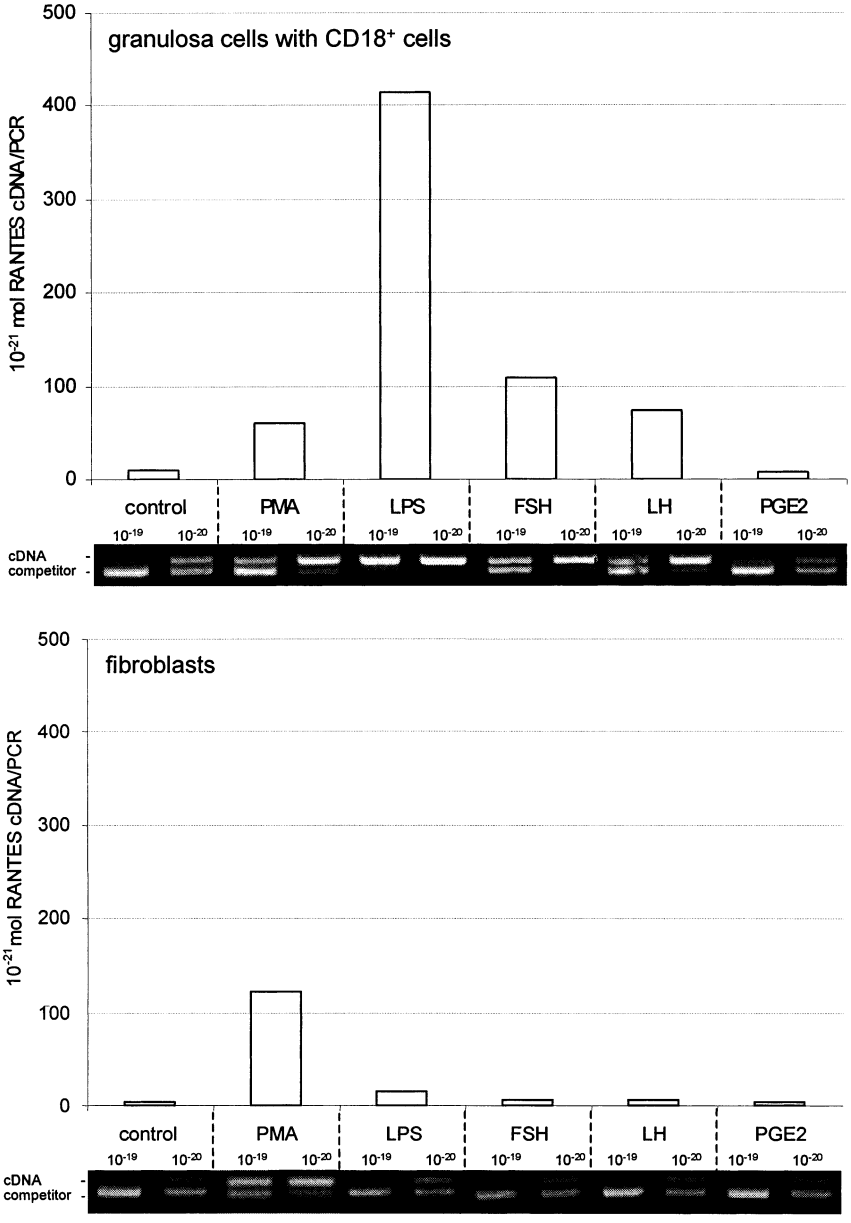

Fig. 2. Semiquantitative competitive RANTES RT-PCR on stimulated ovarian-derived cell types. The RANTES competitors (only shown at 10^{-20} and 10^{-19} mol/PCR) were coamplified with the sample cDNA in the same PCR tube. Both PCR products were separated by gel electrophoresis and quantified. One out of three representative experiments is shown.

RANTES mRNA (data not shown). In a first series of in vitro experiments, unpurified granulosa cells and fibroblasts were stimulated for 6, 12, and 24 h. All further experiments were carried out after 12 h, i.e. the maximum of RANTES mRNA expression. Both cell types expressed RANTES mRNA under basal conditions (all data: mean ± S.E.M.; $\times 10^{-21}$ mol RANTES cDNA/PCR; unpurified granulosa cells: 21.2 ± 5.8; fibroblasts: 3.3 ± 0.6). PMA, which is known to be a RANTES expression regulator and is used as a positive control, significantly stimulated RANTES mRNA levels in both cell types (unpurified granulosa cells: 70.2 ± 16.8, fibroblasts: 66.7 ± 24.7, $P < 0.03$). LH and FSH significantly increased RANTES mRNA in unpurified granulosa cells (LH: 69.1 ± 12.1; FSH 104.5 ± 29.3, $P < 0.03$), but not in fibroblasts (Fig. 2). PGE_2 had no significant effect. Surprisingly, LPS significantly stimulated RANTES mRNA expression in unpurified granulosa cells to up to 20-fold (250 ± 88.8, $P = 0.05$). The experiments were therefore repeated using pure granulosa cells. The basal RANTES mRNA decreased down to the detection limit. Furthermore, none of the stimulating substances brought about any increase in RANTES mRNA (data not shown). At the same time the follicle-derived macrophages showed a basal RANTES mRNA level 100–500 times higher than that of unpurified granulosa cells (5630 ± 940). The significant positive response after stimulating macrophages with LPS (11570 ± 860, $P = 0.05$) indicated that the LPS-increased RANTES mRNA in unpurified granulosa cells was produced by macrophages, not by the granulosa cells alone. LH, FSH and PGE_2 did not significantly stimulate RANTES mRNA in the macrophages.

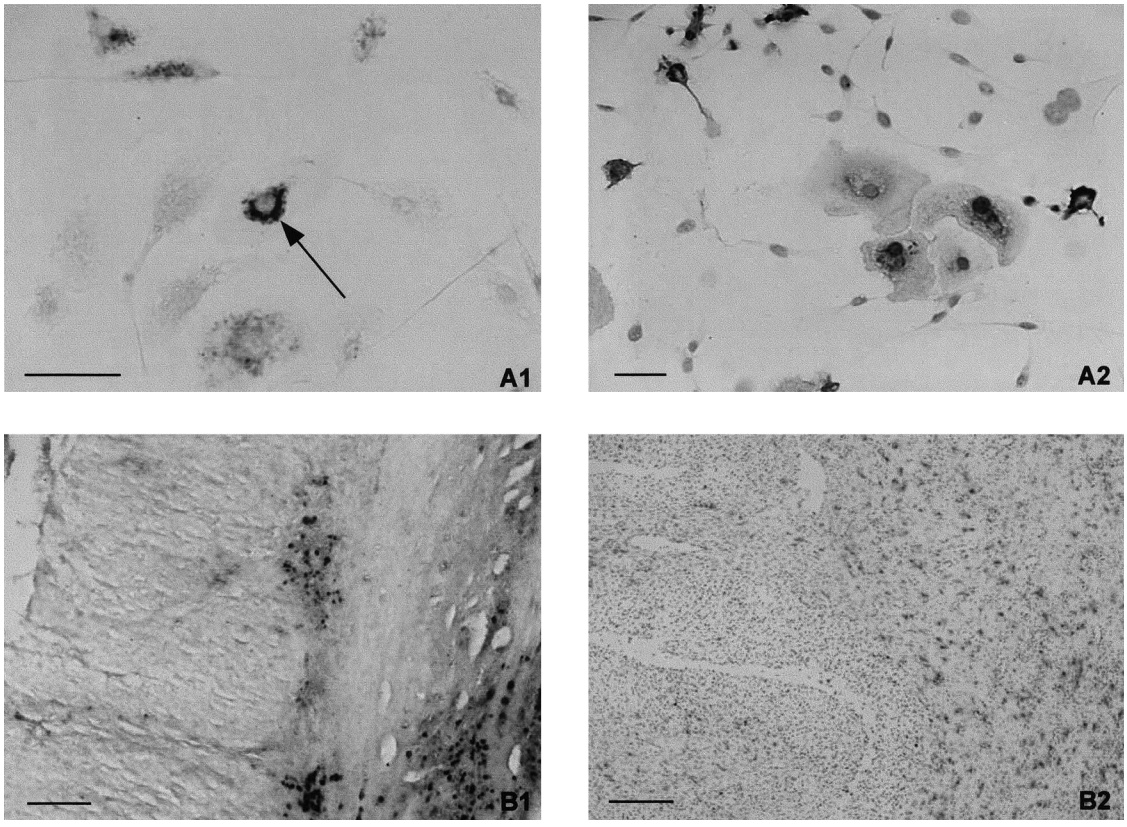

Fig. 3. In situ hybridization of RANTES mRNA in co-cultures of follicle-derived macrophages and granulosa cells (A: A1×320, A2×160; scale bar 50 μm) and a corpus luteum in early development (B: ×40, scale bar 250 μm). The macrophages strongly express RANTES mRNA (→), whereas the granulosa cells lack a signal (A1,B1). Staining with an anti-CD14 monoclonal antibody (A2,B2).

3.3. In situ hybridization

In contrast to the cultured granulosa cells and fibroblasts, the macrophages strongly expressed RANTES mRNA (Fig. 3A). RANTES mRNA positive cells were located in the former thecal layer in the early stage of corpus luteum development (Fig. 3B). The positive cells often accumulated in the infoldings between the heavily gyrated former granulosa layer, the same area where eosinophils are located. Furthermore, RANTES mRNA positive cells were present in the connective tissue capsule of the developing luteal body and adjacent to blood vessels. Large regressing follicles contained few RANTES positive cells. This explains the positive RANTES mRNA RT-PCR found in various regions of the ovary.

4. Discussion

Although Karström-Encrantz et al. [24] recently found RANTES in human follicular fluid in concentrations between 50–400 pg/ml, they argued that the chemokine does not play a role in leukocyte attraction into the ovary, because the serum levels are more than 50 times higher. It is important to emphasize that neither plasma nor serum measurements reflect the actual circulating levels of RANTES, because they largely result from an ex vivo release of the chemokine from the α-granules of platelets during the processing of blood samples [25]. What is more, in humans the dominating neutrophils [26] are attracted by CXC- and not CC-chemokines [24,27].

Cloning and sequencing of RANTES mRNA were essential in order to verify its expression in the bovine ovary around the periovulatory period. RT-PCR demonstrated RANTES mRNA especially in the follicle wall and in the corpus luteum. For the detection of the signal source, we examined unpurified granulosa cells from the follicle harvest and fibroblasts derived from the follicle wall. Following the appearance of a hormone-dependent leukocyte influx during the periovulatory period, we investigated the effect of FSH and LH on the expression of RANTES mRNA. Both hormones increased RANTES mRNA in unpurified granulosa cells, but not in fibroblasts, which are known to lack the hormone receptors. PMA, used as a positive control, leads to a prolonged stimulation of RANTES secretion in unpurified granulosa cells and fibroblasts. In contrast, PGE_2 found to be upregulated in the periovulatory period and acting on granulosa cells in an autocrine fashion, did not stimulate RANTES mRNA.

Surprisingly, LPS treatment caused a significant RANTES mRNA increase in unpurified granulosa cells, which do not express LPS receptors [28]. We assume that cells other than granulosa cells also take part in RANTES mRNA expression. Indeed, pure granulosa cells pretreated with an anti-CD18 monoclonal antibody in order to remove the leukocytes did not react. Among the leukocytes known to contribute to RANTES mRNA expression, macrophages especially are potential candidates. Since they are few in number in the aspirated follicle cells, we obtained macrophages from follicle cell cultures. These cells must have been true macrophages because they expressed (1) the leukocyte common antigen CD45 and CD18, molecules which have been detected on leukocytes only, (2) CD14, an antigen exclusively expressed

on the surface of monocytes and most tissue macrophages and (3) MHC class II, which is typical for monocytes and macrophages under physiological conditions [29].

The ovarian macrophages showed a 100–1000 higher basal RANTES mRNA expression than pure granulosa cells and fibroblasts. Corresponding to our data showing an LPS-induced increase of RANTES mRNA in the macrophages, Shin et al. [30] recently described two LPS-responsive elements in the 5′ flanking region of murine RANTES mRNA from stimulated macrophages. Although LH-receptor positive macrophages have been located adjacent to the oocyte in large preantral follicles and among granulosa cells of medium-sized antral follicles [31], LH had no RANTES mRNA upregulating effect on macrophages in our study. The failure of a RANTES mRNA increase to appear after LH or FSH treatment indicates that interactions between leukocytes and granulosa cells mediate the FSH and LH stimulating effect in unpurified granulosa cells.

Our in vitro experiments were supported by in situ hybridization of the corpus luteum at the early stage of development. Comparing the localization and morphology of RANTES $mRNA^+$ and $CD14^+$ cells in successive sections revealed that macrophages are indeed RANTES mRNA producers. However, a few RANTES mRNA positive cells were also found in the thecal layer of intact or regressing antral follicles and adjacent to blood vessels. The results may indicate that (1) RANTES is also involved in further ovarian events and/or (2) that it is not the only chemokine responsible for attracting leukocytes into the ovary during the periovulatory period. Arici et al. [32] showed that in humans the monocyte/macrophage migration towards and into the dominant follicle may occur through the modulation of monocyte chemoattractant protein-1 (MCP-1). This finding reflects a complex network involving the selective and coordinated production of individual chemokines, and the expression of chemokine receptors to attract selective leukocyte subpopulations into the ovary.

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