PERI-OVULATORY TISSUE REORGANIZATION IN THE HUMAN OVARY

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2011
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Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1222

ISBN 978-82-8264-178-4

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Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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The study would never have succeeded had it not been for the tremendous support I have received from my colleagues, from research funds and from my family. I am truly grateful for their participation and support for this project.

First and foremost I want to thank my supervisor, Peter Fedorcsák PhD, M.D, whose expertise, understanding, and patience, added considerably to my postgraduate experience. I appreciate his vast knowledge and skills in many areas, and his assistance in writing grant proposals, scholarship applications and this thesis. Peter has the ability to grasp all aspects of a problem, keep an overview, and yet still focus on how to solve each of the specific problems. I am indebted to Peter for profound help with performing the experiments, without him many of them would be baffling or impossible to make. I am greatly thankful for sharing his guidance and willingness to provide the right amount of inspiration and motivation when needed. It has been an honor to be his first Ph.D. student.

I owe a dept of gratitude to Professor Tom G. Tanbo, who truly made a difference in my life. He provided me with direction, technical support. It was though his, persistence, understanding and kindness that I completed my postgraduate training. I would like to thank Prof. Tanbo for giving me the opportunity to work with the fascinating topic of human reproduction.

I am also indebted to Professor Thomas Åbyholm for providing facilities, and for his leadership and advice. Very special thanks for his suggestions, and help with revision, motivation and encouragement.

I owe my sincere thanks to the flourishing research atmosphere at the Department of Immunology and Melinda Ráki PhD, M.D for being helpful in constructive discussions, and in practical matters of all kinds. I would not have complete many of the experiments without her great expertise and knowledge.

I appreciate all the help that I received from the nurses and bioengineers at the Section for Reproductive Medicine, Department of Gynecology.

I am greatly thankful for the assistance and advice provided by Tore Henriksen, Guttorm Haraldsen, and John Ødegård.

In conclusion, I recognize that this research would not have been possible without the financial assistance of the National Research Council and the National Resource Center for Women’s Health.
Finally, I am greatly thankful to my family and friends. They have provided eminent support during the study. They have kept me going with their loving support and positive attitude and make me believe everything is possible. They have inspired me in many parts of the project phases and I have enjoyed their ultimate confidence in my capabilities.
List of papers

**Paper 1**

**Paper 2**

**Paper 3**
Abbreviations

Ang-1   angiopoietin-1
Ang-2   angiopoietin-2
α2-AP  α2-antiplasmin
BFA    brefeldin A
CFDA   carboxyfluorescein diacetate
CL     corpus luteum
EDTA   ethylene-diamine tetraacetate
EGF    epidermal growth factor
ECM    extracellular matrix
FGF    fibroblast growth factor
FITC   fluorescein isothiocyanate
FL     follistatin
fMLP   formyl methionyl-leucine-phenylalanine
FSH    follicle-stimulating hormone
FSC    forward scatter
G-CSF  granulocyte colony-stimulating factor
GL     granulosa-lutein cells
GnRH   gonadotropin-releasing hormone
GPCR   G-protein coupled receptor
hCG    human chorionic gonadotropin
HGF    hepatocyte growth factor
HUVEC  human umbilical vein endothelial cells
ICAM-1 inter-cellular adhesion molecule-1
Ig     immunoglobulin
IL     interleukin
IP3    Inositol trisphosphate
IRAK   Interleukin-1 receptor-associated kinase
JAK    Janus kinase
LEP    leptin
LH     luteinizing hormone
MAPK   mitogen-activated protein kinase
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<td>MEK</td>
<td>MAP kinase kinase</td>
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<tr>
<td>Mo</td>
<td>monocytes</td>
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<td>MMP</td>
<td>matrix metalloproteinases</td>
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<td>NFkB</td>
<td>nuclear factor-κB</td>
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<td>OHSS</td>
<td>ovarian hyperstimulation syndrome</td>
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<tr>
<td>PA</td>
<td>plasminogen activator</td>
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<tr>
<td>PAI-1</td>
<td>PA inhibitor-1</td>
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<tr>
<td>PAI-2</td>
<td>PA inhibitor-2</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood-derived mononuclear cells</td>
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<tr>
<td>PECAM-1</td>
<td>platelet endothelial cell adhesion molecule</td>
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<tr>
<td>PCOS</td>
<td>polycystic ovary syndrome</td>
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<td>PDGF-BB</td>
<td>platelet-derived growth factor-BB</td>
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<tr>
<td>PE</td>
<td>phycoerythrin</td>
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<tr>
<td>PGF2-α</td>
<td>prostaglandin F2-α</td>
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<tr>
<td>PLCγ</td>
<td>phospholipase C gamma</td>
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<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
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<td>PN-1</td>
<td>protease nexin-1</td>
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<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription-3</td>
</tr>
<tr>
<td>THP-1</td>
<td>human acute monocytic leukemia cell line (unresolved acronym)</td>
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<tr>
<td>Tie-2</td>
<td>tyrosine kinase with Ig and EGF (epidermal growth factor) homology domains (angiopoietin receptor)</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
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<td>TIR</td>
<td>Toll-IL-1 receptor</td>
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<td>TKR</td>
<td>tyrosine kinase receptor</td>
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<td>TRAF</td>
<td>TIR domain-containing adaptor protein</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Introduction

Ovulation and corpus luteum formation

Throughout reproductive life, ovarian follicles undergo continuous changes. The ovary is already during fetal life populated by primordial follicles, where the oocyte is encircled by a single layer of flattened pre-granulosa cells. In response to a hitherto unknown signal, some of these follicles leave the resting pool and commence growth some months before final follicle maturation. During this development granulosa cells proliferate quickly. In secondary follicles multiple layers of cuboidal granulosa cells can be observed. Later, the follicle forms an antrum and the granulosa cells differentiate into mural granulosa cells and cumulus cells (1-4).

When antral follicles enter the follicular phase of the menstrual cycle, which comprises just a short final period of the follicles’ life-time, the granulosa cells further proliferate and differentiate and the oocytes mature for ovulation and eventual fertilization. In this phase the major secreted steroid is estradiol, which controls proliferation of the uterine endometrium, is necessary to support oocytes in the ovary, and initiates the midcycle surge of luteinizing hormone (LH). The LH surge initiates ovulation and is in turn responsible for corpus luteum (CL) formation.

The ovarian follicle thus undergoes many changes, which are required for successful ovulation and oocyte maturation. These events are regulated by local ovarian factors, including steroid hormones, and the pituitary gonadotropins FSH and LH (4;5). FSH and LH bind their cognate seven-transmembrane Gs protein-coupled receptors (GPCRs) and lead to the rapid phosphorylation and thereby increase intracellular cAMP and activate protein kinase A (PKA) (5;6).

In peri-ovulatory follicles, granulosa and theca cells go through an organized transformation. Activation of the LH receptors (LH-R) initiates a program of terminal differentiation of granulosa cells and theca cells into luteal cells. There are changes, structural and genomic, which lead cells to differentiate into non-dividing cells and allow them to express a new set of molecules, including regulatory proteins, transcription factors, and signaling proteins that will permit luteal cells to respond to a different hormonal environment, among the most important being prolactin and LH. During the luteal phase the granulosa-lutein cells undergo extensive hypertrophy that significantly contributes to growth of the CL, whereas theca cells do not undergo hypertrophy (7).
CL is a heterogeneous gland composed of various steroidogenic luteal cells, including the large and small steroidogenic luteal cells, and other cell types like fibroblasts, endothelial, and immune cells (8). A common view, based on histological evidence shows that the large luteal cells are formed from the granulosa cells, whereas the theca cells differentiate into small luteal cells (4;7-10).

Luteinization further proceeds after follicle rupture. It is a remarkable event involving cell proliferation, cell differentiation, and tissue remodelling and is essential for the success of early pregnancy (7;11). The dominant ovarian steroids produced in this phase are progesterone and estradiol, which control transformation of the proliferative endometrium into a secretory phase. This change is essential for implantation of the fertilized oocyte and initiation of pregnancy. Luteal phase ends with termination of progesterone and estradiol production by CL, and a process of CL regression, known as luteolysis. The decline in expression of the StAR gene and protein, and as well reduced progesterone production is associated with the process of luteal regression. The level of StAR, as well the plasma P and E2 levels can be restored to those found in the midluteal phase CL by hCG administration during the late luteal phase, or increase due to endogenous hCG from and early conception. Several molecules, like prostaglandin F2-α, TNF-α, IL-1β, and estrogens have been implicated in the luteolytic process (12). The regression of CL ensues menstruation and is required for initiation of a new ovarian cycle and follicular development.

The most important changes during these tissue alterations are break down of the follicular basal lamina, neovascularization of the granulosa layer, and migration of various cell types, including immune cells, endothelial cells, fibroblast, and theca cells into the avascular granulosa layer. Subsequent to the ovum ejection, the mural granulosa layer is moving into folds round the follicular antrum, whereas the theca cells are included into the developing CL by invasion of connective tissue (7;8;11). A simplified model of corpus luteum formation is presented in Figure 1.

Wound healing as model of peri-ovulatory events
Ovulation and CL formation share many features with inflammatory reactions, including involvement of chemokines and cytokines, leukocyte migration, angiogenesis, proteolysis, tissue re-organization; indeed, many of these processes are similar to the inflammatory reactions during wound healing (6;13-15).
Healing of skin wounds

Wound healing follows a course that typically starts with blood clotting, activation of thrombocytes, and migration of inflammatory cells into the matrix of the wounded region. In the next phase, vasoactive agents released from activated platelets cause vasodilation and increase capillary permeability. Stimulation of the clotting cascade results in the formation of a fibrin plug, which holds damaged tissues together and provides a provisional matrix for the recruitment of inflammatory cells and later the migration of fibroblasts and other resident cells. Factors derived from blood or released by injured or dead cells induce adhesion of inflammatory cells to the walls of blood vessels and passage of cells between endothelial cells lining these vessels (16-18).

Cell migration during healing of wounds is a fundamental process and occurs in the context of complex changes in the wound environment. In particular, a set of certain cytokines, like IL-1β, IL-8, IL-6, and proteolytic enzymes are highly expressed. These biologically active substances contribute to the spatially and temporally different infiltration of cell subsets and integrate the inflammatory and reparative processes during wound repair (6;18).

Cell migration and its regulation by chemokines and chemokine signaling

The stimulus for directed cell movement during wound healing can be a soluble attractant, a substratum-bound gradient of a particular matrix constituent, or the three-dimensional array of ECM within the tissue. The directed migration of cells in response to a gradient of an attractant is a complex process, which involves modulation of cell-cell adhesion and regulation by several signal transduction pathways, among them mitogen activated protein (MAP) kinase signaling, JAK/STAT signaling, and ligand-regulated Ca²⁺ channels. These intracellular signaling pathways then direct calcium fluxes, activation of phosphoinositide turnover and protein phosphorylation ultimately affecting cell motility (18-20).

Understanding of the precise relationships of signaling pathways directing cell movement during wound healing, in the context of regulation of cell adhesion and signaling function, could also be crucial for understanding the process of corpus luteum formation. Indeed, the same kinds of movement as in wound healing are observed in corpus luteum formation. During inflammation and wound healing, macrophages, local mast cells and endothelial cells release mediators that initiate an inflammatory process, increase blood supply to the affected area, allow migration of white blood cells, mainly neutrophils, followed by a large number of macrophages (19). Attraction of cells to the inflammation site is
controlled by chemoattractants secreted by resident cells, the secretion of which increases during inflammatory response. Chemokine binding to receptors present on the cell surface can increase their adhesion. Chemotaxis occurs along a concentration gradient of chemokines, which act locally and are rapidly induced in response to inflammatory stimuli (17;21).

Directed cell migration is a fundamental element of immune surveillance both in physiological conditions and disease (22). Recruitment of immune cells is also thought to play an important role in luteal function (23). Chemokines are released by endothelial cells, stromal cells, and leukocytes, whereas chemokine receptors are located on the surface of leukocytes; a particular chemokine release pattern and receptor repertoire allows a precise leukocyte targeting during immune response (24;25).

Chemokines secreted by the granulosa-lutein cells bind to receptors on specific leukocyte subsets and increase leukocyte adhesion. One of the well-characterized chemokines that is important during CL formation is interleukin-8 (IL-8), which attracts leukocytes, predominantly neutrophils, to the inflammation site (26-29). IL-8 is a small protein, which is a member of CXC chemokine, also called α chemokines, family. IL-8 binds to seven transmembrane domain G protein-coupled receptors, CXCR1 and CXCR2 (24;27;30;31). The major sources of IL-8 in the corpus luteum are the granulosa-lutein cells, and its release is regulated by hCG, IL-1β, and TNF-α (23;28;32-35).

A second mechanism of promoting directional single-cell migration is haptotaxis, migration along an adhesion gradient, which is particularly relevant during angiogenesis. In contrast to the effect of soluble chemoattractants, mediators of haptotaxis are absorbed to the ECM. Cells direct their migration towards haptotaxis gradient by extending lamellipodia more or less randomly. Each lamellipodium competes for a finite amount of membrane, such that when one lamellipodium facing the attractant gradient adheres, it spreads, and becomes dominant (18). Around the time of ovulation endothelial cells sprout and extend from the theca layer capillaries, then pass through the basal lamina into the granulosa layer. Haptotaxis, which is associated with increased endothelial cell migration in response to integrin binding to the ECM component, may promote lamellipodial extension to granulosa-lutein cells and formation of new ovarian vessels.

**Effects of peri-ovulatory leukocyte infiltration**
The fundamental effect of cell migration during wound healing is the infiltration of damaged tissue by leukocytes. Accumulating leukocytes initiate specific immune responses and trigger production of a battery of growth factors such as platelet-derived growth factor (PDGF),
transforming growth factor β (TGF-β), and basic fibroblast growth factor (bFGF). These factors stimulate the synthesis of extracellular matrix components produced by fibroblasts and the in-growth of blood vessels from the surrounding tissue, which later results in tissue formation and remodeling in the wound area (18).

As main mediators of ovarian inflammatory responses, infiltrating leukocytes have become a major subject of investigation (26;36). Shortly before ovulation, the density of ovarian neutrophils and macrophages increases, and after follicular rupture a large number of neutrophils are observed close to the rupture point (37-39). A rapid infiltration of the luteinizing follicle and progressive invasion of the granulosa-lutein layer by leukocytes is probably necessary to achieve tissue transformation in the early corpus luteum (40). The process of leukocyte accumulation is selective and predominantly macrophages and neutrophils are present in the theca layer of the follicle after the LH surge. Their presence increases ovulation rate and supports tissue reorganization during corpus luteum formation. Activated macrophages produce an array of biologically active substances, including tissue remodeling enzymes and cytokines (1). Ovarian leukocytes also exert a paracrine regulation of corpus luteum, in particular, luteal leukocytes inhibit steroidogenesis (41).

**Angiogenesis**

Angiogenesis is a critical process during wound healing, as it allows improved blood flow and extended access to oxygenized blood (19;21;42). Angiogenesis is also a complex process that involves migration of endothelial cells, proliferation and differentiation of endothelial cells into capillary tubes (18;19;32). The vascular network develops in an interaction with the extracellular matrix. Structural ECM proteins take part in vessel sprouting during angiogenesis by interactions with integrin receptors on endothelial cells, whereas proteolytic enzymes degrade the ECM to permit remodeling and ingrowth of vessels. Exposure of endothelial cells to a certain angiogenic factor can also cause destabilization, leading to a decrease in endothelial cell adhesion and an increase in vascular permeability (43).

Ovarian angiogenesis is regulated by angiogenic factors expressed by both granulosa-lutein cells and ovarian leukocytes. Granulosa cells are a major source of IL-8, which is a cytokine inducing directed cell migration, the angiogenic regulator VEGF, and a multifunctional cytokine TIMP-1. In addition, IL-8 may modulate endothelial cell functions by transactivation of VEGFR2 (43). VEGF can be induced by platelet-derived growth factor (PDGF), TGF-β and TGF-α and IL-1β (19).
Vascular endothelial growth factor (VEGF) contributes to wound healing by inducing proliferation and migration of endothelial cells into surrounding connective tissue, and promoting formation of a cord of endothelial cells, which subsequently develop a lumen. Sprouts from adjacent arterioles and venules fuse to form a network of capillaries (43-45).

Regardless of the site of new vessels formation, new capillaries need to go through maturation and stabilization. Angiopoietin-2 (Ang-2) is involved in maturation of blood vessels by acting as a survival factor for endothelial cells. Furthermore, Ang-2 is involved in stabilization of newly formed capillaries by the recruitment of pericytes and smooth muscles cells (19;44-48). There is a correlation between levels of VEGF and Ang-2, which directs either new blood vessel formation or their stabilization. In particular, in the presence of high VEGF concentrations, high levels of Ang-2 lead to new blood vessel formation, whereas a high Ang-2 in the presence of low VEGF activates vessel stabilization (46;47).

Several studies substantiate the importance of ovarian angiogenesis (21;49). Indeed, new blood vessels are constantly being formed in the ovary to supply the metabolically highly active follicles and corpus luteum. Abundant blood supply is essential for normal ovarian function. Interference with vessel formation in animals inhibits follicle growth (50;51) and a hypoperfused ovarian microenvironment was shown to correlate with oocyte defects (52). The pathophysiological mechanism for these defects may be a reduced vascularity of the theca layer secondary to a reduced synthesis of angiogenic factors by granulosa-lutein cells (49;53). Furthermore, disturbed angiogenesis may also be associated with other reproductive disorders, like dysfunctional uterine bleeding, endometriosis, ovarian hyperstimulation syndrome, polycystic ovary syndrome, and failure of embryo implantation (49;53;54).
Figure 1. From preovulatory follicle to mature corpus luteum. Corpus luteum formation is characterized by a rapid differentiation of granulosa and theca cells into luteal cells and invasion of the follicle by capillaries and immune cells. Towards the left, the wall of the preovulatory follicle is shown with a non-vascularized layer of granulosa cells and the theca interna, which is rich in capillaries. Corpus luteum formation is initiated by the mid-cycle LH surge, which activates LH-receptors and rapidly induces luteinization of granulosa and theca cells and promotes tissue transformation, including leukocyte infiltration and extensive neovascularization of the corpus luteum. Indeed, just before ovulation, the level of neutrophils and macrophages increases and after follicular rupture large numbers of leukocytes are observed close to the rupture point. Leukocytes are thought to migrate to the corpus luteum in response to chemokines released by granulosa-lutein cells. Towards the right, the newly formed CL is shown, where cells mix together and the tissue is perfused by capillaries.

The process of corpus luteum formation is further visualized on the cartoon below. The granulosa layer of the pre-ovulatory follicle is not vascularized, and capillaries are separated from the granulosa-lutein cells by a basement membrane (yellow). After ovulation leukocytes (blue) migrate through the capillary wall and release proteolytic enzymes that disrupt the basement membrane. Direct cell-cell contact with granulosa-lutein cells may activate leukocytes, and both release intracellular chemokines, which further activates leukocyte migration. This process eventually results in re-organization of tissue architecture, including ingrowth of connective tissue and capillaries.
Proteolysis

Proteolysis of matrix components is necessary during cell movement and therefore a crucial factor in tissue repair and CL formation (55-57). Many studies have implicated the plasminogen activator (PA) and MMP system as being important factors during ovarian tissue remodeling (56;58). The main component of the PA system is the zymogen plasminogen, which can be activated by enzymatic cleavage. Two types of plasminogen activators have been characterized, tissue-type PA (tPA), among others present in the ovary and uterus, and urokinase-type PA (uPA), which is present in ECM and on the surface of tumor cells (58;59). Ovarian surface epithelial cells secrete uPA towards the follicular wall in response to LH (60). When it becomes activated, plasminogen is converted into the protease plasmin. Plasmin degrades fibrin and other components of the ECM. Plasmin also activates some members of the MMP system. To prevent excessive tissue degradation, the activity of the PA system is regulated by PA inhibitor-1 (PAI-1), PA inhibitor-2 (PAI-2), α2-antiplasmin (α2-AP), and protease nexin-1 (PN-1) (60-62). Plasmin is known to play a crucial role in tissue remodeling processes, including angiogenesis (59;60;62;63).

Matrix metalloproteinases (MMPs), which also contribute to the process of wound healing, comprise four classes of proteases: collagenases, gelatinases, stromelysins, and membrane-type metalloproteinases (MT-MMPs). These enzymes are produced by granulocytes, macrophages, epidermal cells, and fibroblasts, and are capable of degrading certain components of scar tissue and can be regulated by different factors; for example, VEGF was shown to increase secretion of collagenase by endothelial cells (6;19;64). MMP action is also controlled by specific tissue inhibitors of metalloproteinase (TIMPs), which can rapidly inhibit activated collagenase and other matrix metalloproteinases (6;65).

During angiogenesis, VEGF stimulates expression of MMP-1, MMP-2 and MMP-9 by endothelial cells to promote degradation of collagen (32). In general, VEGF promotes endothelial movement in the extracellular space, and increased blood flow with nutrients to the wound (6;66). Migration starts as keratinocytes at the skin wound edge upregulate their production of MMPs, releasing the cells from the basal lamina. Keratinocytes resting on the basal lamina migrate across the wound site, attaching to fibronectin and vitronectin contained within the clot by upregulating their expression of integrins. While moving through the dense fibrin clot, keratinocytes dissolve the dense fibrin matrix. After migration is complete, the cells behind the wound edge undergo a proliferative burst to replace keratinocytes lost in the injury while also forming additional keratinocyte layers over the basal layer. It is believed that
epidermal growth factor (EGF), keratinocyte growth factor (KGF), and transforming growth factor (TGF-α) may drive cell proliferation and wound closure. Migration and proliferation continue until keratinocytes receive a stop signal. At this time, MMP expression is interrupted, and a new basement membrane is produced whereby new cell-matrix adhesions are established (65;67). Romer et al. demonstrated the importance of plasmin in wound healing by influencing migration of keratinocytes. By using mice with targeted disruption of the plasminogen *plg*”/ gene, they have shown that wound healing may be delayed by lack of plasminogen, and the plasminogen function can eventually be replaced by other factors (68).

The end result of proteolysis, cell migration and new blood vessels formation in the repair process is vitally important to wound healing because it establishes the scaffolding necessary to support and rebuild the damaged tissue.

**Unresolved issues**

Because of their abundant expression, inflammatory chemokines, angiogenic factors, and their receptors have been implicated to direct leukocyte infiltration and angiogenesis during corpus luteum formation (69;70). The ovarian source of chemoattractants and angiogenic factors, because of the variety of cell types that mix during luteogenesis, has been nonetheless uncertain. It has been previously observed that secretion of IL-8 by follicular-fluid derived GL cells was related to the presence of leukocytes among these cells (69). These observations led us to examine the cellular source of inflammatory cytokines and angiogenic factors by co-cultured GL cells and leukocytes, and the impact of co-culturing on release of these factors. As a cellular model of luteinization, we chose to study GL cells that are aspirated from the preovulatory follicle and activated monocytes.
**Aims of the study**
The principal aim of this study was to understand how granulosa-lutein cells and leukocytes interact and how this interaction bolsters recruitment of leukocytes in the early CL. We wished to examine the role of leukocytes during corpus luteum formation, the mechanisms of leukocyte recruitment, and interaction among leukocytes and resident steroidogenic cells. We wished to test the hypothesis whether cell-cell contact between GL cells and leukocytes affects endothelial cell function and angiogenesis *in vitro*. For this purpose we used a model system of follicular fluid-derived granulosa-lutein cells and homologous leukocytes, and also examined the interaction of these cells with human umbilical vein endothelial cells, in order to:

1. determine whether peripheral neutrophils are recruited by ovarian granulosa-lutein cells;
2. examine whether granulosa-lutein cells modify the effect of matrix metalloproteinases released by ovarian leukocytes;
3. study whether ovarian granulosa-lutein cells induce directed migration of monocytes and whether these cells interact in regulating angiogenesis.
Methods

1. Cells
Follicular fluid and peripheral blood samples were collected from consenting women (n = 150) who underwent assisted reproduction treatment. Approval was granted by the Southern Regional Committee of Medical Ethics (license No. S-05058). Controlled ovarian hyperstimulation was initiated using a standard combination of GnRH agonist, human recombinant FSH, and human chorionic gonadotropin (hCG) (71). Granulosa-lutein cells were isolated from follicular fluid and purified using hemolysis, enzymatic dispersion, and gradient centrifugation. Peripheral blood-derived mononuclear cells were derived on the day of follicle aspiration from the same women donating follicular fluid. Monocytes were isolated with gradient centrifugation followed by treatment with CD14-conjugated paramagnetic beads. Human umbilical vein endothelial cells (HUVEC) were isolated by cannulating and flushing the umbilical vein with collagenase. HUVEC were cultured until passage 7, and passage 2 to 7 was used for experiments.

Granulosa-lutein cells were allowed to attach and spread in vitro overnight, washed for removing unattached cells, and were co-cultured according to experimental combinations described in Papers 1-3. Density of GL, Mo and THP-1 cells was kept similar within an experiment. Conditioned medium was saved until assays.

2. Detection of secreted cytokines
Multiplexed assays were used to measure concentration of secreted cytokines, chemokines and MMPs in the cell culture media. These assays allow simultaneous detection of multiple analytes and give a more complex insight into related physiological processes. Cells for these assays were cultured in serum-free medium. In some experiments, in order to assess whether direct cell contact can influence cytokine release, monocytes and granulosa-lutein cells were co-cultured with membrane separation of cell populations.

For cytokine bead array, conditioned medium was mixed with specific immunobeads and analyzed by flow cytometry. Absolute release levels were compared among experimental settings using non-parametric analysis of variance. Conditioned cell culture media were also analyzed to measure levels of angiogenic factors. The test was performed using the Bio-Plex Pro Human Angiogenesis Assay (Paper 3). Release of MMPs and TIMPs were assessed by antibody array. In this test, capture antibodies are arrayed onto a
membrane, and in combination with a biotinylated antibody cocktail, the array allows simultaneous detection and semiquantitative analysis of multiple MMPs and TIMPs (Paper 2).

3. Detection of intracellular cytokines, cell surface receptors and signaling
Since the detection of secreted cytokines could be criticized for not showing which cell is a source of released cytokines, we needed more direct measurement of cellular response and therefore flow cytometry was used to measure intracellular cytokines, cell surface receptors and distal signalling.

For estimation of intracellular IL-6 and IL-8 content, expression of CXCR1 and CXCR2, and downstream activation of p38 MAPK and p44/42 MAPK cells were incubated in various experimental combinations (Paper 1-3). Cells were fixed, permeabilized, and exposed to primary and secondary antibodies. Multi-color flow cytometry analysis was performed with a FACScan instrument.

In addition we also wanted to assess ion channel-dependent calcium fluxes, and for this purpose monocytes were isolated with CD14+ beads and were loaded with fluo3-AM and flura-red-AM (72). Cells were analyzed with FACS for changes of intracellular Ca$^{2+}$ in response to various stimuli (Paper 3).

For visualization of intracellular accumulation of IL-8, MMP-9, TIMP-1, and p38 MAPK translocation, cells were cultured on sterilized coverslips in the presence of appropriate stimulants, fixed, neutralized, and permeabilized. Cells were then washed and blocked for unspecific antibody binding sites, followed by exposure to primary antibodies and appropriate secondary antibodies. Images were taken with an epifluorescent microscope (Zeiss, Jena, Germany), using constant exposure settings in an experiment.

4. Detection of cell migration
To assess the migratory effect of IL-8, VEGF and granulosa-lutein conditioned medium, cell migration assays were performed. In Paper 1, neutrophil chemotaxis was assessed in a 96-well microchemotaxis chamber. In this instrument, test solutions in the bottom chamber are separated from labeled leukocytes in the upper chamber by a 5-µm pore size filter. Directed monocyte migration was assessed according to Zigmond et al. (73). Briefly, three 3-mm wells were cut in a straight line in an agarose gel. Peripheral blood mononuclear cells were placed in the middle well, while the side wells were filled with different experimental combinations of standard chemoattractants or conditioned media. Cell migration was quantified by measuring the distance of the leading edge from the edge of the well on computer screen (Paper 3).
For measurement of HUVEC migration activated by angiogenic factors and granulosa-lutein conditioned media, cell migration assay was performed using Boyden chamber (5 µm pore size). HUVEC were added to the upper chamber and angiogenic factors and granulosa-lutein conditioned media were added to both lower and upper chamber in various combinations, as described in Paper3.

5. **Proteolysis**

An essential requirement for cell movement during corpus luteum formation is proteolysis of matrix components. In order to assess proteolysis, gelatin zymography, reverse zymography, and pericellular gelatinolysis assays were performed.

Concentrated conditioned cell culture media were resolved with SDS-PAGE electrophoresis under denaturing non-reducing conditions using gels co-polymerized with gelatin. After renaturation, gels were developed and stained with 0.25% Coomassie Brilliant Blue (CBB), followed by an extensive destaining until digestion bands have become visible. Dried gels were scanned and analyzed in ImageJ. To characterize gel-resolved enzymes and confirm their identities as MMPs, several experiments were performed (described in details in Paper 2).

TIMPs can be detected by reverse gelatin zymography, where both the gelatin substrate and the MMP enzyme are co-polymerized in the same gel. In reverse zymograms, TIMP activity appears as protein-dense bands because the digestion of copolymerized gelatin by MMPs was inhibited.

Pericellular digestion of gelatin by cell-associated MMPs was examined using coverslips coated with biotin-conjugated gelatin. To distinguish the cultured cells, GL cells and THP-1 cells were labeled with CFDA (green fluorescence) or Paul Karl Horan (PKH)-26 (red fluorescence), respectively. After incubation overnight, the wells were rinsed, fixed and blocked. Alexa-350-conjugated streptavidin (blue fluorescence) was subsequently added. The slides were viewed in epifluorescence microscope to estimate pericellular gelatinolysis.

6. **In vitro angiogenesis assay**

During this project cellular assays that model of angiogenesis were adapted, among them tube formation assay and HUVEC proliferation assay. The tube formation assay was performed by plating HUVEC on growth factor-reduced basal membrane extract Matrigel and exposure to various dilutions of cell-conditioned medium, VEGF and VEGF in addition of VEGFR2 inhibitor. Tube formation was monitored by direct microscopy, and the number of complete and broken tubes was counted (Paper 3).
To assess HUVEC proliferation in response to known angiogenic factors, HUVEC were cultured with different concentrations of FGF, EGF, and VEGF, and proliferation was measured by radiolabelled thymidine incorporation (Paper 3).
Summary of papers

**Paper 1**
Experiments described in this paper indicate that human granulosa-lutein cells synthesize the neutrophil chemoattractant IL-8, accumulate IL-8 in the ER-Golgi and secretory vesicles, but do not secrete IL-8 when cultured alone. When GL cells are co-cultured together with monocyte-like THP-1 cells, synthesis and secretion of IL-8 was significantly increased. Determination of secreted cytokine concentration in the cultured medium showed a significant increase in IL-8 secretion in the GL-THP-1 cells co-culture system, compared to IL-8 secretion by either GL cell or THP-1 cells alone. An increased secretion of IL-8 by co-cultured GL and THP-1 cells suggests that GL cells and monocytes/macrophages mutually induce chemokine secretion, presumably by inducing degranulation of cellular storage vesicles.

**Paper 2**
Here we examined the cellular source of MMPs and TIMPs in cells derived from the preovulatory ovarian follicle. We have found that ovarian leukocytes secrete matrix metalloproteinases (MMP), whereas granulosa-lutein cells release TIMPs that may so control proteolytic activity.

**Paper 3**
In this paper we reported that ovarian granulosa-lutein cells release IL-8 that attracts monocytes, and the presence of monocytes afflicts release of cytokines and angiogenic factors in vitro, including secretion of IL-1β, IL-6, TNF-α and VEGF. We described that ovarian granulosa-lutein cells also release angiogenic factors, predominantly follistatin, VEGF and IL-8. GL cell-conditioned medium induces activation of p38 MAPK in HUVEC and promotes angiogenesis *in vitro*. 
Discussion

The granulosa layer of the pre-ovulatoy follicle is avascular and capillaries are separated from the granulosa lutein cells by a basement membrane. After the LH surge, and preceding follicular rupture, a large number of neutrophils can be observed next to the rupture region (74), and leukocyte products are thought to contribute to dissolution of basement membrane and subsequent cell migration. A variety of leukocyte subsets which include eosinophils, neutrophils, monocytes/macrophages, and lymphocytes have been described in the ovary (1;37). In the early corpus luteum, neutrophiles and macrophages are present in the theca layer, and further development of corpus luteum converges with the appearance of large numbers of leukocytes, which would eventually represent 20-52% of luteal cells (37). Ovulation and corpus luteum formation resemble therefore inflammatory reactions, and, in particular, may have features similar to wound healing.

While the mechanisms of interaction between GL cells and leukocytes are not fully elucidated, GL cells are known to secrete chemokines, such as IL-8, which may regulate invasion of leukocytes and angiogenesis during CL formation (23;29). Presumably, signaling to initiate leukocyte homing is mediated primarily by chemokines produced by GL cells. Indeed, several studies demonstrate that leukocytes exit circulation in response to undefined signals that are hormonally driven (11;37).

In order to further explore the cellular interaction in the peri-ovulatory ovary and its effect on secretion of inflammatory cytokines and angiogenic factors, we studied a co-culture model of leukocytes and GL cells that are aspirated from the preovulatory follicle.

Cell-cell communication during corpus luteum formation

Luteinization of GL cells implies increased release of progesterone, as wells as inflammatory cytokines and chemokines. In Paper 1 we found that ovarian granulosa-lutein cells synthesize and secrete the leukocyte chemoattractant IL-8 causing increased migration of neutrofiles in vitro, which has been also observed by other authors (27;28;75). In addition, in Paper 3, we report that GL cell-derived IL-8 induces migration of monocytes. These findings suggest that multiple leukocytes may be targeted to, and ultimately come into contact with, peri-ovulatory GL cells. Co-localization of leukocytes and GL cells may allow mutual cell activation, involving among other processes calcium influx in leukocytes and activation of cytokine secretion.
**Figure 2. A model of cell-cell communication during corpus luteum formation.** Granulosa-lutein (GL) cells secrete a range of inflammatory mediators, including IL-1β, IL-6, IL-8, which activate cognate receptors on monocytes. Inflammatory cytokines induce downstream p38 mitogen activated protein (MAP) kinase and JAK/STAT signaling, and result in an increased intracellular Ca²⁺ concentration. Activated monocytes migrate towards IL-8 secreted by GL cells, and themselves release inflammatory mediators, such as MMPs, IL-1β, IL-6, IL-8. A co-activation of GL cells and monocytes further stimulates synthesis and secretion of inflammatory cytokines and angiogenic factors, including VEGF. Inflammatory cytokines and angiogenic factors secreted by GL cells and monocytes increase adhesiveness and haptotaxis of endothelial cells causing directed cell migration. For example, IL-8 activates both cognate receptors CXCR1 and CXCR2 on endothelial cells, which use MAPK signal transduction cascades. Activation of p44/42 MAPK takes part in cell proliferation and activation of p38 MAPK takes part in cell migration by endothelial cells. Furthermore, VEGF secreted by GL cells and monocytes increases endothelial cell secretion of matrix metalloproteinases, stimulates endothelial cell movement in the extracellular space, and angiogenesis (64;66). VEGF stimulates an expression of MMP-1, MMP-2 and MMP-9 in endothelial cells to promote degradation of collagen (32;65;76). GL cells release tissue inhibitors of metalloproteinase (TIMPs), which can rapidly inhibit activated matrix metalloproteinases, released by leukocytes.
A key observation of Papers 1 and 3 is that GL cells and monocytes appear to co-activate the other mutually, resulting in enhanced cytokine release. Although the nature of this co-activation is uncertain, novel insights into signaling pathways suggests that activation of one cytokine receptor can result in many complex patterns in the cell and different signaling pathways can interact to provide more complex regulation of cell processes. Cross-talk between receptors can result in desensitization or enhancement of function (4;27;77).

Inflammatory cytokines secreted by activated leukocytes may activate various responses in granulosa-lutein cells and endothelial cells, which may induce increased synthesis and secretion of inflammatory and angiogenic factors. Interactions between leukocytes and luteal cells and endothelial cells result in functional changes in these cells, suggesting that cross-talk between cells is an important component in overall regulation of tissue function during CL formation and regression (4;78). A simplified model of these putative interactions is presented in Figure 2.

Papers 1 and 3 support the theory that IL-8 is a ubiquitous cytokine with diverse effects in ovarian physiology. Initially synthesized and released by GL cells, IL-8 activates monocytes, so that both cell types proceed to release increased levels of IL-8, which further stimulates cells to produce range of different factors, like IL-1β, IL-6, TNF-α and VEGF. These cytokines and growth factors may modulate cell-cell interactions during corpus luteum formation (28;34;79;80). Ujioka and colleagues injected mature female rabbits with anti-IL-1β antiserum, anti-IL-8 antiserum, or anti-IL-1 receptor antagonist antiserum before an ovulatory dose of hCG. Rabbits injected with anti-IL8 and/or anti-IL1β exhibited a reduced hCG-induced ovulation rate. Ujioka and colleagues also observed that neutralization of IL-1β reduces ovulation rate without affecting functions considered essential for ovulation, like production of prostaglandins and collagenase activation. Whereas, IL-8 neutralization decreased ovulation rate in linkage with a reduced number of neutrophiles in the ovaries (34). Likewise, Brännström and Arici reported that TNF-α and IL-1β regulate release of IL-8 by ovarian stromal cells (28;80). Pellicer et al. shown that IL-6 can directly influence ovarian function and thereby can be important regulator of reproductive function (81).

Cytokines released by inflamed tissue play a crucial role in increasing expression of adhesion molecules on endothelial cells and also in up-regulation of corresponding receptor expression on leukocytes, eventually increasing migration of leukocytes to the target tissue. Leukocytes recruited to the inflammation site release chemokines and cytokines that further accelerate recruitment and function of leukocytes (26). Oakley et al. studied peri-ovulatory leukocyte infiltration, type of infiltrating leukocytes, and site of leukocyte infiltration in the rat
ovary. They used cycling adult and gonadotropin-stimulated immature rats, and collected ovaries at different stages of estrous cycle in the adult rats, and at different time points in superovulated immature rats. To affirm the origin of infiltrating leukocytes, Oakley and colleagues assessed leukocyte count in the spleen and the ovary. They observed a similar trend of peri-ovulatory rise of leukocyte numbers in cycling rats and in the superovulated immature rat model. In addition, Oakley and colleagues observed a strong inverse relationship between leukocyte numbers in the ovary and spleen, and significant reduction of leukocyte infiltration in the splenectomized rats. These results indicate that the spleen may serve as an immediate supplier of leukocytes to the peri-ovulatory ovary in rats (26).

Ahuja et al. described the multiple activation patterns of IL-8 receptor CXCR2. CXCR2 has two different binding forms, which have distinct coupling specificities in response to the same ligand. The low-affinity form of CXCR2 receptor can mediate elevation of intracellular Ca\(^{2+}\), while the high-affinity form of IL-8 receptor mediates neutrophil chemotaxis (31;82-86). Results presented by Wilson et al. show that rising intracellular calcium levels induce IL-8 gene expression, protein synthesis and secretion in monocytic cell lines. This finding may suggest that an increase in intracellular calcium concentration can be a mechanism by which the IL-8 gene can be activated (87). The rise in intracellular Ca\(^{2+}\) induced by agonists like fMLP or IL-8 depends on two mechanisms: the release of Ca\(^{2+}\) from intracellular stores and the influx across the plasma membrane (31;84;88). Binding of chemoattractants like IL-8 to their receptors stimulates direct cell migration or secretory functions. IL-8 has been shown to induce transient increases in cytosolic free calcium (27;89). Binding of IL-8 to specific receptor on human monocytes generates changes in intracellular calcium levels and stimulates the secretory functions of the cell, suggesting a link between the regulation of cell migration and cytokine production (90). Several studies suggest the importance of the cytoplasmic calcium level for leukocytes migration in chemoattractant gradients (91).

**Peri-ovulatory reorganization of the extracellular matrix**

In addition to regulating chemokine release and leukocyte migration, cell-cell interaction between GL cells and leukocytes may regulate tissue re-organization in the peri-ovulatory follicle. The proposed mechanism of follicle rupture is that cells in the follicular wall produce multiple proteases, including enzymes of the PA system as well as MMPs, which then act together and degrade ECM of the follicular wall (59;60;92). Bodén and colleagues studied CL formation in a gonadotropin-primed immature mouse model. In this study they followed the
development of CL and luteal angiogenesis by expression of the endothelial cell marker CD31. They have tested whether the MMP system and the PA system can cooperate or if they can replace each other during follicular development and ovulation, using plasminogen deficient mice treated with synthetic MMP inhibitor galardin (GM6001) (58). Bodén and colleagues found that plasminogen and MMP, both alone and in combination, are dispensable for ovulation and CL formation. Reduced serum progesterone levels, observed in study of Bodén et al., did not appear to be a result of defective CL formation (58;93).

In Paper 2 we report an intriguing pattern of synthesis and secretion of MMPs and TIMPs by cultured cells derived from the preovulatory follicle. MMPs may act as matrix degrading proteases during follicular development and later, just prior to ovulation, as activators of MMPs in the theca-interstitial cells surrounding the peri-ovulatory follicles (6;94). Follicular development is associated with a coordinated and cell-specific regulation of different MMPs, which are secreted as inactive proenzymes requiring activation, and the active enzymes are selectively inhibited by specific inhibitors present in the ECM. We described that the matrix metalloproteinases (MMPs) are strongly related to presence of infiltrating leukocytes and levels of TIMPs are related to granulosa-lutein cells.

Leukocytes may release MMPs to cleave ECM and activate cytokines to promote further leukocytes infiltration. MMPs are not constitutively expressed by most cell types, but several factors such as hormones, growth factors and cytokines can modulate their expression (1;6). Several growth factors induce expression of MMPs while other factors down-regulate MMP expression and up-regulate TIMPs (32;56;95). TIMPs regulate cell growth and apoptosis independently of inhibiting MMPs (19). Their temporal and spatial expression suggests that they might be involved in the degradation of the follicular wall at the time of ovulation and CL formation. MMP-2 and TIMP-1 were expressed during luteal development indicating that these proteases and inhibitors are associated with neovascularization and tissue remodeling during CL formation. We report in Paper 2 that TIMP secretion was inducible by hCG and phorbol ester. Balance between MMP and TIMP activity may play an important role during corpus luteum formation, and their preovulatory release is associated with leukocytes and luteinizing granulosa cells, respectively.

Kliem and colleagues examined whether expression of angiogenic factors correlated with protease expression during CL formation and luteolysis (59). For this purpose, they investigated how mRNA expression of different MMPs, their inhibitors and PAs is changed during bovine CL formation and induced luteolysis. They found that expression of proteases and angiogenic factors was induced by the LH surge at the beginning of CL development, and
that the expression of angiogenic factors decrease, but the expression of proteases increases during induced CL luteolysis (59).

**Angiogenesis during corpus luteum formation**

After ovulation, as the corpus luteum begins to form from the wall of the ruptured follicle, it grows and vascularizes extremely rapidly. Luteal angiogenesis is regulated by angiogenic factors released by both granulosa-lutein cells and ovarian leukocytes (21;96). To estimate the release pattern of angiogenic factors in the corpus luteum, we have evaluated angiogenic activity in our co-culture model of leukocytes and GL cells aspirated from the preovulatory follicle. In Paper 3 we examined whether interaction with infiltrating leukocytes would affect granulosa cell-regulated angiogenesis in vitro. As shown in Papers 1 and 3, ovarian GL cells synthesize and secrete the chemokine IL-8, activate IL-8 receptor-specific signaling in leukocytes. This induce a directed migration of monocytes, and an increased IL-8 release by co-cultured GL cells and monocytes suggested that initial monocyte recruitment further enhances synthesis and secretion of IL-8. The release pattern of several angiogenic factors was also found to be altered, and we observed an increased secretion of angiopoietin-2, follistatin, G-CSF, and HGF by co-cultured cells. Huang and colleagues studied inhibition of angiogenesis during tumor development by inhibiting effects of IL-8, using the anti-IL-8 antibody ABX-IL8 to neutralize the IL-8 secreted by melanoma cells. They grafted one group of nude mice with the human melanoma cell line A375SM, which secretes high levels of IL-8, and second group with TXM-13 cells, which release intermediate IL-8 levels. Mice were subsequently treated with ABX-IL8. Huang and colleagues found that IL-8 significant affects angiogenesis, and ABX-IL8 was to inhibit MMP-2 activity in vitro, decrease expression of MMP-2, and decrease vascularization of tumors in vivo. Moreover, they found that ABX-IL8 directly interfered with capillary tube formation by human umbilical vein endothelial cells, and the angiogenic effect of IL-8 was negated by ABX-IL8. ABX-IL8 only disrupted formation of new capillary tubes and did not affect existing vessel-like tubes in vitro (97).

Wulff and colleagues have described the localization and changes of expression of angiopoietins Ang-1 and Ang-2 and their Tie-2 receptor in the human CL (47;98). They observed a uniform, low level of Ang-1 expression in the CL, high levels of Ang-2 expression by granulosa-lutein, theca-lutein, and endothelial cells after CL rescue with hCG. Angiopoietin receptor Tie-2 was expressed by endothelial cells, whereas VEGF exclusively by granulosa-lutein cells. Based on these data, Wulff et al. claimed that VEGF and angiopoietins regulate angiogenesis and are responsible for stabilization of blood vessels
during CL rescue (47;99). These findings are corroborated by studies showing that hCG induces VEGF secretion by human granulosa-lutein cells (99;100). Based on these results, it is reasonable therefore to propose that VEGF and angiopoietins play a major role in human CL regulation (47;99).

Since GL cells were shown to promote specific endothelial cell functions \textit{in vitro}, including directed cell migration and capillary tube formation (Paper 3), altered release of angiogenic cytokines may imply that leukocytes modulate ovarian angiogenesis. In Paper 3 we describe that ovarian leukocytes also release angiogenic factors, so that angiogenic factors released by infiltrating leukocytes may participate in control of angiogenesis by influencing the global ovarian pattern of angiogenic factors. Confirming the role of these substances in vessel formation, we and others found that ovarian granulosa-lutein cells promote specific angiogenic behavior of endothelial cells in vitro, including capillary tube formation and directed cell migration (79). Results presented in Paper 3 indicate that IL-8 indirectly regulates angiogenesis, since IL-8 receptors, CXCR1 and CXCR2, have been observed on endothelial cells and have been shown to play a role in cell proliferation-related signaling. Similar results have been presented in other studies (101-103). IL-8 activates both its cognate receptors CXCR1 and CXCR2 on endothelial cells, which induce multiple signal transduction cascades that result in increased vascular permeability, adhesiveness, migration and proliferation during angiogenic process (27;31;104-107). We present in Paper 3 that IL-8 promotes activation of p44/42 MAPK, which takes part in cell proliferation and p38 MAPK, which takes part in cell migration by HUVEC. Petreaca and colleagues studied signal transduction pathways involved in IL-8-induced endothelial permeability, using a Transwell system and FITC-dextran tracker to assess permeability of microvessel endothelium (43). In this interesting study, it was found that IL-8 induces permeability of endothelial cell monolayer, and that trans-activation of VEGFR2 by IL-8 is required for and increased endothelial permeability (43). According to results of Petreaca and colleagues, VEGFR2 transactivation is associated with the interaction of VEGFR2 with the IL-8 receptors CXCR1 and CXCR2 (43).

\textbf{Synthesis}

Our findings affirm that multiple cell-cell interactions direct ovulation and corpus luteum formation. Although our data may give a limited insight into the sequence of these peri-ovulatory events, based on the well-described progress of wound healing we propose the following model, presented in schematically in Figure 2.
Activated by the LH surge, GL cells undergo luteinization, which implies increased release of progesterone, inflammatory cytokines and chemokines, which induce directed migration of leukocytes, first neutrophiles, and then monocytes. Leukocytes recruited to the peri-ovulatory follicle release a range of chemokines that increase further leukocyte recruitment.

The dynamic nature of changes during CL formation including communication between infiltrating leukocytes and stromal cells increases production of various inflammatory cytokines, among them IL1-β, IL-6, IL-8, FL, VEGF, and alters the pattern of angiogenic factors, like angiopoietin-2, follistatin, G-CSF, and HGF. Infiltrating leukocytes may release MMPs to cleave ECM and activate cytokines to promote further leukocyte infiltration. TIMPs released by GL cells inhibit MMPs. Several growth factors may induce expression of MMPs while other factors down-regulate MMP expression and up-regulate TIMPs. Balance of MMP/TIMP and their preovulatory release are maintained by leukocytes and luteinizing granulosa cells. These events may culminate in ovulation and the follicle’s rupture that allows infiltration of further leukocytes, as well as fibroblasts and endothelial cells. Luteal angiogenesis may be mediated via intracellular and intercellular signaling that ultimately leads to expression of factors that enhance endothelial cell proliferation and survival. GL cells were shown to promote specific endothelial cell functions in vitro, including directed migration and capillary tube formation. Altered release of angiogenic cytokines may imply that leukocytes modulate ovarian angiogenesis.

**Clinical considerations**

Even though Papers 1-3 apply a model of co-cultured human cells as a proxy of peri-ovulatory events, we still wish to consider clinical relevance of these findings. In particular, we propose possible application for luteal phase support and pathophysiology of ovarian hyperstimulation syndrome (OHSS).

Embryo implantation requires secretory transformation of the endometrium, induced by corpus luteum-derived progesterone, which is therefore crucial for implantation and for supporting the pregnancy during the early stages (108). Although partly autonomous, progesterone production by the corpus luteum requires some stimulation by LH, which in pregnancy is provided by the action of hCG on LH receptors. Insufficient luteal phase progesterone production, probably because of disruption of the physiological luteal phase or aspiration of granulosa cells during oocyte retrieval, is a major problem during IVF that compromises treatment success (109). Clinically, there are two common ways to provide
luteal phase support: direct progesterone supplementation or by hCG to stimulate progesterone production. Pabuccu and colleagues reviewed recent data on the options available for luteal support in assisted reproduction. They concluded that optimal duration of luteal phase support is still not clarified. Progesterone and estradiol have a crucial role in the maintenance of early pregnancy. Until luteoplacental shift, at about 7 weeks’ of gestation, the ovarian production of these hormones is critical, after that period, the placenta normally secretes sufficient levels of these hormones. Studies examining luteal support indicate a beneficial effect of supplementation with hCG over progesterone alone. However, the risk of ovarian OHSS increases with the use of hCG, and administration of hCG for luteal phase support is not routinely recommended (108). Therefore, progesterone rather than hCG supplementation should be used for luteal phase support in women at risk from developing OHSS (108).

Because of widespread use of drugs that can modulate their release, prostaglandins also need to be mentioned as possible mediators of leukocyte-granulosa cell interactions (110-112). Prostaglandins are synthesized by the cyclooxygenase (COX) enzyme, COX-1 is expressed in most cell types, and COX-2 is induced in response to inflammatory factors, in particular by leukocytes (113). In the human ovary, COX-2 is expressed by interstitial, but not by granulosa cells, and it was suggested that COX-2 is mainly produced in the follicles before ovulation (114;115). COX-2 is important for female reproduction and its deficiency leads to multiple reproductive defects in mice. Indeed, high doses of indomethacin, an inhibitor of both COX- and COX-2, blocks ovulation in rats, and targeted disruption of Cox2 gene, but no Cox1, in mice leads to defects in ovulation, fertilization, and implantation (110;116;117). After the LH surge and just before ovulation, cumulus cells expand, which can be important for efficient exit of the COC from the ovary and fertilization (118). Prostaglandins are thought to play a role in maintenance and function of the COC (119), as well as regulation of progesterone synthesis (120-122).

Because of the putative importance of immune cells during CL formation, manipulation of immune cells or their secretory products may provide a way to modulate ovarian function. Ovarian hyperstimulation syndrome (OHSS) is a serious, potentially life-threatening complication of ovarian stimulation. OHSS is characterized by an increased capillary and increased mesothelial permeability, which leads to fluid shift out of the intravascular space (123;124). Doxycycline is a tetracycline antibiotic that also inhibits matrix metalloproteinase activity. Presumably by inhibiting release of matrix-bound VEGF, doxycycline may also inhibit angiogenesis. Fainaru and colleagues hypothesized that
doxycycline could also suppress VEGF-induced vascular hyperpermeability caused by gonadotropin stimulation and therefore prevent OHSS (124). They induced OHSS in mice and quantified ascites accumulation. Gonadotropin treatment led to weight increase compared with the untreated mice, and doxycycline treatment decreased weight gain in a dose-dependent fashion. Doxycycline dose-dependently reduced peritoneal vascular leakage. Fainaru et al. observed no difference in level of ovarian response to stimulation or in the microvascular density in the corpora lutea. Their results indicate that doxycycline directly inhibits vascular hyperpermeability in a mouse model of OHSS. Importantly, the results of this study suggest that doxycycline does not affect angiogenesis in the corpus luteum and consequently doxycycline may, during the course of gonadotropin stimulation, prevent vascular hyperpermeability and the development of OHSS (124). In his fascinating article, Fainaru et al. do not revile exact pattern of doxycycline action. Doxycycline is known to have an inhibitory effect on MMPs therefore it is possible for us to assume that activity of MMPs may be as well related to suppression of OHSS.

**Conclusion**

In conclusion, we propose that a direct cell-cell contact with granulosa lutein cells activates leukocytes and both release intracellular chemokines. This further activates leukocytes migration, which eventually results in re-organization of tissue architecture, including ingrowth of capillaries.
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Tissue reorganization during ovulation and corpus luteum formation involves a coordinated action of matrix metalloproteinases (MMPs) and tissue MMP inhibitors (TIMPs). In this study we investigated the cellular source of ovarian MMPs and TIMPs. Cells isolated from the preovulatory human follicle were cultured after immunobead depletion of CD45-expressing cells, which allowed differential assessment of leukocyte and granulosa-lutein cell fractions. Secretion of MMP-9 by follicular fluid-derived cells was associated with the presence of leukocytes. Granulosa-lutein cells synthesized low levels of MMP-9 but failed to secrete this enzyme that presumably accumulated in the cytoplasm, indicated by an increased MMP-9 expression of luteinized cells in sectioned midluteal phase corpora lutea. Synthesis and secretion of TIMP by follicular fluid-derived cells was associated with granulosa-lutein cells. TIMPs derived by granulosa-lutein cells failed to inhibit MMP-related pericellular proteolysis. The findings support a two-cell model of periovulatory MMP/TIMP release, in which leukocytes secrete MMPs and granulosa-lutein cells release TIMP, suggesting that there exists an intriguing interaction among cells that intertwingle during ovulation and corpus luteum formation. (Endocrinology 151: 0000–0000, 2010)

Follicle growth, ovulation, corpus luteum formation, and regression compel a periodic reorganization of the ovarian extracellular matrix (ECM) (1). The preovulatory follicle is enclosed in the basal lamina, which consists of type IV collagen, laminin-α1β2γ1, nidogen, and perlecan; patches of this material are also deposited among the granulosa cells (2, 3). These ECM components provide polarity for and maintain a degree of specialization of granulosa cells and regulate the entry of proteins into the intrafollicular milieu. Composition and integrity of ECM regulates cell shape, communication, steroidogenesis, and survival in vitro (4, 5). During luteinization the basement membrane-like ECM components are replaced by distinct subendothelial and interstitial matrices (6), which is thought to abolish polarity of luteinizing cells (7) and promote progesterone synthesis (8, 9). Abnormal ovarian ECM composition may contribute to states of altered hormone secretion, such as the postmenopause or polycystic ovary syndrome (10).

Rearrangement of ECM requires a coordinated action of proteases and protease inhibitors. Matrix metalloproteinases (MMPs) are a family of soluble and membrane-associated extracellular proteolytic enzymes with common structural features, including the zinc-containing catalytic domain, the autoinhibitory propeptide that is removed during enzyme activation and the hemopexin domain that allows interactions with other proteins. MMPs cleave a wide variety of substrates, including structural proteins of the ECM, other MMPs, growth factor binding proteins, and cytokines/chemokines (11, 12). The more than 23 members of the MMP family are divided according to their main substrate specificity; the gelatinase

Abbreviations: APMA, 4-Aminophenylmercuric acetate; BFA, brefeldin A; CBB, Coomassie Brilliant Blue; CFDA, carboxyfluorescein diacetate; CL, corpus luteum; DMEM/F12, DMEM with Ham’s F12; ECM, extracellular matrix; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; GL, granulosa-lutein; hCG, human chorionic gonadotropin; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; PKH, Paul Karl Horan; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinase.
Materials and Methods

Reagents

DMEM with Ham’s F12 (DMEM/F12), fetal calf serum (FCS), RPMI 1640, carboxyfluorescein diacetate (CFDA) were purchased from Invitrogen (Carlsbad, CA); GM6001 and negative control of GM6001 were obtained from Calbiochem (La Jolla, CA). Monoclonal antibodies were purchased from the following suppliers: antihuman TIMP-1, R&D Systems (Abingdon, UK); antihuman MMP-9, clone GE-213, Chemicon (Millipore, Billerica, MA); clone 56-2A4, Oncogene Research Products (Cambridge, MA); and antihuman CD45, Dako (Glostrup, Denmark). Anti-CD45-conjugated immunobeads were from Dynal (Oslo, Norway). Antimouse Cy3-conjugated IgG was purchased from Jackson (West Grove, PA). Other reagents were purchased from Sigma-Aldrich (Oslo, Norway).

Cell culture

Human follicular fluid-derived cells were separated from aspirates of preovulatory follicles of women (n = 66) who underwent assisted reproduction treatment. Patients received controlled ovarian hyperstimulation using a standard combination of GnRH agonist, human recombinant FSH, and human chorionic gonadotropin (hCG) (19). Although women were approached irrespective of infertility diagnosis, patients with poor response to stimulation indicative of ovarian pathology were excluded. Collection of clinical samples was approved by the Regional Committee for Medical and Health Research Ethics, Health Region South (no. S-05058). All findings were confirmed in at least three independent experiments, unless otherwise specified.

Methods for follicular fluid cell isolation and processing were described in detail earlier (20). Briefly, cells were enriched by hemolysis, enzymatic and mechanical dispersion, and density gradient centrifugation. When appropriate, contaminating leukocytes were depleted with CD45-conjugated supramagnetic beads. The proportion of CD45+ cells was determined by fluorescence-activated cell sorter (FACS) analysis. Cells were plated on gelatin-coated culture dishes in 10% FCS in DMEM/F12, and cultured overnight at 37 °C in humidified air with 5% CO2. The cell cultures were extensively washed with DMEM/F12 to remove serum traces and were cultured for additional 48 h in serum-free DMEM/F12. The conditioned media were subsequently collected, clarified for particulate matter with centrifugation, and stored at −80 °C until assays. Total cellular protein content was determined with the bicinchoninic acid assay (21).

Peripheral blood-derived mononuclear cells were derived on the day of follicle aspiration from the same women donating follicular fluid. Heparinized blood was subjected to Ficol gradient centrifugation and cells in the interphase were cultured in serum-free RPMI 1640.

The human acute monocytic leukemia cell line THP-1 (obtained from DSMZ, Braunschweig, Germany), was propagated in RPMI 1640 with 10% FCS. When indicated, THP-1 cells were exposed to 80 nM phorbol 12-myristate 13-acetate (PMA) to induce cell transformation and MMP release. The human osteosarcoma cell line OHS, maintained in DMEM/F12 with 10% FCS, was a kind gift of Professor Mælandsmo (Radiumhospitalet, Oslo, Norway).

Gelatin zymography

Conditioned cell culture media were concentrated 10 times by lyophilization and redissolution in distilled water. The samples were further diluted with water to normalize for the total cellular protein content of samples from the same experiment. Ten microfilters of this solution were mixed with 10 μl gel loading buffer and resolved under denaturing nonreducing conditions using a 7% sodium dodecyl sulfate-polyacrylamide gel copolymerized with 2 mg/ml gelatin. After renaturation with 2.5% Triton X, gels were developed in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl2, 1 μM ZnCl2, and 0.02% Brij-35 for 48 h at 37 °C. Gels were stained with 0.25% Coomassie Brilliant Blue (CBB), followed by an extensive destaining until digestion bands have become visible.

Dried gels were scanned together with an OD calibrator (SilverFast IT8; Lasersoft, Sarasota, FL) in a high-end desktop scanner (Expression 1680 Pro; Epson, Hovik, Norway). Digitalized images were imported in ImageJ (version 1.4; http://rsbweb.nih.gov/ij), and calibrated OD was calculated for each digestion band. The OD so obtained correlated linearly (r2 =
0.98, n = 5) with expected enzymatic activity of serial dilutions of collagenase.

A standard mixture of collagenase (type I) and trypsin was used as a positive control and molecular weight marker on all gels, and this internal standard allowed comparison of MMP content across gels. In some gels, conditioned media of OHS and THP-1 cells were separated because these cells are known sources of MMP-2 and MMP-9, respectively.

To further characterize gel-resolved enzymes and confirm their identities as MMPs, the following experiments were performed. First, samples were preincubated before electrophoresis with 1 μg/ml 4-aminophenylmercuric acetate (APMA) or 25 μg/ml trypsin at 37°C for 1 h. APMA and trypsin facilitate pro-MMP activation by cleavage of propeptide, which results in a protein product that migrates as a lower molecular weight band. Second, gels were developed in the presence of 1,10-phenanthroline (10 mM), leupeptin (500 ng/ml), phenylmethanesulfonyl fluoride (170 μg/ml), or soybean trypsin inhibitor (100 μg/ml). Characteristically, MMP activity, but not trypsin activity, was selectively inhibited by 1,10-phenanthroline, whereas the other protease inhibitors selectively inhibited trypsin. And third, conditioned media were resolved with standard 7% gels under denaturing nonreducing conditions and were transferred onto a polyvinyl difluoride membrane by Western blot. The membrane was probed with primary anti-MMP-9 antibody (GE-213), followed by detection of immunocomplexes by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

Reverse gelatin zymography

TIMPs can be detected by reverse gelatin zymography, so termed because both the gelatin substrate and the MMP enzyme are copolymerized in the same gel. For this assay, standard 12% sodium dodecyl sulfate-polyacrylamide gels were poured with 1 mg/ml gelatin and 0.1% (vol/vol) conditioned medium of PMA-treated THP-1 cells as the source of gelatinases. Before loading the samples, the gels were prerun for 1.5 h at 120 V to remove traces of THP-1-derived TIMP. Subsequent sample preparation, electrophoresis, gel development, and CBB staining were as for gelatin zymography above. In reverse zymograms, TIMP activity appears as protein-dense bands because the digestion of copolymerized gelatin by MMPs was inhibited. To affirm specific detection of TIMP, reverse zymography was modified as follows. First, gels were prepared with fluorescein isothiocyanate-gelatin and viewed after development under UV light without CBB staining. Detection of fluorescence-dense bands indicated that TIMP bands were not due to unspecific abundant proteins. Second, gels were prepared without gelatin but with THP-1-conditioned medium and processed as above. Absence of protein bands indicated that TIMP bands were not due to unspecific proteins detected by CBB staining.

Immunofluorescence

For immunodetection of intracellular MMP-9 and TIMP-1, granulosa-lutein (GL) cells were cultured on flame-sterilized glass coverslips. Brefeldin A (BFA; 10 μg/ml) was added to the culture media to inhibit release of MMP-9 and TIMP-1 from the cells. Monolayers were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and exposed to blocking buffer (1% goat serum in 0.2% Tween 20 and PBS). Primary antibodies diluted in blocking buffer were added (1:100 dilution; anti-TIMP-1), followed by secondary Cy3-conjugated antibody (1:600 dilution). Nuclei were counterstained with bisbenzimide and the coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). PMA-stimulated THP-1 cells were used as positive control. Images were taken with an epifluorescent microscope (Zeiss, Jena, Germany), using constant exposure settings in an experiment.

Pericellular gelatinolysis

Pericellular digestion of gelatin by cell-associated MMPs was examined using gelatin-coated coverslips. Gelatin was biotinylated by mixing 10 mg/ml gelatin with biotin, followed by filtration through a Sephadex-G50 column. Coverslips were coated with 50 μg/ml poly-l-lysine and 1:10 dilution of biotin gelatin for 30 min. The gelatin film was fixed to the coverslip with 4% paraformaldehyde for 5 min, which was extensively neutralized with 10% FCS in DMEM/F12.

To distinguish the cultured cells, GL cells and THP-1 cells were labeled with CFDA (green fluorescence) or Paul Karl Horan (PKH)-26 (red fluorescence), respectively (21). CFDA-labeled GL cells were first seeded onto biotin-gelatin-coated coverslips at the density of 400,000 cells/well in 10% FCS in DMEM/F12. After overnight incubation, the wells were washed with serum-free DMEM, and PKH-26-labeled THP-1 cells were added (12,000 cells/well) in addition to PMA that facilitated transformation of THP-1 cells. The number of GL cells and THP-1 cells, which was optimal for observing pericellular proteolysis, was determined in pilot experiments. Some cultures were treated with the broad-spectrum MMP inhibitor GM6001 (5 μg/ml).

After additional incubation overnight, the wells were rinsed with PBS, fixed with 4% paraformaldehyde, and blocked with 1% BSA in PBS. Alexa-350-conjugated streptavidin (blue fluorescence) was subsequently added (1:60 dilution in BSA/PBS), followed by PBS wash and mounting of coverslips in Vectashield (Vector Laboratories). The slides were viewed in epifluorescence microscope to estimate pericellular gelatinolysis. Only clearance areas that were unambiguously associated with a red or green cell were considered indicative of gelatinolysis to distinguish from cell detachment or unevenly set gelatin film.

Antibody array

The antibody array of Ray Biotech (Norcross, GA) consists of duplicate dots of capture antibodies arrayed on a membrane; in combination with a biotinylated anticytokine antibody cocktail, the array allows simultaneous detection and semiquantitative analysis of multiple MMPs and TIMPs. Conditioned cell culture media of leukocyte-depleted granulosa-lutein cells, derived from six women as well as conditioned media of peripheral leukocytes from three of these women were collected. Conditioned media were subjected to antibody array as described by the manufacturer. The arrays were scanned together with an OD calibrator, quantitated in ImageJ, and analyzed as recommended by the manufacturer.

FACS

THP-1 cells were differentially labeled with PKH-26 and were cocultured with GL cells in the presence of PMA and BFA. Trypsinized cell cultures were fixed, permeabilized, and intracellular MMP-9 was determined with indirect immunofluorescence and FACS analysis, as previously described (21).
Immunohistochemistry

Formalin-fixed, paraffin-embedded ovariectomy specimens of three women were retrieved from the archives of the Division of Pathology, Rikshospitalet Medical Center. The specimens were selected by a database search for the presence of corpus luteum. Sectioned specimens were first reexamined to confirm the presence of mature CL or regressing CL.

Sections were stained using the Dako EnVision System, peroxidase (3-diaminobenzidine tetrahydrochloride) (K4007; Dako), and Dako autostainer. Deparaffinized sections for CD45 staining were microwaved in 10 mM citrate buffer (pH 6.0). The sections were incubated with primary antibodies (1:100; anti-MMP-9 and anti-CD45), horseradish peroxidase-labeled polymer conjugated to goat antimouse and 3-diaminobenzidine tetrahydrochloride. Nuclei were counterstained with hematoxylin, dehydrated, and mounted in Diatex. Negative controls included mouse myeloma protein of the same subclass and concentration as the monoclonal antibody.

Statistics

Linear regression and Student’s t test were performed where appropriate. \( P < 0.05 \) was considered statistically significant.

Results

Release of MMP by follicular fluid-derived cells

Cells isolated from the aspirates of preovulatory ovarian follicles released various gelatinases in vitro. Based on migration in gelatin zymography and immunoreaction with anti-MMP-9 antibody, these enzymes were identified as MMP-9 species (Fig. 1A). A major 95-kDa band of pro-MMP-9 as well as minor species corresponding to putative MMP-9/NGAL complexes [115, 125, and 135 kDa; neutrophil gelatinase-associated lipocalin (NGAL)] was observed (22) and pro-MMP-9 dimer (196 kDa). The identity of these enzymes as MMPs was further confirmed by observing cleavage by APMA and sensitivity to the protease inhibitor phenantroline, but not trypsin (Fig. 1B). Release of MMP-9 was consistently detectable in all individual samples (\( n = 8 \)), whereas minor MMP-9 species could be discerned only in follicular fluid cells derived from some...
women. MMP-9 species were also detected in extracts of cells (not shown). Lower molecular weight gelatinases (putative pro-MMP-2 and MMP-2 of 68 and 58 kDa, respectively) were detected in one sample.

Cellular source of MMP-9 in follicular fluid

The fluid of the preovulatory follicle contains, beyond GL cells, a heterogeneous mixture of leukocytes (20). To examine whether MMP-9 was secreted by leukocytes or GL cells, follicular fluid cells derived from seven women were subjected to depletion with CD45-conjugated immunobeads (Fig. 1C). The population of CD45−/H11002 cells expressed cytokeratin and the steroidogenic acute regulatory protein, consistently with a granulosa-lutein cell phenotype (supplemental data, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org).

Conditioned media of follicular fluid cells either depleted or not for leukocytes were resolved with gelatin zymography, which indicated that concentration of pro-MMP-9 correlates with the proportion of CD45− cells present (β = 0.63, P = 0.03; Fig. 1D), suggesting that either leukocytes release pro-MMP-9 directly or leukocytes induce pro-MMP-9 release by other cells indirectly.

Qualitative comparison of minor MMP species argued for that leukocytes were the main source of MMP-9 in follicular fluid cells. The release of MMP-9/NGAL complexes was associated with the presence of nonlymphoid cells because the mean proportion of nonlymphoid leukocytes (identified by CD45 expression and side scatter properties in FACS analysis) was 36% (SD 17, n = 5) in samples in which release of MMP-9/NGAL complexes was detected compared with 15% (SD 5, n = 3) in samples where MMP-9/NGAL was not detected (P = 0.05). Furthermore, the release of pro-MMP-9 by GL cells was stimulated by PMA but was unaffected by hCG, implicating leukocytes as MMP sources (Fig. 1F).

Release of TIMP-1 by GL cells

Follicular fluid-derived cells were found to secrete TIMPs that migrated as proteins of approximately 20 kDa molecular mass. TIMP release was unaffected by depletion of CD45+ cells (Fig. 1E), but was stimulated by hCG (Fig. 1, F and G), suggesting that GL cells are the main source of TIMP.

Intracellular accumulation of MMP-9 and TIMP-1 in isolated GL cells and luteinized cells of the CL in situ

To further examine the cellular source and pattern of MMP-9 and TIMP-1 release, cultured GL cells (n = 4) and sections of corpora lutea (n = 3) were examined for the presence of specific immunoreactivity. In cultured GL cells, strong accumulation of TIMP-1 and occasional accumulation of MMP-9 was observed in the ER-Golgi, characteristic perinuclear vesicular-lamellar structures that become disrupted by BFA (Fig. 2A). Weak MMP-9-positive cells were detected among PMA-treated GL cells by FACS analysis (see Fig. 4B).

MMP-9 accumulation in the mature CL was examined by immunostaining of paraffin-embedded corpora lutea. Cytoplasm of large luteal cells was strongly positive for MMP-9. Leukocytes that were scattered among luteal cells did not appear to preferentially accumulate MMP-9 (Fig. 2B). Nonluteinized ovarian cortex and medulla were MMP-9 negative (not shown).

Release of MMP and TIMP species by GL cells and mononuclear leukocytes

To compare the pattern of MMP and TIMP release, conditioned media of leukocyte-depleted GL cells (n = 6) and homologous peripheral mononuclear leukocytes (n =
3) were subjected to antibody array analysis (Fig. 3), which allowed simultaneous detection of multiple MMPs (1, 2, 3, 8, 9, 10, and 13) and TIMPs (1–4). GL cells preferentially expressed TIMP-1, -2, and -3, whereas leukocytes derived mostly MMP-3, -8, -9, -10 as well as TIMP-4. Secretion of MMP-1, -2, and -13 was comparable between GL cells and leukocytes.

**Pericellular gelatinolysis by cocultured GL cells and monocytes/macrophages**

To examine the integrated effect on pericellular proteolysis by GL cell-derived TIMPs and leukocyte-derived MMPs, the following experiment was devised. Follicular fluid cells were depleted for leukocytes (n = 6) and peripheral mononuclear leukocytes (n = 3) were cultured in vitro. Conditioned media were exposed to antibody array, in which substrate-specific antibodies are blotted in duplicate on a membrane, as indicated on the lowest panel. M1–M13, MMPs; T1–T4, TIMPs; + and −, positive and negative control dots. Amount of MMP and TIMP released by GL cells and leukocytes was quantified by image analysis of dot intensities (mean ± se; graph).

![FIG. 3. Release of multiple MMPs and TIMP by GL cells and leukocytes. GL cells depleted for leukocytes (n = 6) and peripheral mononuclear leukocytes (n = 3) were cultured in vitro. Conditioned media were exposed to antibody array, in which substrate-specific antibodies are blotted in duplicate on a membrane, as indicated on the lowest panel. M1–M13, MMPs; T1–T4, TIMPs; + and −, positive and negative control dots. Amount of MMP and TIMP released by GL cells and leukocytes was quantified by image analysis of dot intensities (mean ± se; graph).](image)

Pericellular gelatinolysis was completely inhibited by a broad-spectrum MMP inhibitor (GM6001), indicating that gelatin digestion was due to MMPs released from or associated with the monocytes/macrophages. Without PMA treatment, pericellular gelatinolysis by THP-1 cells was absent.

**Discussion**

Immune cells periodically infiltrate the ovary and reach especially high tissue density around ovulation and during regression of the CL (23, 24). Appearance of leukocytes coincides with periods of major ECM reorganization, implicating leukocyte products, in particular MMPs, in ovarian tissue remodeling (25, 26).

In this study, we describe an intriguing pattern of synthesis and secretion of MMPs and TIMPs by cultured cells derived from the preovulatory follicle. In this model of luteinization, MMP-9 was predominantly secreted by leukocytes. GL cells exhibited a constitutive low level of MMP synthesis but absent or low level of MMP release. TIMPs were, however, mainly secreted by GL cells, and TIMP secretion was both constitutive and inducible by hCG and phorbol ester. Antibody array data indicate that this secretion pattern can be extended to several leukocyte-derived MMPs (MMP-3, stromelysin 1; MMP-8, collagenase 2; MMP-10, stromelysin 2) as well as to granulosa-lutein cell-derived TIMP-1, -2, and -3. The findings suggest that the MMP-TIMP system is compartmentalized during luteinization so that leukocytes preferentially secrete MMPs, whereas luteinizing granulosa cells derive TIMPs. Because the phenotype of luteinizing cells is changing rapidly, the data are probably applicable only to the immediate periovulatory period, and granulosa cell-leukocyte interactions may differ during later stages of the luteal phase.

Leukocytes are recruited to the periovulatory follicle in response to chemokines secreted by GL cells (21). Infiltrating leukocytes may release MMPs to cleave extracellular matrix proteins, thus promoting further leukocyte infiltration, angiogenesis, and luteinization of granulosa cells; resident GL cells may release TIMPs to control MMP action or confine it to the immediate pericellular space, thus balancing the effect of leukocyte-derived MMPs. Notably, ovarian TIMP expression peaks around ovulation (27), and an imbalanced MMP/TIMP release has been linked to states of altered ovarian function, including the...
polycystic ovary syndrome (17, 28), and an altered MMP/TIMP secretion (1) along with increased perifollicular leukocyte infiltration (24, 29) were described in follicular atresia. Physiological importance of the ovarian MMP/TIMP system is also indicated by disruption of individual genes coding for MMPs and TIMPs in mice, which were shown to induce reproductive disturbance of variable severity, such as smaller litter size in mice lacking the Mmp-9 gene (30), impaired ovarian progesterone release in response to hCG in mice lacking the Timp-1 gene (31), and sterility due to impaired CL angiogenesis in mice with dysregulation of TIMP-1 release by microRNAs (32).

Certain features of MMPs and TIMPs may nonetheless amend the two-cell model of periovulatory MMP-TIMP release. MMPs have overlapping substrate specificity that gives rise to enzymatic redundancy among MMPs and also with other proteases (12). Redundancy of MMPs implies that it is difficult to attribute significance to a single cellular source because proteases released by other cells may substitute a lost function. Furthermore, GL cells may come into contact with several blood components on ovulation; interaction with platelets in particular was shown to influence granulosa cell shape, steroidogenesis, and promotion of endothelial cell migration (33).

The present experiments cannot attribute a single mechanistic role to GL cell-derived TIMPs as inhibitors of MMP-induced proteolysis. We found that MMP-mediated (i.e. sensitive to chemical MMP inhibition) pericellular gelatinolysis by monocytes/macrophages was not inhibited by coculturing with GL cells, which were found to release copious amounts of TIMPs. It is possible that monocytes/macrophages can circumvent an inhibitory TIMP action in the pericellular microenvironment by adherence to and sealing off the subcellular space, tightly binding MMP substrates, or overwhelming inhibitors (25). Alternatively, granulosa cell-derived TIMPs may act independently of proteolysis inhibition. Supporting this latter concept, experiments on plasminogen-deficient mice treated with MMP inhibitor indicate that normally vascularized corpora lutea can form in the absence of gelatinolytic activity (34). Indeed, MMPs and TIMPs are involved in many processes beyond ECM reorganization. For example, MMPs cleave and thereby activate or inhibit cytokines and induce shedding of cytokines from ECM binding sites (12). TIMPs regulate cell growth and apoptosis independently of inhibiting MMPs, probably by binding to specific cell surface receptors (35). Furthermore, TIMP-1 regulates steroid synthesis in concentrations lower than necessary for MMP inhibition (36).

The present data are based on cells derived from the preovulatory follicle and thus provide a temporally limited insight into cyclically changing ovarian MMP and TIMP expression (27). We found that periovulatory GL cells constitutively synthesize MMP-9, but these cells show a low or absent enzyme release in vitro, which may explain

![FIG. 4. Pericellular proteolysis by MMPs derived by cultured GL cells and monocytes/macrophages (Mo/M). A, GL cells were isolated from the follicular fluid after depletion of leukocytes and were labeled with CFDA (green), whereas the THP-1 monocyteid cells were labeled with PKH-26 (red). Cells were cultured on biotinylated gelatin-coated coverslips alone or were cocultured in the presence of PMA (80 nM). Pericellular gelatin digestion was visualized with fluorochrome-conjugated streptavidin (blue). Digestion areas around monocytes/macrophages were either shaped as cell-wide tracks along the path of moving cells (arrow) or as pericellular clearance (asterisk). The broad-spectrum MMP inhibitor GM6001 (5 μg/ml) completely inhibited gelatin clearance (right panels). B, To establish the cellular source of MMPs in this model, differentially labeled GL cells and THP-1 monocyteid cells were cultured with BFA to inhibit MMP release and were subsequently stained for intracellular MMP-9 and analyzed by FACS. Cell cultures were performed in the absence of presence of PMA (80 nM). Monocytes/macrophages and GL cells were distinguished by gating on forward scatter vs. PKH-26 plot (leftmost plot). Histograms show MMP-9-associated immunofluorescence for the gated cells in monoculture of GL cells and monocytes/macrophages as well as for cocultured cells.](endo.endojournals.org/...)

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MMP-9 accumulation by lutein cells of the midcycle CL. Such gradual sequestration of MMP-9 in specific gelatinase granules is a prominent feature of neutrophils, in which a rapid MMP degranulation during inflammation is thought to promote extravasation and diapedesis (37). A concerted exocytosis of MMP-9 from lutein cells and cessation of TIMP release may be a key event during luteolysis (38).

A further limitation of the findings stems from the fact that ovarian stimulation may itself influence ovarian MMP and TIMP release because follicular fluid derived from women undergoing in vitro fertilization tends to have lower levels of MMP-2 and MMP-9 but higher levels of TIMP-1 compared with control women (39).

It is notable that active MMPs were not detected in the conditioned media of follicular fluid-derived cells. Most MMPs are secreted from the cells aszymogens that are activated by cleavage of propeptide by other MMPs or plasmin (11). However, MMP activation may be temporally and spatially separated from MMP release from the cells; for example, secreted pro-MMP-9 promotes tumor growth compared with cell-associated active MMP-9 (40).

In conclusion, periovulatory release of MMPs and TIMPs in the ovarian follicle is associated with leukocytes and luteinizing granulosa cells, respectively, which further supports that there exists an intriguing interaction between cells that mix during corpus luteum formation.

Acknowledgments

We thank Rolf Gaustad, Parvin Mahzonnii, and Ellen Hellesylt for technical assistance and Jan-Olof Winberg (University of Tromso) and Bente Halvorsen (University of Oslo) for advice.

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Disclosure Summary: The authors have nothing to disclose.

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