CYCLOOXYGENASE-2 IN MYOMETRIUM DURING PARTURITION AND IN OVARIAN CARCINOMA

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Academic Dissertation

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HELSINKI 2005

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ISBN 952-91-8283-X (nid)

ISBN 952-10-2303-1 (pdf)

http://ethesis.helsinki.fi

Yliopistopaino

Helsinki 2005

To Antti, Antero, Suvi, and ...

TABLE OF CONTENTS

ORIGINAL PUBLICATIONS

I Erkinheimo T-L*, Saukkonen K*, Narko K, Jalkanen J, Ylikorkala O, and Ristimäki A. Expression of cyclooxygenase-2 and prostanoid receptors by human myometrium. The Journal of Clinical Endocrinology and Metabolism 85: 3468-3475, 2000.

II Erkinheimo T-L, Lassus H, Finne P, van Rees BP, Leminen A, Ylikorkala O, Haglund C, Butzow R, and Ristimäki A. Elevated cyclooxygenase-2 expression is associated with altered expression of p53 and SMAD4, amplification of HER-2/neu, and poor outcome in serous ovarian carcinoma. Clinical Cancer Research 10: 538-545, 2004.

III Erkinheimo T-L, Lassus H, Sivula A, Sengupta S, Furneaux H, Hla T, Haglund C, Butzow R, and Ristimäki A. Cytoplasmic HuR expression correlates with poor outcome and with cyclooxygenase-2 expression in serous ovarian carcinoma. Cancer Research 63: 7591- 7594, 2003.

IV Erkinheimo T-L, Sivula A, Lassus H, Heinonen M, Furneaux H, Haglund C, Butzow R, and Ristimäki A. Cytoplasmic HuR expression correlates with epithelial cancer cell but not with stromal cell cyclooxygenase-2 expression in mucinous ovarian carcinoma. Submitted.

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ABBREVIATIONS

ASA	acetylsalicylic acid
AA	arachidonic acid
ARE	AU-rich element
AU	adenylate-uridylate
cDNA	complementary deoxyribonucleic acid
CISH	chromogenic *in situ* hybridization
COX	cyclooxygenase
CRE	cyclic adenosine monophosphate response element
CRM1	chromosome maintenance region 1
EIA	enzyme-linked immunoassay
ELAV	embryonic lethal abnormal vision
FAP	familial adenomatous polyposis
FISH	fluorescence *in situ* hybridization
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hCG	human chorionic gonadotropin
HNPCC	hereditary nonpolyposis colorectal cancer
IHC	immunohistochemistry
IL	interleukin
IVF	*in vitro* fertilization
kb	kilobase
kDa	kilodalton
LH	luteinizing hormone
LMB	leptomycin B
LMP	low malignant potential
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
NF	nuclear factor
NK	natural killer
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
PBS	phosphate buffered saline
PG	prostaglandin (e.g., PGD_2, PGE_2, $PGF_{2\alpha}$, PGG_2, PGH_2)
PDGF	platelet-derived growth factor
PGI_2	prostacyclin
PL	phospholipase
PMA	phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator-activated receptor
RIA	radioimmunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
siRNA	small interfering ribonucleic acid
α-SMA	α-smooth muscle actin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TBST	tris-buffered saline and Tween
Tx	tromboxane
TNF	tumor necrosis factor
UTR	untranslated region
VEGF	vascular endothelial growth factor

ABSTRACT

Prostanoids (prostaglandins, prostacyclin, and thromboxane) play an important role in several physiological and pathological processes in the female reproductive organs. Cyclooxygenase (COX) enzymes are the rate-limiting enzymes in the conversion of arachidonic acid (AA) to prostanoids. COX-2, the inducible COX isoenzyme, is expressed at low levels in basal conditions, but is induced by cytokines, tumor promoters, and hormones. The aim of this study was to investigate COX-2 expression in gynecological organs during parturition and in ovarian carcinoma.

Prostanoids are important mediators of human parturition at term and in preterm conditions. Fetal membranes have been previously shown to express elevated levels of COX-2 at the onset of labor. We investigated the expression of COX-2 in myometrial samples during parturition by Northern blot analysis, and observed a 15-fold elevation in COX-2 mRNA expression at the onset of labor. Cultured human myometrial cells expressed low levels of COX-2 mRNA in baseline condition, but stimulation of these cells with a proinflammatory cytokine interleukin-1β (IL-1β) caused a 17-fold induction of COX-2 mRNA expression. In addition, IL-1β induced expression of COX-2 protein, and thereafter the production of prostacyclin, which was blocked by a COX-2 selective inhibitor, NS-398. These data suggest that COX-2 is an important inducible prostanoid-producing enzyme in the myometrium during parturition. However, although selective COX-2 inhibitors may be effective in prevention of premature birth, their use is limited by fetal side-effects, such as oligohydramnions and constriction or premature closure of the fetal ductus arteriosus.

Prostanoids have been connected to carcinogenesis because they can reduce apoptosis and host immune response and increase angiogenesis and invasion. Furthermore, the use of acetylsalicylic acid (ASA) and other nonsteroidal anti-inflammatory drugs is associated with the reduced risk of cancer-related deaths especially in the digestive tract. COX-2 is frequently expressed in human carcinomas, and inhibition of COX-2 suppresses tumor formation in various animal models of carcinogenesis. Importantly, COX-2 selective inhibitors have recently been found to reduce tumor burden in patients with familial adenomatous polyposis. In order to investigate the role of COX-2 in gynecological malignancies, the most lethal gynecological carcinoma, ovarian carcinoma, was studied. Serous and mucinous types of ovarian carcinoma were selected because of their different pathogenesis and outcome. COX-2 protein expression was investigated by immunohistochemistry in 442 serous and 56 mucinous ovarian carcinoma specimens. COX-2 immunoreactivity localized mainly to the epithelial cancer cells, but also in the stromal compartment of the tumor, especially in

the mucinous carcinomas. COX-2 expression was elevated in the epithelial cancer cells more often in the serous (70%) than in the mucinous (39%) tumors ($P<0.0001$). Elevated epithelial expression of COX-2 associated with high histological grade in both histologies ($P<0.0001$ in serous and $P=0.0285$ in mucinous) and with reduced survival in serous carcinoma ($P=0.0011$). When COX-2 expression was compared to other molecular markers related to carcinogenesis of serous ovarian tumors, it was found to correlate with altered expression of tumor suppressors *p53* and *SMAD4*, and with amplification of the *HER-2* oncogene. Furthermore, cytoplasmic expression of the mRNA stability factor HuR correlated with epithelial COX-2 expression in both histologies, and was associated with reduced survival in serous ovarian tumors. Thus, HuR is the first mRNA stability protein, the expression of which has been linked to reduced survival in carcinoma patients. This property of HuR may in part depend on its ability to induce COX-2 expression. We have observed that inhibition of HuR by use of small interfering RNA (siRNA) technology reduced COX-2 expression in serous ovarian carcinoma cells under tissue culture conditions.

In conclusion, our study suggests that expression of COX-2 plays an important role in the synthesis of prostanoids during parturition in the myometrium. In serous ovarian carcinoma, elevated COX-2 expression is associated with reduced survival, which may indicate that COX-2 selective inhibitors might prove to be effective in the treatment of this malignancy. However, clinical trials are needed to test the safety and efficacy of COX-2 inhibitors in these diseases.

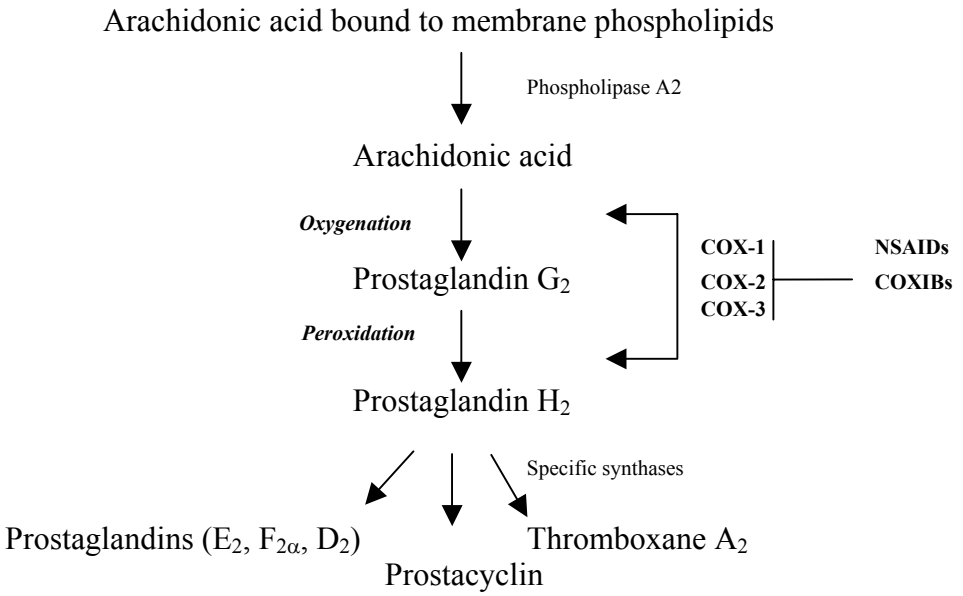
REVIEW OF THE LITERATURE

1. COX ENZYMES

1.1. Synthesis of prostanoids

Prostanoids such as prostaglandins (PGs), prostacyclin (PGI_2), and tromboxanes (Txs) are 20-carbon polyunsaturated fatty acids synthesized from AA. AA is liberated from membrane phospholipids by phospolipase A_2 (PLA_2) and to some extent by PLC (Smith and Marnett 1991). Cyclooxygenase is an enzyme, the COX activity of which catalyzes the conversion of AA to PGG_2 by introducing two molecules of O_2 into AA, and the peroxidase activity then reduces PGG_2 to PGH_2, which is further converted into various biologically active prostanoids such as PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA_2 by tissue-specific synthases (Dempke *et al*. 2001) (Fig.1).

Figure 1. The prostanoid biosynthetic pathway. COX=cyclooxygenase; NSAIDs=non-steroidal anti-inflammatory drugs, COXIBs=COX inhibitors.



Prostanoids are short-lived and act as local hormones in an autocrine or paracrine manner. They act through seven transmembrane receptors which belong to the G protein-linked receptor family, or through the nuclear peroxisome proliferator-activated receptors (PPARs) (Dussault and Forman 2000, Sugimoto *et al*. 2000). Prostanoids have many physiological functions such as regulation of platelet aggregation, cytoprotection of the stomach, regulation of kidney perfusion, reproduction, and parturition. In addition, their biosynthesis is related to inflammation and carcinogenesis as well (Smith and Langenbach 2001).

1.2. COX enzymes

Two COX genes have been cloned, *COX-1* and *COX-2*. COX-1 complementary deoxyribonucleic acid (cDNA) was originally cloned from sheep seminal vesicles (DeWitt and Smith 1988, Merlie *et al*. 1988, Yokoyama *et al*. 1988) and the human homolog was characterized in 1991 from platelets (Funk *et al*. 1991). COX-2 cDNA was first cloned from chicken embryo fibroblasts in the late 80s (Simmons *et al*. 1989), and its murine homolog was identified as a tumor promoter- and serum-inducible immediate early gene product from Swiss 3T3 fibroblasts (Kujubu *et al*. 1991) and from C127 fibroblasts (O'Banion *et al*. 1992). Human COX-2 cDNA was originally cloned from human umbilical vein endothelial cells by Hla and Neilson (1992). COX enzymes are composed of three domains: an epidermal-growth factor-like domain, a membrane-binding region, and a large catalytic domain. The catalytic domain contains both cyclooxygenase and peroxidase activity sites (Luong *et al*. 1996). The size of the human *COX-1* gene is larger than the human *COX-2* gene mainly due to the different sizes of the introns. Most of the exons are conserved between *COX-1* and *-2*, with the exception of the absence of exon 2 in *COX-2*. The *COX-2* mRNA is approximately 4.5 kb, and the *COX-1* mRNA is 2.7 kb (Williams and DuBois 1996). Recently, Chandrasekharan *et al*. (2002) identified from canine cerebral cortex COX-3 and PCOX-1α, which are formed from the *COX-1* gene by alternative splicing. COX-3 may be the target of acetaminophen, also known as paracetamol, which is an antipyretic and analgesic drug with very weak anti-inflammatory activity (Chandrasekharan *et al*. 2002). Table 1 depicts the basic characteristics of *COX-1* and *COX-2* genes.

The promoter region of the *COX-1* gene contains no TATA box but possesses high GC content, which is a characteristic of a housekeeping gene (Wang *et al*. 1993). The *COX-2* promoter region contains a TATA box and a number of transcriptional regulatory sequences including nuclear factor

(NF) -κB, the NF-IL-6, and the cyclic AMP response element (CRE). The COX-2 gene has an extensive 3´-untranslated region (UTR) having several AU-rich elements (AREs), which have been associated with rapid degradation of the transcript (Appleby *et al*. 1994). No differences exist in the subcellular location of COX-1 and -2, and they are both expressed in endoplasmic reticulum and nuclear envelope (Morita *et al.* 1995, Spencer *et al.* 1998).

Table 1: Comparison of *COX-1* and *COX-2* genes and gene products.

Parameter	**COX-1**	**COX-2**
Human chromosome	Chromosome 9	Chromosome 1
Gene size	22 kb	8.3 kb
TATA box	No	Yes
Promoter	GC rich	NF-κB, NF-IL-6, CRE
Regulation	Constitutive	Inducible
mRNA size	2.7 kb	4.5 kb
Molecular mass	72 kDa	72 kDa
Protein localization	Endoplasmic reticulum and nuclear envelope	Endoplasmic reticulum and nuclear envelope

1.3. Function of COX-1 and COX-2

Although COX-1 and COX-2 are enzymatically quite similar isoenzymes, regulation of gene expression differs drastically. Whereas COX-1 is constitutively expressed in most tissues and the expression of COX-1 is usually not regulated, COX-2 expression is low or non-detectable in most tissues but can be readily induced by hormones, cytokines, growth factors, and tumor promoters, e.g., phorbol 12-myristate 13-acetate (PMA). For this reason, it is thought that COX-1 controls normal physiological functions such as cytoprotection of the gastric mucosa, platelet aggregation, and vasodilatation in the kidney, whereas the function of COX-2 has been associated with inflammation and carcinogenesis (Needleman and Isakson 1997). Inhibition of COX-2 is thus thought to mediate the great majority of the anti-inflammatory properties of NSAIDs. However, recent studies suggest that COX-2 may also play an important role in various physiological processes such as regulation of renal medullary blood supply, salt excretion, and systemic blood pressure (Harris and Breyer 2001). COX-2 is also important in reproductive functions, since it is involved in ovulation, implantation, and in the angiogenesis needed for establishment of the placenta. Animal studies have suggested that both COX enzymes are expressed in the ductus, with COX-1 preceding COX-2 in the course of gestation (Guerguerian et al. 1998, Coceani *et al.* 2001).

Hence, both COX-1 and -2 have been implicated in PGE_2 formation in fetal ductus at term, while COX-1 has been assigned a predominant role in the premature fetus. COX-2 is essential for initiation of the closure of the ductus arteriosus at term (Hinz and Brune 2002).In the mouse ductus, the PGE_2 synthesis comprises both COX-1 and -2. When the function of COX is suppressed, the production of vasodilator nitric oxide (NO) is upregulated. PGE_2 and NO can function synergistically in keeping the ductus patent (Baragatti *et al.* 2003).

Many factors associated with inflammation have been shown to induce COX-2: These factors include bacterial lipopolysaccharide (LPS) and also cytokines such as IL-1 and tumor necrosis factor (TNF)-α (Vane *et al.* 1998). Human and animal models of inflammatory arthritis strongly suggest that increased expression of COX-2 is responsible for the increased production of PGs found in inflamed joint tissues (Anderson *et al.* 1996, Amin *et al.* 1997, Crofford *et al.* 1994). In carcinogenesis, many effects of COX-2 can be facilitated by either PGE_2 or $PGF_{2\alpha}$, including stimulation of proliferation, inhibition of apoptosis, enhanced production of matrix metalloproteinases, promotion of angiogenesis, and induction of immunosuppression. COX-2-derived prostanoids can suppress apoptosis by upregulating anti-apoptotic Bcl-2 (Sheng *et al.* 1998). COX-2 also contributes to the production of pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), since treatment of COX-2-overexpressing colorectal cancer cells with a COX-2 selective inhibitor, NS-398, diminished secretion of these factors (Tsujii *et al.* 1998). Furthermore, in a rat corneal model, bFGF-induced blood vessel formation was suppressed by another COX-2 selective inhibitor, celecoxib, but not by a COX-1 selective inhibitor, SC-560 (Masferrer *et al.* 2000). COX-2 can also modulate invasive properties of human cancer cells (Tsujii *et al.* 1997), for instance by upregulating the expression of CD44, which leads to increased invasiveness of tumor cells (Dohadwala *et al.* 2001) and increases immunosuppression by inhibiting T and B cell proliferation, cytokine synthesis and diminishing the activity of natural killer (NK) cells (Rhind *et al.* 1999). All this suggests that COX-2 affects multiple steps of carcinogenesis and that its inhibition could be an effective modality in treatment of cancer patients.

1.4. Gene-deletion studies of COX enzymes

Cox-1 and *-2* knockout mice were generated in the mid 90s (Langenbach *et al*. 1995, Morham *et al*. 1995). *Cox-1*-deficient mice showed reduced inflammatory response to AA, and their platelet aggregation was impaired. Homozygous female mice produced litters of normal size, but they had

delayed parturition, and thereafter most of the pups were born dead or died shortly after birth (Langenbach *et al*. 1995). However, the *Cox-1*-deficient mice which survived were generally quite healthy. They did not have spontaneous stomach ulcers, and they showed less indomethacin-induced gastric ulceration, although Cox-1 is thought to be responsible for production of cytoprotective PGs .

In contrast to *Cox*-1-deficient mice, genetic deletion of the *Cox-2* gene caused abnormalities more severe. Most homozygous mutant animals died at approximately 8 weeks after birth, due to abnormalities in their kidneys. Kidneys from newborn homozygous mice were normal, but those of 8-week-old homozygous mutant mice were small and pale. The number of glomeruli was reduced below that of wild-type kidneys. Hence, the renal pathology of *Cox-2*-deficient mice was considered to be due to defective postnatal development of the glomeruli (Dinchuk *et al*. 1995, Morham *et al*. 1995). Another neonatal event in *Cox-2*-deficient mice was defective closure of the ductus arteriosus. Although the ductus showed normal closure in *Cox-1* null mice, about 35% of *Cox-2* null mice died with a patent ductus within 48 hours after birth. However, the fact that 65% of *Cox-2*-null mice survived to weaning suggests that Cox-1 can play a compensatory role (Loftin *et al*. 2001).

The genetic disruption of *Cox-2* produced multiple failures in female reproductive processes. *Cox-2* knockout females were largely infertile due to ovulation failure. Their ovaries were small, owing to a virtual absence of the corpora lutea, but ovarian follicular development was apparently normal (Dinchuk *et al*. 1995). In addition, their implantation and decidualization processes were defective (Langenbach *et al*. 1995, Lim *et al*. 1997). Lim *et al*. (1997) found that in homozygous *Cox-2* knockout female mice, the principal early reproductive defect involved uterine receptivity to implantation. This finding was demonstrated by transplanting wild type (*Cox-2* +/+) murine blastocysts into homozygous mutant *Cox-2*-deficient pseudopregnant mice. In recipient mothers, blastocyst implantation was impaired, a defect which could not be reversed by injection of progesterone. The Cox-2 selective inhibitor Dup697 could prevent implantation also in wild type and heterozygous (*Cox-2* -/+) mice. These results indicate that the uterine epithelium of homozygous *Cox-2* mutant mice is largely responsible for the implantation failure. Lim *et al.* (1997) showed that a stable analog of PGI_2 could restore the decidualization of *Cox-2*-deficient mice, and thereafter showed the importance of PGI_2 and inducible prostaglandin synthesis in early stages of implantation.

Compensatory Cox expression may, at least in part, explain why Cox-deficient mice are able to survive as well as they do (Kirtikara *et al*. 1998). However, these experimental observations on *Cox*-deficient mice clearly indicate that the original hypothesis that Cox-1 is involved in homeostatic functions, while expression of Cox-2 modulates inflammatory reactions, is too simple. It appears more likely that Cox-1 and -2 have distinct as well as overlapping actions in maintaining homeostasis in mice.

1.5. Regulation of COX-2 mRNA stability

COX-2 gene expression is regulated at both transcriptional and post-transcriptional levels (Ristimäki *et al.* 1994, Hla *et al.* 1999, Smith *et al.* 2000). In eukaryotic cells, the half-life of mRNA can vary from 20 minutes to 24 hours (Ross *et al.* 1995) and thus regulation of mRNA stability is an important component of gene expression (Chen and Shyu 1995, Brennan and Steiz 2001). This is especially evident for short-lived transcripts, including certain proto-oncogenes, cytokines, and cytokine-response-genes that are inherently unstable. These genes are usually transcribed in a rapid but transient manner. This combination of regulatory steps of gene expression facilitates tight control over these potentially harmful proinflammatory and transforming factors. Degradation of mRNA can be regulated by specific cis-elements and trans-acting factors. The best-studied cis-element is the adenylate- and uridylate-rich (AU-rich) element (ARE) found in the 3'untranslated regions (UTRs) of many unstable mammalian mRNAs (Chen and Shyu 1995). An AU-rich consensus sequence was first observed in the 3'UTR of murine and human TNF-α mRNAs as well as in the mRNAs encoding human lymphotoxin (TNF-β), colony- stimulating factor (CSF), human and mouse IL-1, and human and rat fibronectin (Caput *et al.* 1986). Comparison of the ARE sequences of many oncogene and cytokine mRNAs led to the identification of motifs often present in multiple copies within the 3'UTR and constituting an AU-rich stretch of 50 to 150 nucleotides. When these AUUUA motifs are present within a functional ARE, they have a destabilizing role (Akashi *et al*. 1994; Chen *et al.* 1994). AREs are also potent stimulators of both decapping and deadenylation processes (Xu *et al.* 1997, Gao *et al.* 2001). AREs differ considerably in size, AU content, and number of AUUUA motifs. Databases have been developed which contain eucaryotic mRNA UTR sequences and specific regulatory signals.

Trans-acting factors are RNA-binding proteins that selectively bind to mRNA's 3'UTRs AU-rich areas. Several ARE-binding proteins have been identified. The Hu family of proteins are a group of RNA-binding proteins related to embryonic lethal abnormal vision (ELAV) proteins, essential in

Drosophila for neuronal development (Campos *et al.* 1985). Hu proteins have been shown to stabilize or to activate translation of target mRNA or both. The Hu family consists of three members that are neuronal tissue-specific: Hel-N1 (also known as HuB), HuC, and HuD. HuR (or HuA) is a Hu family member which is ubiquitously expressed (Ma *et al.* 1996). Other proteins capable of binding ARE are heat shock protein 70, nuclear ribonucleic proteins (nRNP) like human nRNP (hnRNP) A1, hnRNP C, and hnRNP D, also known as AUF-1 (Brewer 1991), RNA-binding proteins with enzymatic activity, such as glyseraldehyde-3-phosphate dehydrogenase (GAPDH), and AUH. HuR is the best-characterized ARE-binding protein (Brennan and Steiz 2001). It is predominantly expressed in the nucleus, but has been shown to shuttle between nucleus and cytoplasm (Fan and Steiz 1998). HuR binds to ARE-containing mRNAs in the nucleus, and this complex is then transported to the cytoplasm. In the cytoplasm, mRNAs are released to the translational apparatus or released for rapid degradation (Gao and Keene 1996), after which HuR returns to the nucleus or degrades. Use of cell-permeable peptides has allowed identification of two distinct nucleocytoplasmic export mechanisms for c-fos mRNA (Gallouzi and Steiz 2001). One of these export proteins is the export receptor chromosome maintenance region 1 (CRM1), which binds to leucine-rich nuclear export signals present in two protein ligands of HuR: pp32 and APRIL. Shuttling of pp32 and APRIL is totally blocked by inhibition of CRM1 with a fungal metabolite called leptomycin B (LMB). LMB can cause G1 cycle arrest in mammalian cells and is a potent anti-tumor agent against experimental tumors in mice (Yoshida *et al.* 1990, Komiyama *et al.* 1985). However, LMB has been shown to cause toxic side-effects in phase I trials for treatment of cancer (Kau and Silver 2003). In the case of RNA export, CRM1 binds to ribonuclear proteins containing a nuclear export sequence (NES). CRM1 inhibition produced an increased association of HuR with pp32 and with APRIL, and an increase in HuR ability to bind nuclear poly(A)+RNA *in vivo*. LMB inhibits the stabilization of COX-2 mRNA in human mammary cancer cells and suppresses IL-1β-induced COX-2 expression in human colon cancer cells and umbilical vein endothelial cells (Jang *et al.* 2003).

HuR is known to stabilize several inducible mRNAs such as c-fos and TNF-α (Ma *et al.* 1997) and recently it was demonstrated that COX-2 mRNA contains HuR-binding AREs in its 3'UTR (Nabors *et al.* 2001, Dixon *et al.* 2001, Sengupta *et al.* 2003, Subbaramaiah *et al.* 2003, Cok *et al.* 2003). Consistent with its function as an mRNA stability protein, overexpression of HuR led to increased COX-2 expression in colorectal cancer cells (Dixon *et al.* 2001) and the down-regulation of HuR inhibited COX-2 expression in breast cancer cells (Sengupta *et al.* 2003). IL-1β was the first agent

shown to increase the half-life of the COX-2 transcript (Ristimäki *et al.* 1994), and recently IL-1β was shown to increase the cytosolic content of HuR protein/COX-2 mRNA complexes (Cok *et al.* 2003). Previously, two staining patterns of HuR have been observed in human tumors: nuclear and cytoplasmic. There are no studies in which expression of HuR would have been connected with any clinical parameters in human tumors.

2. NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

2.1. Mechanism of NSAIDs

Reduction in prostanoid production by inhibition of COX enzymes appears to be the main mechanism of most NSAIDs. These drugs compete with AA for binding to the active site of the COX enzyme but do not inhibit the peroxidase activity (Smith and Marnett 1991). The classical NSAIDs, such as ASA, are nonselective, inhibiting both COX-1 and -2. Acetylation of serine 530 in COX-1 and serine 516 in COX-2 by ASA causes an irreversible inactivation of the COX enzymes (O'Banion *et al*. 1992). The substrate-binding site in COX-2 is larger and has a slightly different shape than that of COX-1. This allows the COX-2 selective inhibitors access to a side pocket located off the main substrate-binding site (Luong *et al*. 1996). Inhibition of COX-1 is thought to cause undesirable gastric adverse effects such as ulcers and reduction in renal blood flow. The analgetic, anti-pyretic, and anti-inflammatory effects of NSAIDs are thought to follow the inhibition of COX-2 activity. However, in rats, neither the COX-1 selective inhibitor SC-560 nor the COX-2 selective inhibitor celecoxib sufficed to induce ulcers, but gastric damage occurred when these two inhibitors were administered in combination, suggesting that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric injury (Wallace *et al*. 2000). In the platelet, the only detectable isoform is COX-1, and reduced platelet aggregation is not only a well-established adverse effect of ASA, but also the therapeutic tool against thromboembolic diseases (Vane *et al*. 1998).

Celecoxib is selective for COX-2, but at high concentrations it also affects COX-1 activity (McAdam *et al.* 1999). Selective COX-2 inhibitors, rofecoxib (Langman *et al.* 1999) and celecoxib (Emery *et al.* 1999), cause fewer gastrointestinal side-effects than do nonselective NSAIDs. These kinds of results has been observed also in clinical trials in which selective COX-2 inhibitors, celecoxib, rofecoxib, valdecoxib, and lumiracoxib have been approved for use by the US Food and Drug Administration. In the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (Bombardier

et al. 2000), in the Celecoxib Long-Term Arthritis Safety Study (CLASS) (Fizgerald and Patrono 2001, Fizgerald 2003), and in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET) (Schnizer *et al.* 2004, Farkouh *et al.* 2004) the incidence of serious gastrointestinal events was reduced significantly in patients receiving coxibs in comparison with traditional NSAIDs. However, according to the recent Adenomatous Polyp Prevention on Vioxx (APPROVe) study, rofecoxib led to an increased risk for thromboembolic events that can cause myocardial infarction and stroke (Merck announcement 2004). The main mechanism behind this event is the depression of PGI_2 production while the production of Tx remains stable, which can elevate blood pressure by increasing vasoconstriction and induce thrombotic events by enhancing the aggregation of platelets (Fizgerald 2004). The higher a patient's intrinsic risk for cardiovascular disease, the more likely that these events may cause myocardial infarction and stroke.

2.2. NSAIDs in benign gynecologic and obstetric processes

High concentrations of prostanoids have been observed during normal menstruation, but especially in connection with menorrhagia (Ylikorkala 1994), dysmenorrhea (Bieglmayer *et al.* 1995), and endometriosis (Karck *et al.* 1996, Garzetti *et al.* 1998), all of which directly correlate with painful menstruation (Koike *et al.* 1992). COX activity has been implicated in the pathogenesis of polycystic ovary syndrome. Polycystic ovaries release greater amounts of PG than do normal ovaries (Navarra *et al.* 1996). Elevated levels of PGs have also been connected with infection and inflammation as a consequence of high levels of cytokines. In all these states, COX-2-selective inhibitors are at least as effective in pain relief and reduction of inflammatory reactions as are conventional NSAIDs, but offer a better gastrointestinal safety profile. Use of NSAIDs in women attempting to become pregnant has been associated with reversible female infertility (Smith *et al.* 1996, Akil *et al.* 1996, Mendonca 2000, Norman 2001, Norman and Wu 2004). The use of selective or non-selective COX-inhibitors may cause luteinized unruptured follicle syndrome, a condition characterized by the ability of a normally growing preovulatory follicle to luteinize, but not rupture, in response to the luteinizing hormone (LH) surge. This results in entrapment of the oocyte and in infertile cycles. This syndrome has developed also after use of COX-2 selective inhibitor rofecoxib in normal daily amounts during the ovulation period (Pall *et al.* 2001). NSAIDs have served as tocolytic agents, among beta-2 agonists, calcium-channel inhibitors, and oxytocin antagonists. However, dose-dependent fetal side-effects limit their use. Oligohydramnions and constriction or premature closure of the fetal ductus arteriosus are frequent with traditional NSAIDs, but also occur after short-term use of the COX-2 selective inhibitor nimesulide (Sawdy *et al.* 2003a). These side-

effects might be diminished by reduction in the dose of nimesulide without reduction in effectiveness (Sawdy *et al.* 2003b). After long-term use of nimesulide, no constriction of fetal ductus arteriosus could be observed in one study (Sawdy et al. 2004). It has been shown that tocolytic effects may be due to COX-2 inhibition, whereas fetal side-effects may be due to COX-1 inhibition (Sawdy *et al.* 1997, Yousif and Thulesius 1998).

2.3. NSAIDs and cancer

Record-linkage studies have shown a lower incidence of gastrointestinal cancers among patients with rheumatoid arthritis, and use of NSAIDs was suggested to be responsible for this phenomenon (Laakso *et al*. 1986, Gridley *et al*. 1993). Consistent with these early observations, several epidemiological studies have shown an association between prolonged use of ASA and reduced risk for colon cancer (Thun 1994). Furthermore, the nonselective NSAID sulindac and the COX-2 selective inhibitor celecoxib have reduced polyp burden in familial adenomatous polyposis (FAP) patients (Giardiello *et al*. 1993, Steinbach *et al*. 2000, Phillips *et al*. 2002). Clearly, NSAIDs have the potential to suppress carcinogenesis in the large bowel. Use of ASA has been associated with reduced incidence of cancer in the stomach and esophagus (Coogan *et al*. 2000; Farrow *et al*. 1998), and in the lung and breast (Harris *et al*. 1996; Schreinemachers and Everson, 1994).

In ovarian cancer, regular use (three or more times per week for at least 6 months) of ASA was observed to associate with a reduced risk, after adjustment for age at menarche, parity, oral contraceptive use, and family history of first-degree breast cancer (Akhmedkhanov *et al.* 2001). However, in case-control studies, acetaminophen was the only NSAID significantly associated with reduced risk for ovarian cancer (Cramer *et al.* 1998, Rosenberg *et al.* 2000, Moysich *et al.* 2001), and the difference was most significant with the greatest frequency of use (Cramer *et al.* 1998, Moysich *et al.* 2001) and the longest duration (Rosenberg *et al.* 2000, Moysich *et al.* 2001). In cohort studies (Rodriguez *et al.* 1998, Fairfield *et al.* 2002, Sorensen *et al.* 2003), use of ASA, acetaminophen, or other NSAIDs has been inversely associated with ovarian carcinoma, and there was direct association of duration of use with cancer risk (Sorensen *et al.* 2003). There have also been studies reporting no association of ovarian cancer risk with NSAIDs (Tavani *et al.* 2000, Friis *et al.* 2002, Meier *et al.* 2002, Lacey *et al.* 2004). Acetaminophen is a poor COX inhibitor, it has only weak anti-inflammatory activity, and it has not been demonstrated to have any protective effect against colorectal carcinoma. It has, however, an antigonadotropic effect, which may explain the reduction in risk for ovarian cancer after its long use (Cramer *et al.* 1998).

There exist no studies on association of selective COX-2 inhibitors with risk for ovarian cancer. *In vitro*, the selective COX-2 inhibitor NS-398 inhibited production of PGE_2 and proliferation of an ovarian carcinoma cell line (Rodriguez-Burford *et al.* 2002, Denkert *et al.* 2003). The cell proliferation was inhibited at concentrations of 50 to 500 μM, which are above the concentrations needed for inhibition of PGE_2 production and which are 10 to 100 times as high as circulating levels of the drug during *in vivo* administration (Denkert *et al.* 2003). Furthermore, that inhibition of cell proliferation could not be reversed by addition of exogenous PGE_2 suggests that NS-398 can reduce ovarian cancer cell proliferation, but the effect is independent of COX-2. The significance of this finding is unclear, due to the relatively high dose used in this *in vitro* setting. To this end, it is uncertain whether the use of NSAIDs or COX-2 selective drugs can act as chemopreventive agents in women at high risk for ovarian cancer. On the other hand, since COX-2 selective inhibitors are tested as neoadjuvant and adjuvant treatment modalities in various types of cancer, it would be important to know the extent of COX-2 expression also in ovarian carcinoma and whether it correlates with clinical parameters including survival.

3. COX-2 DURING PHYSIOLOGICAL GYNECOLOGICAL CONDITIONS

3.1. COX-2 during the menstrual cycle

The relationship between PG biosynthesis and ovulation was first observed during the early 70's (Armstrong 1981). Indomethacin-dependent inhibition of ovulation was initially reported in rats and rabbits (Armstrong and Grinwich 1972), but was subsequently observed in numerous animal species and humans (Armstrong 1981). The relationship between PGs and ovulation was further strengthened by investigations in which PGs were able to restore ovulation in indomethacin-treated animals (Tsafriri *et al.* 1973). By immunohistochemical methods, COX-2 expression has been discovered in the granulosa cell layer of human secondary and developing follicles (Tokuyama *et al.* 2003) and the corpus luteum (Matsumoto *et al.* 2001b), but not in primary or Graafian follicles (Matsumoto *et al.* 2001b, Tokuyama *et al.* 2003), in corpus albicans or the stromal cell layer of the ovary (Matsumoto *et al.* 2001b), in normal ovarian surface epithelium (Matsumoto *et al.* 2001b, Denkert *et al.* 2002, Li *et al.* 2004, Shigemasa *et al*. 2003) or in the epithelial cells of inclusion cysts (Matsumoto *et al.* 2001b, Shigemasa *et al*. 2003). Cox-2 induction appears immediately after the LH surge in mice (Richards 1995). The time between surge and induction of COX-2 varies between animal species and affects the length of the ovulatory process (Richards 1997). Studies on the regulation of COX-2 in human pre-ovulatory follicles have been limited. However, the COX-2 transcript in granulosa cells and the COX-2 protein in preovulatory follicular fluid have been detected in women enrolled in *in vitro* fertilization programs (Narko *et al.* 1997, Tokuyama *et al.* 2001, 2003). In primates, COX-2 protein and intrafollicular PG levels can be observed 24 and 36 hours after human chorionic gonadotropin (hCG) injection, which mimics the endogenous LH surge, and COX-2 mRNA as early as 12 hours post-hCG treatment in both granulosa and theca cell layers (Duffy and Stouffer 2001). This COX-2 induction accompanies normal oocyte development and seems to be necessary to produce the proteolytic enzymes that rupture the follicles (Tsafriri 1995). COX-2-negative follicles were not luteinized and contained a compact cumulus-oocyte complex, suggesting an apparent failure to respond to the gonadotropin pre-ovulatory signal (Liu and Sirois 1998). The inductive trigger for increased COX-2 expression during ovulation may involve LH, follicle-stimulating hormone (FSH), transforming growth factor-α (Li *et al.* 1996), and/or IL-1β (Narko *et al.* 1997). In addition, oocyte-derived factors enhance PG synthesis during the ovulatory process (Elvin *et al.* 1999), and PGs act as inducers of COX-2 expression in luteal cells (Nakamura and Sakamoto 2001, Tsai and Wiltbank 2001).

In human endometrium during the normal menstrual cycle, COX-2 is mainly localized to the perivascular cells and in the basal cell layer of proliferative glandular endometrium, but not in secretory phase glandular endometrium (Marions and Gemzell 1999, Uotila *et al.* 2002). The expression of COX-2 increases premenstrually (Jones *et al.* 1997), but no COX-2 expression is found in endometrium of postmenopausal women after two years' use of continuous combined hormone replacement therapy (Hsu *et al.* 2003).

Analysis of COX-2 expression in the cervix uteri of rodents and primates suggests that normally COX-2 is absent or only weakly expressed in the cervix during the normal menstrual cycle but increases during labor and parturition (Dong *et al.* 1996, Wu *et al.* 2000). In humans, although no immunoreactive COX-2 protein has been detectable in the histologically normal cervix (Kulkarni 2001, Sales 2001), Landen *et al.* (2003) could find positive COX-2 expression in 50% of human papillomavirus infected cervix samples.

3.2. COX-2 during pregnancy

During pregnancy the uterine myometrium relaxes and accomodates the growing fetus. However, when labor commences, the uterine myometrium have to contract cyclically to give birth to the fetus. The quiescence of pregnant uterus is depending on the critical balance of oxytocin and its receptors (Zeeman *et al.* 1997), steroid hormones progesterone and estrogen (Zervou *et al.* 2002), gap-junction protein connexin (Chow *et al.* 1994) and synthesis of PGs and its receptors (Challis *et al.* 1997). Increased prostaglandin synthesis is thought to play a key role in the onset of labor and progress of parturition.

Synthesis of PGs is suppressed in the ovary and in the endometrium after ovulation (Smith *et al.* 1996), but it is increased again during the implantation period, which is an inflammatory-like reaction important for the increase in endometrial vascular permeability. When progesterone levels are maximal (i.e., during pregnancy), basal PG production falls (Maathuis 1978). In the human endometrium, PG synthesis and COX-2 are up-regulated by gonadotropins at the time of decidua formation (Han et al 1999) and with the start of pregnancy in the luminal epithelium and the perivascular cells of human endometrium (Marions and Gemzell 1999). In animals, COX-2 expression occurs in the stromal cells of endometrium at the site of implantation (Kim *et al*. 1999, Chakraborty *et al.* 1996, Charpigny *et al.* 1997). In mice, Gross *et al.* (1998) found Cox-1 to be

important for normal entry into parturition and that its absence led to peripartal fetal death; Cox-2 could not compensate for the loss of Cox-1, thus supporting the role of Cox-1 in early stages of labor in this model. They also showed, however, that their pregnant knockout mice with the Cox-1 and oxytocin null mutation had a normal onset of labor, although duration of labor was much longer than in wild type mice (Gross *et al.*1998). This indicates that Cox-1 may play some role in parturition. Because the Cox-1 has not been shown to be an inducible enzyme in this system, it may mediate the initial stages of labor onset, and when it undergoes suicide inactivation, the inducible Cox-2 may assume most of the responsibility for sustaining myometrial contractility and cervical ripening.

The fetal membranes are thought to be the main source of PGs within the human uterus during parturition, and with labor a large increase occurs in production of PGs in fetal membranes (Skinner and Challis 1985). This increase in PG synthesis is thought to be due to induction of COX-2 expression in the fetal membranes (amnion, chorion, deciduas), while COX-1 expression levels remain constant (Teixeira *et al.* 1994, Hirst *et al.* 1995, Slater *et al.* 1995, Fuentes *et al.* 1996, Mijovic *et al.* 1997, Slater *et al.* 1998). In contrast to fetal membranes, myometrial COX-2 expression decreased (Zuo *et al*. 1994) or showed no change (Moore *et al.* 1999, Sparey *et al.* 1999) at the onset of labor as detected by *in situ* hybridization, reverse-transcriptase polymerase chain reaction (RT-PCR), or immunoblotting. However, Slater *et al.* (1999) found COX-2 mRNA and protein to increase at term in myometrium prior to the onset of labor, while COX-1 expression stayed constant or decreased. This would imply COX-2 plays a role during labor onset.

Bacterial products can induce cytokine and PG biosynthesis. Increased expression of COX-2 is a consequence of the stimulatory effect of bacterial lipopolysaccharides (LPS) and proinflammatory cytokines. IL-1β, TNF-α, and other cytokines are elevated in fetal membranes and in the amniotic fluid of women with intra-amniotic infection (Romero *et al.* 1989), but also in spontaneous term labor (Romero *et al.* 1990). IL-1β stimulates the production of PGs in human myometrial cells (Pollard and Mitchell 1996, Gramatopoulos and Hillhouse1999) and induces COX-2 expression in an immortalized human myometrial smooth muscle cell line (Belt 1999). The effect of PGs on the function of myometrium is dependent in part on the diversity of PG receptors. The EP_3 receptor is more prominently expressed in the fundal part of the primate uterus, whereas the relaxatory EP_2 and EP_4 receptors dominate in the lower part (Smith *et al.* 1998). As myometrial cells mainly produce PGI_2 and PGE_2, their main function in the lower part of the uterus is relaxation rather than contraction. Myometrial PG receptors are also dependent on gestational age, and in early pregnancy

the contractile EP_3 and FP receptors are down-regulated (Matsumoto *et al.* 1997), whereas during labor at term, FP receptor expression increases (Brodt-Eppley and Myatt 1999).

4. COX-2 AND CARCINOMAS

4.1. Ovarian carcinoma

4.1.1 Statistics on ovarian cancer

Ovarian cancer is the second most common gynecological malignancy in the Western world, and it is the sixth most common cancer in females in Finland (Finnish Cancer Register 2001). Ovarian carcinoma is the most fatal malignancy among gynecological cancers and was the fifth most lethal cancer type after breast, lung, pancreas, and colon cancers in females in 2001. In Finland 478 new cases of ovarian cancer were diagnosed in 2001. Ovarian carcinomas, i.e., tumors originating from the surface epithelium, account for approximately 85 to 90% of all malignant ovarian tumors and exhibit several histological subtypes with different pathogenesis and outcome. The most common histological subtypes of epithelial ovarian carcinoma are serous, endometroid, and mucinous carcinoma, representing 40 to 50%, 15 to 20%, and 5 to 15% of all cases. Less common histological subtypes include clear cell (mesonephroid), and undifferentiated carcinoma, malignant mixed epithelial tumor, and transitional cell carcinoma (malignant Brenner tumor) (Heinz *et al.* 2001).

Table 2. Mean annual numbers, incidence, deaths, and mortality from gynecological cancers in 2001 and predicted number of cases for 2004 in Finland (Finnish Cancer Register 2001).

Primary site	No. of cases 2001	Incidence per 100,000	Deaths in 2001	Mortality per 100 000	Predicted for 2004
Corpus uteri	633	12.7	168	2.6	750
Ovary	478	10.2	262	4.7	471
Cervix uteri	160	3.8	61	1.2	142
Vagina, vulva, tuba	115	2.0	56	0.7	

4.1.2 Origin of ovarian cancer

The Müllerian duct system forms the fallopian tubes, the uterus, and the upper part of the vagina, and this celomic epithelium differentiates into the mesothelial lining of the ovarian surface and the epithelial lining of the fallopian tubes, endometrium, and endocervix. The ovary itself is thought to

be of mesonephroid origin, but during adult life its surface mesothelium frequently undergoes Müllerian metaplasia. Serous ovarian carcinomas are therefore morphologically similar to epithelial tumors arising in the fallopian tube, the mucinous arising in the endocervix, and the endometroid in the endometrium (Salazar *et al.* 1995).

According to the currently favored theory, ovarian carcinomas arise from the ovarian surface epithelium, usually in the context of invaginations that create intraovarian cysts (Scully 1995, Aoki *et al.* 2000, Okamura and Katabuchi 2001). The etiology and early events in the progression of these carcinomas are not well understood compared to other human malignancies. This is due to lack of access to preneoplastic tissues because of the difficulty in diagnosing ovarian carcinoma at an early stage (Scully 1995). However, Salazar *et al.* (1996) have observed numerous atypical features in ovaries removed prophylactically from cancer-prone women when compared with those at no known increased risk for disease. These features included surface epithelial pseudostratification, surface papillomatosis, epithelial inclusion cysts with epithelial hyperplasia, stromal or hilar hyperplasia, and increased follicular activity, all of which are rarely seen in normal ovaries. Multiparity, lactation, tubal ligation, hysterectomy, and use of oral contraceptives are associated with decreased risk for ovarian cancer (Whittemore *et al.* 1992, Hankinson *et al.* 1993). Risk factors have been reported to differ between histological subtypes; the protective effects of parity and of oral contraceptives appear not to involve mucinous carcinoma (Kvale *et al.* 1988, Risch *et al.* 1996). An estimated 5 to 10% of ovarian carcinomas are of an inherited predisposition. Known cancer-predisposing syndromes linked to ovarian carcinoma include breast and ovarian cancer syndrome (*BRCA1/BRCA2* genes) and the hereditary non-polyposis colorectal cancer (HNPCC) syndrome (Boyd and Rubin, 1997).

4.1.3 Prognosis of ovarian cancer

Of ovarian cancers, 70% are diagnosed with widespread intra-abdominal disease or distant metastases, which partially accounts for the poor prognosis. Stage according to the International Federation of Gynecology and Obstetrics (FIGO) staging system and grade of this disease at diagnosis are important determinants of prognosis. The five-year overall survival rate is 48%, ranging from 80 to 90% at stage I, 64 to 70% at stage II, and 29 to 59% at stage III, down to 17% at stage IV (Heinz *et al.* 2001). The different histological types of ovarian carcinoma show different clinical behavior and prognosis. The overall prognosis for mucinous ovarian carcinoma is relatively good (68%) due to late dissemination of the tumor cells, but the outcome in primarily advanced

disease is particularly poor due to resistance to the chemotherapeutic agents commonly used (Omura *et al.* 1991, Makar *et al.* 1995, Heinz *et al.* 2001). The 5-year overall survival rate for serous and endometroid carcinomas is 40% and 60% (Heinz *et al.* 2001). Serous ovarian tumors are more often malignant and bilateral than are mucinous tumors. In addition to stage and grade, other prognostic factors of ovarian carcinoma are residual tumor size, age, and performance status, but not primary tumor size (Friedlander 1998).

Stage and grade of ovarian carcinoma are the main determinants in decisions about treatment methods. Radical surgery or at least maximal debulking is the cornerstone of effective treatment of ovarian cancer. It is essential for accurate staging, histological classification, and grading, and it is a good basis for selection of adjuvant chemotherapy. Carboplatin-paclitaxel chemotherapy has been the first-line chemotherapy in Finland for ovarian carcinomas of stage Ib and advanced stages since 1996. Based on the previous reports, adjuvant chemotherapy may improve overall survival and recurrence-free survival also in patients with early stage ovarian carcinomas.

4.1.4 Prognostic markers in ovarian cancer

Cytogenetic analyses have revealed abnormal karyotypes in approximately 50 to 90% of ovarian carcinomas. Cytogenetic abnormalities and their complexity are correlated with grade of ovarian carcinoma. Cytogenetic alterations exist most often in ovarian carcinomas of the serous type (Pejovic *et al.* 1992, Taetle *et al.* 1999). Aberration of the tumor-suppressor gene *p53* are the most frequent molecular alteration detected in ovarian carcinoma (Marks *et al.* 1991, Klemi *et al*. 1995), and it has been predicted to confer poor prognosis (Hartmann *et al.* 1994, Klemi *et al*. 1995, Reles 2001), whereas other studies have found no correlation between p53 status and survival (Marks *et al.* 1991, Fallows *et al.* 2001). Normal surface epithelium of ovaries and normal serous epithelium of the fallopian tube express only weak immunopositivity for the tumor suppressor gene p53, but this expression is either lost or enhanced in 59% of serous ovarian carcinomas (Lassus *et al.* 2003). Lassus *et al.* (2003) observed that prognosis of the ovarian carcinoma patients was reduced in p53-negative or -high groups when compared to the p53-low group. Aberrant p53 expression was associated with poor response to therapy, high risk of recurrence, and poor overall survival also in well-differentiated, early-stage serous ovarian carcinomas. Thus, these two altered patterns of p53 expression (negative or high) most likely represent loss of p53 tumor suppressor gene function, negative expression reflecting either loss or inactivation of the gene, and high expression represents

a mutation of the gene that led to stabilization of the nonfunctional p53 protein. Alterations in p53 have been as usual in familial ovarian carcinomas as in sporadic carcinomas (Auranen *et al.* 1997).

The distal half of chromosome 18q is among the genomic regions most frequently lost in serous ovarian carcinoma (Arnold *et al.* 1996, Tapper *et al.* 1998, Lassus *et al.* 2001). Expression of the tumor suppressor gene SMAD4, located at 18q21, is usually present in the normal surface epithelium of the ovary, but is absent or weak in 28% of serous ovarian carcinomas (Lassus *et al.* 2001). In addition to inactivation of tumor suppressor genes, activation or overexpression of several proto-oncogenes has been detected in ovarian cancer. For example, amplification of the proto-oncogene *Her-2/neu* (also known as *ErbB2*) occurs in approximately 30% of sporadic ovarian carcinomas (Hellström *et al*. 2001) and in nearly 70% of familial ovarian carcinomas (Auranen *et al.* 1997). The *Her-2* gene encodes for a 185-kDa cell surface glycoprotein which has tyrosine kinase activity and belongs to the epidermal growth factor receptor family. In breast cancer, *Her-2* amplification is associated with aggressive behavior of the tumor, and with poor outcome (Ross and Fletcher 1999). The human anti-Her-2 monoclonal antibody (mAb) trastuzumab (Herceptin) has been used successfully in metastatic breast cancer with Her-2/neu amplification to gain longer survival for these patients (Cobleigh *et al.* 1999, Slamon *et al.* 2001). The prognostic role of Her-2/neu in ovarian carcinoma remains controversial. In several studies, Her-2/neu overexpression or amplification or both have been associated with poor clinical outcome (Slamon *et al.* 1989, Berchuck *et al.* 1990, Meden *et al.* 1994, Fajac *et al.* 1995, Lassus *et al.* 2004), whereas others have shown no effect (Singleton *et al.* 1994, Skirnisdottir *et al.* 2001) or even an increased effect on survival (Haldane *et al.* 1990). In clinical samples, Her-2/neu overexpression can be determined by immunohistochemistry, but detection of the gene copy number by fluorescence *in situ* hybridization (FISH) is necessary to confirm *Her-2/neu* amplification (Press *et al.* 2002). An alternative method, the chromogenic *in situ* hybridization (CISH), using conventional bright-field microscopy, has been developed for detection of the amplified *Her-2/neu* gene (Tanner *et al.* 2000). Lassus *et al*. (2003) found that *Her-2/neu* amplification identified by CISH in serous ovarian cancer was associated with poor survival.

4.2. COX-2 in ovarian carcinoma

By immunohistochemistry COX-2 expression is low or undetectable in normal ovarian surface epithelium (Denkert *et al.* 2002, Landen *et al.* 2003, Shigemasa *et al.* 2003, Li *et al.* 2004). The expression of COX-2 is increased when benign ovarian tumors (COX-2 positivity 0-64%)

(Matsumoto *et al.* 2001b, Klimp *et al.* 2001, Denkert *et al.* 2002) and tumors with low malignant potential (COX-2 positivity 37-67%) (Matsumoto *et al.* 2001b, Klimp *et al.* 2001, Denkert *et al.* 2002) are compared with invasive carcinomas (COX-2 positivity 31-83%, Table 3) (Matsumoto *et al.* 2001b, Klimp *et al.* 2001, Denkert *et al.* 2002, Ferrandina *et al.* 2002a and b, Shigemasa *et al.* 2003, Ali-Fehmi *et al.* 2003, Li *et al.* 2004, Raspollini *et al.* 2004, Seo *et al.* 2004). This relatively high variation in COX-2 positivity may depend on the use of different antibodies, scoring criteria, and selection of patients. Only Shigemasa *et al.* (2003) and Seo *et al.* (2004) examined COX-2 expression in different histological subgroups, whereas other researchers considered all ovarian cancer histologies as a single entity. Shigemasa *et al.* (2003) found a significant difference in frequency of COX-2 expression between endometrioid (56% of 16) versus mucinous (17% of 18), and endometrioid versus clear cell (14% of 14) carcinomas, but not between serous (34% of 38) ovarian carcinomas versus other histological subtypes. According to the Seo *et al.* study (2004), the COX-2 expressing tumors were more common among nonmucinous (65% of 46) than mucinous (6% of 18) ovarian carcinoma histologies. Methods other than immunohistochemistry have also been used to observe the expression of COX-2. Denkert *et al.* (2002) and Shigemasa *et al.* (2003) used the reverse transcriptase polymerase chain reaction (RT-PCR), and Ferrandina *et al.* (2002a) and Li *et al.* (2004) used Western blotting to investigate COX-2 expression. Expression of COX-2 appeared in 42 to 100% of ovarian tumors by immunoblotting and in 72 to 88% by RT-PCR. According to most studies COX-2 expression occurs in epithelial carcinoma cells of ovarian tumors, but only a few studies have shown stromal COX-2 expression in macrophages (Klimp *et al.* 2001) or in lymphoid cells (Ferrandina *et al*. 2002 a and b).

Elevated expression of COX-2 in epithelial cancer cells is associated with reduced survival (Ferrandina *et al.* 2002a and b, Sigemasa *et al.* 2003, Ali-Fehmi *et al.* 2003, Seo *et al.* 2004, Raspollini *et al.* 2004), especially in patients over 60 years old (Denkert *et al*. 2002). COX-2 expression is an independent prognostic factor for poor survival (Denkert *et al.* 2002) and reduced likelihood of response to chemotherapy in multivariate analysis (Ferrandina *et al.* 2002a and b, Raspollini *et al.* 2004). Seo *et al.* (2004) observed the high COX-2 expression to associate with clinical factors such as histological type, stage, grade, presence of ascites, and residual tumor size. However, many studies show no correlation between COX-2 expression and clinical factors (Matsumoto *et al.* 2001, Denkert *et al.* 2002, Ferrandina *et al.* 2002a, Shigemasa *et al.* 2003, Li *et al.* 2004).

Table 3. Expression of COX-2 in benign, low malignant potential (LMP) and malignant ovarian tumors.

Method	COX-2 positive tumors % (n[1])			Reference
	Benign	LMP[2]	Malignant	
Immunohistochemistry	0% (3)	0% (3)	0% (13)	Dore *et al.* 1998
	50% (8)	67% (21)	79% (28)	Matsumoto *et al.* 2001b
	64% (14)	67% (15)	83% (18)	Klimp *et al.* 2001
	0% (14)	37% (19)	42% (86)	Denkert *et al.* 2002
	ND[3]	ND	47% (76)	Ferrandina *et al.* 2002a
	ND	ND	45% (87)	Ferrandina *et al.* 2002b
	0% (6)	ND	14-56%[4] (86)	Shigemasa *et al.* 2003
	ND	ND	68% (125)	Ali-Fehmi *et al.* 2003
	0% (9)	ND	71% (137)	Li *et al.* 2004
	ND	ND	69% (78)	Raspollini *et al.* 2004
	ND	ND	6-65%[5] (64)	Seo *et al*. 2004
Western blotting	ND	ND	100% (2)	Ferrandina *et al.* 2002a
RT-PCR[6]	ND	100% (1)	88% (8)	Denkert *et al.* 2002
	ND	ND	72% (36)	Shigemasa *et al.* 2003
Immunofluorecence	0% (13)	ND	89% (9)	Landen *et al.* 2003

[1] Total number of tumors investigated

[2] Low malignant potential

[3] Not determined

[4] Percentage of COX-2-positive tumors (%) in different ovarian carcinoma histologies; clear cell carcinoma 14% (n=14), mucinous carcinoma17% (n=18), serous carcinoma 34% (n=38), and endometrioid carcinoma 56% (n=16).

[5] Percentage of COX-2-positive tumors (%) in mucinous 6% (n=18) and in nonmucinous 65% (n=46) ovarian carcinomas.

[6] Reverse transcriptase polymerase chain reaction

4.3 COX-2 in other gynecological malignancies

4.3.1 Cervical cancer

COX-2 protein expression appeared in 25 to 100% of invasive cervical carcinomas as detected by immunohistochemistry (Table 4) (Ruy *et al.* 2000, Kulkarni *et al.* 2001, Gaffney *et al.* 2001, Sales *et al.* 2001, Ferrandina *et al.* 2002c, Kim *et al.* 2002, Landen *et al.* 2003). COX-2 expression is especially high in the invasive edge of the tumor (Ruy *et al.* 2000). In severe cervical intraepithelial dysplasia (carcinoma in situ) (Kulkarni *et al.* 2001) and in cervical carcinoma, COX-2 expression is localized in the transformed epithelial cells (Ruy *et al.* 2000, Sales *et al.* 2001, Kulkarni *et al.* 2001,

Gaffney *et al.* 2001, Ferrandina *et al.* 2002c, Kim *et al.* 2002, Landen *et al.* 2003) and also in stromal cells (Kim *et al.* 2002, Ferrandina *et al.* 2002c) and in vascular endothelial cells (Sales *et al.* 2001). Elevated COX-2 expression is associated with high age (Ferrandina *et al.* 2002c), large tumor size (Gaffney *et al.* 2001, Ferrandina *et al.* 2002c), adenocarcinoma-like histology (Ferrandina *et al.* 2002c), and parametrial invasion (Ruy *et al.* 2000, Ferrandina *et al.* 2002c), but not with stage or grade (Ferrandina *et al.* 2002c, Kim *et al.* 2002). Patients with COX-2-positive cervical cancer show significantly shorter overall survival than do patients with the COX-2-negative carcinoma (Gaffney *et al.* 2001, Ferrandina *et al.* 2002c, Kim *et al.* 2002). The percentage of COX-2-positive tumors is higher among patients not responding to treatment than among patients with partial or complete response (Ferrandina *et al.* 2002c, Kim *et al.* 2002).

Table 4. Expression of COX-2 in normal uterine cervix, carcinoma *in situ* of the uterine cervix, and cervical carcinoma detected by immunohistochemical methods.

Elevated Cox-2%/ (n)			Reference
Normal	**Carcinoma in situ**	**Carcinoma**	
ND[1]	ND	100% (36)	Ruy *et al.* 2000
0% (13)	100% (1)	92% (13)	Kulkarni *et al*. 2001
ND	ND	83% (24)	Gaffney *et al.* 2001
0% (5)	ND	100% (10)	Sales *et al*. 2001
ND	ND	43% (8)	Ferrandina *et al.* 2002c
ND	ND	30% (75)	Kim *et al.* 2002
50% (14)	ND	25%(12)	Landen *et al*. 2003

[1] Not determined

4.3.2 Endometrial cancer

COX-2 is overexpressed in 39 to 92% of endometrial adenocarcinomas as detected by IHC (Table 5) (Tong *et al.* 2000, Uotila *et al.* 2002, Cao *et al.* 2002, Fujiwaki *et al.* 2002, Ferrandina *et al.* 2002d, Landen *et al.* 2003). In endometrial adenocarcinoma, COX-2 expression localized in epithelial carcinoma cells (Fujiwaki *et al.* 2002, Uotila *et al.* 2002) but not in stromal cells (Cao *et al.* 2002). COX-2 expression correlated with high grade and stage of endometrial carcinoma, but there was no correlation of COX-2 expression with survival of patients (Ferrandina et al. 2002d, Cao et al. 2002).

Table 5. Expression of COX-2 in normal endometrium and endometrial adenocarcinoma detected by immunohistochemical methods.

Elevated COX-2 %/ (n)		
Normal	**Carcinoma**	**Reference**
86% (7)	92% (12)	Uotila *et al.* 2002
0% (3)	73% (11)	Tong *et al.* 2000
ND[1]	39% (69)	Ferrandina *et al.* 2002
ND	ND	Cao *et al.* 2002
ND	ND	Fujiwaki *et al.* 2002
7% (15)	69%(13)	Landen *et al.* 2003

[1] Not determined

4.4 COX-2 in other malignancies

Several different types of carcinomas express COX-2 and produce PGs. Numerous studies have concentrated on colorectal carcinogenesis. Elevated levels of COX-2 mRNA and protein have been detected in 75 to 100% of human colorectal cancers and approximately 50% of preneoplastic lesions (Eberhart *et al.* 1994, Sano *et al.* 1995, DuBois *et al.* 1998). Expression of COX-2 correlates with poor survival in colorectal carcinoma (Masunaga *et al.* 2000), in adenocarcinoma of the esophagus (Buskens *et al.* 2002), and in breast carcinoma (Ristimäki *et al.* 2002). Finally, expression of COX-2 and its role in cancer formation may not be restricted to the carcinomas, since COX-2 is expressed in glioma (Deininger *et al.* 1999), melanoma (Denkert *et al.* 2001), and retinoblastoma (Karim *et al.* 2000).

AIMS OF THE STUDY

Regulation of PG synthesis is known to play a role in many physiological processes and is involved in tumor formation. The purpose of this study was to investigate the expression of COX-2, an inducible enzyme of PG synthesis, in gynecological organs in benign and malignant processes. Myometrial smooth muscle of the uterus was selected as a representative benign tissue type and serous and mucinous types of ovarian carcinoma as a model of the malignant process.

The specific aims were:

To investigate the expression of COX-2 protein in myometrium of the term pregnant uterus in labor in comparison with the uterus not in labor and to study the regulation of COX-2 expression in cultured human myometrial cells. (Study I)

To observe the significance of COX-2 protein expression in ovarian carcinoma and study correlations of expression of COX-2 with clinically meaningful parameters and patient survival in serous and in mucinous ovarian carcinoma. (Studies II and IV)

To study whether mRNA-stabilizing HuR protein is connected with COX-2 expression in serous and mucinous ovarian carcinoma. Finally, to investigate whether expression of HuR itself correlates with clinically meaningful parameters including patient survival (Studies III and IV).

MATERIALS AND METHODS

1. HUMAN TISSUE SPECIMENS (I-IV)

All myometrial tissues and neoplastic and nonneoplastic ovarial tissues were obtained from the Department of Obstetrics and Gynecology of Helsinki University Central Hospital, Finland. The study was approved by the local Ethics Committee. Myometrial tissues were collected in gestation weeks 37 to 42 from 25 healthy women, of which 10 were in labor and 15 not in labor at the time of cesarean section. The cesarean section was done because of disproportion or breech presentation. Ten women in labor had been in labor for 6 to 22 h, and the time elapsed from the rupture of fetal membranes was 4 to 24 h, after which mostly all had their cervix opened. Fifteen women not in labor had no contractions, and their fetal membranes were intact. Preterm cesarean sections were excluded because of the possibility of subclinical infections or maternal health problems, which may cause induction of PG synthesis. All myometrial samples from pregnant women were collected from the upper edge of the uterine incision during lower-segment cesarean section. Myometrial control sample came from fertile woman during hysterectomy for myomatous uterus.

All serous ovarian cancer specimens were collected between the years 1964 and 2000 and mucinous specimens between the years 1989 and 2000 in primary operations before patients had received any chemotherapy (Table 6 and 7). The histology was determined by a gynecological pathologist and was verified by another pathologist (R.B.). The clinical information of the patients was extracted from the patient records and survival information from the Population Register Center. The median age of serous ovarian carcinoma patients was 57 years (range, 15-88 years) and of mucinous carcinoma patients 54 years (range, 24-84 years) at the time of diagnosis. The median follow-up time of patients alive at the end of study period was 5.2 years (range, 0.4-36.1 years) in serous and 4.4 years (range, 0.06 -10.28 years) in mucinous ovarian carcinoma patients. The clinical staging and grading were performed according to the FIGO. New treatment regimens were adopted as follows: platinum-based chemotherapy at the beginning of the 1980s; radical surgery at the end of the 1980s; and paclitaxel/platinum chemotherapy after 1996.

Tissue microarrays were constructed by selecting a representative tumor area from hematoxylin-eosin-stained sections of each tumor by our pathologist (R. B.). Four tissue cores (diameter 0.8 mm) were obtained from each tumor block, and replaced into a recipient paraffin block with a custom-

built precision instrument (Beecher Instruments, Silver Spring, MD). These samples were cut with a microtome into 5 µm-sections, and the presence of cancer cells was verified on hematoxylin-eosin-stained sections.

Table 6. Summary of human tissue samples and methods used in studies

Tissue	Methods used (number of samples analyzed)	Study
Myometrium		
In labor	Northern blot (10) Immunohistochemistry (2)	I
Not in labor	Northern blot (15)	I
Not pregnant	Northern blot (1)	I
Ovary		
Normal	Immunohistochemistry (6)	II, IV
Serous carcinoma	Immunohistochemistry (474 array, 12 histological) Western blot (12) RT-PCR[1] (12)	II, III
Mucinous carcinoma	Immunohistochemistry (64 array, 12 histological) Western blot (3) RT-PCR (3)	IV NP[2]

[1] Reverse transcriptase polymerase chain reaction
[2] Not published

Table 7. Clinical features of 474 serous and 64 mucinous ovarian carcinoma patients.

Clinical feature	Serous ovarian carcinoma n / total (%)	Mucinous ovarian carcinoma n / total (%)
Age		
≤ 57 (serous)/ ≤ 54 (mucinous)[1]	238/474 (50%)	33/64 (52%)
> 57(serous)/ > 54 (mucinous)	236/474 (50%)	31/64 (48%)
Grade		
1	181/466 (39%)	50/64 (78%)
2	121/466 (26%)	13/64 (20%)
3	164/466 (35%)	1/64 (2%)
Stage		
I	101/471(21%)	50/64 (78%)
II	64/471 (14%)	3/64 (5%)
III	251/471 (53%)	10/64 (16%)
IV	55/471 (12%)	1/64 (2%)
Status after follow-up[2]		
deceased	262/474 (55%)	9/64 (14%)
living	212/474 (45%)	55/64 (86%)
Tumor size		
≤ 10 cm	152/467 (33%)	ND[3]
> 10 cm	315/467 (67%)	ND
Residual tumor size		
≤ 1cm	216/423 (51%)	ND
> 1 cm	207/423 (49%)	ND
p53		
negative	69/460 (15%)	0/50 (0%)
normal	193/460 (42%)	40/50 (80%)
overexpression	198/460 (43%)	10/50 (20%)
SMAD4		
negative	33/457 (7%)	ND
low	117/457 (26%)	ND
moderate	255/457 (56%)	ND
high	52/457 (11%)	ND
HER-2/neu		
normal	300/381 (79%)	35/39 (90%)
amplified	81/381 (21%)	4/39 (10%)
Inhibin-α		
negative	ND	7/58 (12%)
positive	ND	51/58 (88%)

[1] Mean age at diagnosis in serous carcinoma was 57 years and 54 years in mucinous carcinoma.

[2] Mean follow-up time for serous carcinomas 5.2 years (range, 0.4-36.1 years) and for mucinous carcinomas 4.4 years (range, 0.06-10.28 years).

[3] Not determined

2. CELL CULTURE EXPERIMENTS (I-IV)

Myometrial cells were isolated from tissues obtained from either lower-segment elective cesarean sections at term (three separate cell lines) and a control cell line from the uterus of a nonpregnant patient who underwent hysterectomy for myomatous uterus. The specimens were digested with collagenase (0.2%, type IV, Sigma, St. Louis, MO, USA) and trypsin (0.05%, Life Technologies, Inc., Paisley, Scotland) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Life Technologies, Inc.), 2 mmol/L L-glutamine, and antibiotics for 2h in 37˚C at 5% CO_2 in air. Primary cultures were grown in DMEM supplemented with 10% fetal calf serum (FCS, Hyclone Laboratories, Inc.), L-glutamine, and antibiotics in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). The cells were used at passages 2 to 7, and exhibited positive staining for monoclonal antihuman α-smooth muscle actin antibody (α-SMA, 1:1000, DAKO Corp., Klostrup, Denmark), which stains smooth muscle cells, but not fibroblasts. These cells grew in a "hill and valley" pattern, and their proliferation rate was quite low, which differs from that of fibroblasts. Cultured smooth muscle cells have been described to retain their morphological and biochemical properties through 16 passages and for at least one year (Casey *et al.* 1984). All this indicates that the cells used in our experiments represented myometrial smooth muscle cells. Before the experiments the cells were starved for 48 h in 0.5% FCS in DMEM, and all incubations were carried out in 0.5% FCS without (control) or with IL-1β, PMA, oxytocin, TNF-α, dexamethasone, and COX-2 selective inhibitor NS-398 (Table 8).

We had three human serous ovarian carcinoma cell lines: OVCAR-3, CaOV-3, and ES-2 (ATCC, Rockville, MD, USA), and EFO-27 that originated from a mucinous ovarian carcinoma (DSMZ, Frankfurt, Germany). OVCAR-3 cells were cultured in DMEM and CaOV-3, ES-2, and EFO-27 in RPMI cell culture medium supplemented with 10% FCS, L-glutamine, and antibiotics. The expression of COX-2 in OVCAR-3 (Table 8), CaOv-3, ES-2, and EFO-27 was studied by stimulating these cell lines with IL-1β or PMA.

The small interfering (si)RNA duplexes against HuR and β-actin were synthesized by Dharmacon Inc. (Lafayette, CO, USA), and the sequences were: HuR Sense 5'-AAC AUG ACC CAG GAU GAG UUA dTdT-3' and HuR Antisense 5'-UAA CUC AUC CUG GGU CAU GUU dTdT-3'. The β-actin siRNA sequence were: β-actin Sense 5'-AAU GAA GAU CAA GAU CAC UGC dTdT-3' and β-actin Antisense 5'-GCA AUG AUC UUG AUC UUC AUU dTdT-3'. The day before transfection, OVCAR-3 cells were trypsinized and diluted 1:20 with optiMEM 1-medium (Life

Technologies, Inc. Invitrogen) supplemented with 10% FCS without antibiotics and transferred to 12-well plates, 1 ml per well (final split ratio 1:2). Transient transfection of siRNAs was carried out using Oligofectamine Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. First, 8 µl OptiMEM medium and 2 µl Oligofectamine were mixed, and at the same time 85 µl OptiMEM was mixed with 5 µl of HuR or β-actin siRNA (20 µM) duplexes. These two reactions were incubated separately for 10 min at room temperature, after which they were combined and incubated for 20 min at room temperature for complex formation. The cells were washed once with serum- and antibiotic-free OptiMEM, and 400 µl of OptiMEM and 100 µl of the transfection mixture was added to the cells. The cells were incubated for 4 hours at 37°C at 5% CO_2 in a cell incubator after which 250 µl of 3x serum medium (30% FCS in OptiMEM with penicillin and streptomycin) was added. The final siRNA concentration in 500 µl was 200 nM. The cells were incubated with the siRNA molecules for 72h, and IL-1β (10 ng/ml) was added for the last 24 h of the incubation period. In the LMB (Sigma) experiment, the cells were incubated with LMB (5-10 ng/ml) in combination with IL-1β for 24 h.

Table 8. Agents and concentrations in cell culture experiments.

Cell line	Agents	Substance concentration	Parameter analyzed	Study
Myometrial	IL-1β	0.01-10 ng/ml	COX-2 protein	I
	IL-1β	10 ng/ml	COX-2 protein, 6-keto$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, and mRNAs for EP_2 and EP_4	I
	PMA	10 ng/ml	COX-2 protein, 6-keto$PGF_{1\alpha}$, and mRNAs for EP_2 and EP_4	I
	Oxytocin	0.4 nM	COX-2 protein	I
	TNF-α	100 ng/ml	COX-2 protein	I
	Dexamethasone	1 μM	COX-2 protein	I
	NS-398	15 μM	COX-2 protein , 6-keto $PGF_{1\alpha}$	I
	Indomethacin	10 μM	6-keto $PGF_{1\alpha}$	I
OVCAR-3	IL-1β	10 ng/ml	Proteins for COX-2, HuR, β-actin	III
	HuR siRNA	200 nM	Proteins for COX-2, HuR, β-actin	III
	LMB	5 and 10 ng/ml	Proteins for COX-2, HuR, β-actin	III

3. ENZYME-LINKED IMMUNOASSAY (EIA) AND RADIOIMMUNOASSAY (RIA) (I)

Myometrial cells were incubated after 48 h starvation in 10-cm^2 tissue culture dishes (Nunc, Roskilde, Denmark) with test agents for 6 hours, after which the cells were washed and incubated with AA (10 μM, Sigma) for 10 min. PGE_2 and $PGF_{2\alpha}$ were analyzed by EIA according to the manufacturer's protocol (Cayman Chemical Co., Ann Arbor, MI, USA) and 6-keto $PGF_{1\alpha}$, a stable hydrolysis product of PGI_2 by RIA (Amershamn Pharmacia Biotech, Uppsala, Sweden) employing specific antibodies and a tritiated 6-keto-$PGF_{1\alpha}$.

4. IMMUNOHISTOCHEMISTRY (I-IV)

Formalin-fixed, paraffin-embedded specimens were deparaffinized and antigen retrieved with microwave oven (4x5 min in 700 W in 0.01 M Na-citrate buffer, pH=6). The slides were then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After that the slides were immersed in blocking solution (1.5:100 normal horse serum in PBS) for 15 min to block unspecific binding sites. Immunostaining was performed with a COX-2-specific antihuman monoclonal antibody (160112; Cayman) at a dilution of 1:200 (2.5 μg/ml) or with a HuR monoclonal antibody 19F12 (Wang *et al*. 2000, Clonegene LCC, Hartford, CT, USA) at a dilution of 1:10 000 (1.0 μg/ml) in PBS containing 0.1% sodium azide and 0.5% bovine serum albumin (Table 9). HuR antibody which was raised against a unique peptide from the NH_2-terminus of HuR. The specimens were incubated with the antibody at room temperature overnight. The sections were then treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and avidin-biotin peroxidase complex (Vectastain ABComplex, Vector). The peroxidase staining was visualized with 3-amino-9-ethylcarbazole (Sigma), and the sections were counterstained with Mayer's hematoxylin. To confirm the specificity of the COX-2 staining, twelve sections with serous and mucinous ovarian cancer and six nonneoplastic ovarian samples were stained with and without human COX-2 control peptide (20 μg/ml, Cayman) for one hour at room temperature prior to the staining procedure. To confirm the specificity of the HuR staining, nine histological sections with serous and four sections with mucinous ovarian carcinomas were stained with and without the antigenic peptide for 19F12 (10 μg/ml) for one hour at room temperature prior to the staining procedure.

Table 9. Antibodies used in immunohistochemistry (IHC).

Primary Antibody	Dilution used in IHC[1]	Dilution used in WB[2]	Source	Study
Rabbit anti-mouse Cox-2 affinity-purified polyclonal IgG (160126)	1:250	1:1000	Cayman	I
Mouse anti-human COX-2 monoclonal antibody (160112)	1:200 (2.5μg/ml)	1:1000	Cayman	II-IV
Mouse HuR monoclonal antibody (19F12)	1:10 000 (1.0μg/ml)	1:100 000	Clonegene	III, IV

[1] Immunohistochemistry
[2] Western blot

5. SCORING OF IMMUNOHISTOCHEMISTRY (II-IV)

The intensity of COX-2 staining in both epithelial and stromal cells was scored independently and in a blinded manner by three investigators (T-L. E., B. v R., and A. R.) from 474 serous and by two investigators (T-L. E., A. S.) from 64 mucinous ovarian carcinoma tissue cores on the scale presented in Table 10. Only those tumors that exhibited at least three tissue cores were included in analysis: serous 93% (442 of 474) and mucinous 88% (56 of 64). Since one mucinous tumor contained no stroma, the final scores for mucinous stromal COX-2 expression were obtained from 55 tumors. All specimens with discordant scores were re-evaluated with a multiheaded microscope, and the consensus score was used for further analyses.

The intensity of staining of HuR protein was scored in cancer cells and in non-neoplastic stromal cells by two independent and blinded investigators (T-L. E. and A. S.) in the serous and mucinous ovarian specimens. Only those tumors from which at least three cores remained analyzable after the immunostaining procedure were scored (serous 96%, 445 of 474 and mucinous 89%, 57 of 64). Since one mucinous tumor contained no stroma, the final scores for mucinous stromal HuR expression were obtained from 56 tumors. The localization and intensity of immunoreactivity in nucleus and cytoplasm were scored separately on the scale presented in the Table 10. In HuR immunohistochemical analysis, the independent investigators scored every immunopositive region, and the score of the tumor was the most common score found. The percentage-agreement between the two independent and blinded investigators in allocation of the tumors into cytoplasm-negative and cytoplasm-positive categories was 96% (serous) and 89% (mucinous).

When immunoreactivity of inhibin-α was under 10% of all stromal cells, the inhibin-α score was 1. When immunoreactivity was over 10%, but under 50% of all stromal cells, the score was 2, and otherwise 3. Only those tumors that exhibited at least three cores were included to the analysis (55/64, 86%). The scoring of immunoreactivity for p53 (Lassus *et al.* 2003), SMAD4 (Lassus *et al.* 2001), and HER-2/neu (Lassus *et al.* 2004) has been described in previous studies.

Table 10. Scoring criteria for COX-2, HuR, and inhibin-α immunoreactivity.

Protein	Score	Protein expression	Used in
COX-2	0	no staining	II, III, IV
	1	weak diffuse, moderate to strong <10%	
	2	moderate, strong granular 10-50%	
	3	strong granular > 50%	
	Low	scores 0-1	
	High	scores 2-3	
HuR	0	negative	III, IV
	1	only nuclear	
	2	only cytoplasmic	
	3	nuclear > cytoplasmic	
	4	cytoplasmic > nuclear	
	Nuclear	score 1	
	Cytoplasmic	scores 2-4	
Inhibin-α (stromal)	0	negative	IV
	1	< 10%	
	2	10-50%	
	3	> 50%	

6. RNA ISOLATION AND NORTHERN BLOT (I)

Total RNA was extracted by the guanidine isothiocyanate-cesium chloride method from myometrial tissues (Chirgwin *et al*. 1979) and from cultured myometrial cells with TRIzol reagent (Life Technologies, Inc.). Tumor samples (100 mg) of 12 serous and 3 mucinous ovarian carcinoma specimens were crushed in 1 ml of TriZol by the Fast Prep instrument, after which RNA was purified by isopropanol precipitation following the phenol/chloroform extraction. For Northern blot analysis, 20 μg of RNA was denaturated and electrophorezed through 1.2% agarose gel. The RNA was transferred to nylon membranes (Micron Separation, Inc., Westborough, MA, USA), and immobilized by heating at 80˚C for 1 h and cross-linked under UV light for 6 min (Reprostar II UV, Camag, Muttenz, Switzerland). Purified cDNA fragments of human COX-2 open reading frame (ORF) (1.8 kb), and GAPDH (0.8kb) were labeled with (α-^{32}P) deoxy-CTP (DuPont-NEN life Science Products, Boston, MA, USA) and Prime-a-Gene kit (Promega Corp., Madison, WI, USA). cDNAs from human ORF of EP_1, EP_3, EP_4, FP, and IP receptors were gifts from Merck Frosst Canada Inc, and the cDNA for human EP_2 receptor ORF was a gift from Dr. John W. Regan, University of Arizona (Tucson, AZ, USA). Northern blots were visualized by autoradiography and were quantitated with NIH Image 1.57 on a Macintosh personal computer.

7. RT-PCR (III)

Total RNA (1 μg) was converted to cDNA with Moloney murine leukemia virus (M-MLV) reverse transcriptase RNase H minus, RNasin (Promega), 2'deoxynucleoside 5'triphosphates (dNTP) (Pharmacia), and random primers (Invitrogen). The reverse transcriptase reaction mix of 5 μl was PCR amplified in a reaction mixture of 45 μl that contained 10x Dynazyme buffer, 2 units of Dynazyme II polymerase (Finnzymes, Espoo, Finland), and antisense and sense primers for COX-2 (0.5 μg) enzyme or GAPDH (0.15 μg). The nucleotide sequences of the primer for COX-2: 5'-TTC AAA TGA GAT TGT GGG AAA AT-3' (sense) and 5'-AGA TCA TCT CTG CCT GAG TAT CTT-3' (antisense); and for GAPDH: 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense) and 5'- TCT AGA CGG CAG GTC AGG TCC ACC-3' (antisense) (Ristimäki *et al.* 1994). The PCR reaction mixture was heated to 94°C for 3 min followed by amplification for COX-2 by 40 and for GAPDH by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. After the last cycle, the reactions remained at 72°C for an additional 15 min. Amplified cDNAs were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining. The amplified products were visualized under UV transillumination in MultiImage™ FC Light Cabinet by use of digital imaging FluorChem™ 8800 software (Alpha Innotech Corporation, San Leandro, CA, USA).

8. WESTERN BLOT (I-IV)

Tumor samples (50 mg) of 12 serous (Study II) and 3 mucinous (not published) ovarian carcinoma specimens were crushed in 1 ml of radioimmunopresipitation assay buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris pH 8.0) supplemented with a Complete mini protease inhibitor mixture tablet (Boehringer Mannheim, Germany) by the Fast Prep homogenization instrument (Qbiogene Inc., Carlsbad, CA, USA). The OVCAR-3 cells from the HuR siRNA experiment (Study III) were lysed in 400 μl (95°C) lysis buffer (60 mM Tris-HCl pH 6.8), 2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.002% bromophenol blue). Alternatively, the myometrial cells (Study I) and OVCAR-3 cells from the LMB experiments (Study III) were lysed in 1 ml of radioimmunopresipitation assay buffer. The amount of proteins was determined by BSA protein assay (Pierce, Rockford, IL, USA). From tumor samples, 100 μg of protein and 75 μg of protein from cell culture experiments were separated by SDS-PAGE (12%), and transferred electrophoretically to Hybond-C extra nitrocellulose membranes (Amersham, Buckinghamshire, UK). Nonspecific binding was blocked by Tris-buffered saline nonidet P40 (TBS-NP40) and 5% low-fat dry milk solution overnight at 4˚C . For immunodetection, the

membrane was incubated with anti-mouse COX-2 polyclonal immunoglobulin (1:1000, Cayman, Study I), the monoclonal COX-2 antibody (1:1000 dilution) with or without the COX-2 blocking peptide (20 μg/ml, 360 107, Cayman Chemical Co., Studies III, IV), with 19F12 monoclonal HuR antibody (1:100 000, Study III), or with β-actin antibody (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA; Studies II- IV). The blocking procedure abolished the signal for COX-2 protein, thus confirming the specificity of the COX-2 antibody.

The secondary antibodies for polyclonal COX-2 were goat anti-rabbit antibodies (1:2000), for monoclonal COX-2 and HuR, sheep antimouse antibodies (1:2000 dilution), and for β-actin, donkey antigoat antibodies conjugated to horseradish peroxidase (1:2000 dilution, Santa Cruz Biotechnology). COX-2 protein was visualized by enhanced chemiluminescence (ECL) with the Super Signal West Femto Maximum Sensitivity Substrate (Pierce) according to the manufacturer's protocol by MultiImage^{TM} FC Light Cabinet (Promega) and the digital imaging FluorChem^{TM} 8800 software (Alpha Innotech Co., San Leandro, CA, USA).

9. STATISTICAL ANALYSIS

For statistical analysis, COX-2 scores 0 and 1 were combined as low COX-2 expression, and scores 2 and 3 represented high COX-2 expression. The correlation between COX-2 and HuR staining intensity and clinically relevant and prognostic variables were assessed by Chi-square test or by Fisher's exact test. Probability of survival was estimated by the Kaplan-Meier method. Survival probabilities were compared between groups by using the log-rank test or log-rank test for trend. Disease-specific survival time was defined as the time from primary surgery to death of the patient from ovarian cancer or to the end of follow-up. Deaths due to causes other than ovarian cancer were treated as censored cases, as were those patients alive at the end of follow-up. Multivariate survival analysis was performed by the COX proportional hazards model, entering the variables significant for survival of patients in univariate analysis.

Table 11. Statistical analyses.

Analysis	Used for	Study
Student's t-test	Correlation between variables	I
Mann-Whitney U-test	Correlation between variables	I
Spearman correlation	Correlation between variables	I
Descriptive statistics	Frequency of phenomenon	II, III, IV
Chi-square test	Correlation between variables	II, III, IV
Fisher's exact test	Correlation between variables	II, III, IV
Kaplan-Meier test	Survival analysis	II, III, IV
Log-rank test or log-rank test for trend	Correlation between variables	II, III, IV
COX proportional hazard	Multivariate analysis	II, III

RESULTS

1. COX-2 EXPRESSION IN MYOMETRIUM (I)

1.1. COX-2 expression in pregnant myometrium in vivo

Myometrial tissues were collected at cesarean section from 25 healthy pregnant women at gestation weeks 37 to 42. Of these women, 10 were in labor, they had had regular contractions for 6 to 22 h (mean 14.3±1.9 h), the cervix of each was open (6.6±0.9cm) except for one patient with a closed cervix, and 4 to 24 h (12.9±2.1 h) had elapsed following rupture of the membranes. All these patients received oxytocin treatment to increase contractile activity. Of these 25 women, 15 underwent elective cesarean section, and these did not have regular contractions, their fetal membranes were intact, and they had no infection.

Myometrial samples collected from women in labor expressed elevated levels of COX-2 mRNA (15.2±3.5 -fold, $p<0.0001$) compared to myometrial specimens collected from women not in labor as detected by Northern blot analysis. All non-labor specimens contained either low or undetectable levels of the transcript. Expression level of COX-2 mRNA was not associated with the duration of regular contractions, rupture of fetal membranes, or cervical situation. Immunohistochemically detected COX-2 protein occurred in the cytoplasm of the smooth muscle cells, and there was also weak COX-2 staining in the smooth muscle cells of the veins.

1.2. Regulation of COX-2 expression in myometrial cells in vitro

Myometrial cells isolated from the pregnant uterus during cesarean section and from the non-pregnant uterus during hysterectomy were cultured for passages 2 to 7. COX-2 mRNA expression was low or undetectable under baseline conditions. The expression was, however, induced with IL-1β, TNF-α, PMA, but not with oxytocin. IL-1β-induced COX-2 expression was concentration- and time-dependent. The peak induction was achieved by stimulation of myometrial cell lines with IL-1β (10ng/ml) for 6 h, at which time COX-2 mRNA expression was stimulated by 17.1±1.5-fold in comparison with the basal expression in these cell lines ($p<0.0001$). An identical pattern of induction of COX-2 mRNA was found also in myometrial cell cultures isolated from the non-pregnant uterus. Dexamethasone, but not the selective COX-2 inhibitor NS-398, could block the inductive effect of IL-1β on COX-2 mRNA expression. IL-1β and PMA also induced expression of COX-2 protein as detected by immunofluorescence staining and Western blot analysis. Consistent

with our mRNA data, IL-1β, TNF-α, and PMA but not oxytocin induced release of 6-ketoPGF$_{1\alpha}$, a stable hydrolysis product of PGI$_2$. Synthesis of 6-ketoPGF$_{1\alpha}$ was blocked by the selective COX-2 inhibitor NS-398, dexamethasone, and the nonselective NSAID indomethacin. Indomethacin could also suppress the basal, presumably COX-1-dependent, release of 6-ketoPGF$_{1\alpha}$. Cultured myometrial cells expressed high levels of PGE$_2$ receptor subtype EP$_2$ mRNA, but only low levels of EP$_1$, EP$_3$, EP$_4$, FP, and IP receptors.

2. *COX-2 EXPRESSION IN SEROUS AND MUCINOUS OVARIAN CARCINOMA (II, IV)*

2.1. COX-2 expression in serous and mucinous ovarian carcinomas in vivo and in vitro

COX-2 protein expression, as detected by immunohistochemistry, localized mainly to the cytoplasm and to the perinuclear region of the epithelial cancer cells in both serous and mucinous ovarian carcinomas (99%, 439 of 442 and 95%, 53 of 56 respectively). The stromal compartment was positive in only 7.9% (35 of 442) of serous, but in 62% (34 of 55) of mucinous cases (Table 12). The nonneoplastic ovarian surface epithelium (n=6) was negative or only weakly positive for COX-2 immunostaining. Elevated COX-2 immunoreactivity (scores 2-3) in the epithelial carcinoma cells clearly differed from the staining pattern of the non-neoplastic surface epithelium and it was seen in 70% (310 of 442) of the serous and in 39% (22 of 56) of the mucinous tumors. In serous ovarian carcinoma, there was no elevated (scores 2-3) stromal COX-2 expression, but it occurred in 24% (13 of 55) of mucinous tumors (Table 12).

COX-2 expression was detected in 12 serous and 3 mucinous ovarian cancer specimens by RT-PCR and Western blot analysis. Expression of COX-2 mRNA was apparent by RT-PCR in 75% (9 of 12) of serous and in 67% (2 of 3) of mucinous ovarian carcinoma specimens. COX-2 protein was detected by the Western blot method in 42% (5 of 12) of the serous and in 33% (1 of 3) of the mucinous ovarian carcinoma specimens. All tumors that expressed COX-2 protein by Western blot were also positive for COX-2 mRNA by RT-PCR. The expression of COX-2 was investigated also in three human serous, OVCAR-3, CaOV-3, and ES-2, and in one mucinous EFO-27 ovarian carcinoma cell lines. At the basal level, no COX-2 expression appeared in any of the ovarian carcinoma cell lines, but IL-1β did induce COX-2 expression in OVCAR-3 cells. In the CaOV-3

cell line, COX-2 was induced with PMA, but not with IL-1β, and the expression was not as strong as in the OVCAR-3 cell line. Neither ES-2 nor EFO-27 expressed any detectable basal or inducible COX-2.

Table 12 . Expression of COX-2 protein in epithelial and in stromal cells of mucinous and serous ovarian carcinoma specimens *in vivo* as detected by immunohistochemistry.

Epithelial Cox-2 immunopositivity in ovarian carcinomas			
Scores	**Positive/n (%) Serous**	**Positive/n (%) Mucinous**	**P-value[1]**
1-3	439/442 (99%)	53/56 (95%)	NS[2]
2-3	310/442 (70%)	22/56 (39%)	< 0.0001
Stromal Cox-2 immunopositivity in ovarian carcinomas			
Scores	**Positive/n (%) Serous**	**Positive/n (%) Mucinous**	**P-value[1]**
1-3	35/442 (7.9%)	34/55 (62%)	< 0.0001
2-3	0/442 (0%)	13/55 (24%)	< 0.0001

[1] Chi-square test between serous and mucinous tumors inside same scores
[2] Not significant

2.2. Correlation of COX-2 expression with clinical parameters

In serous ovarian carcinoma, elevated expression of COX-2 was associated with age over 57 years (P=0.0099), high histological grade (P<0.0001), and residual tumor size over 1 cm (P=0.0111). No correlation appeared between COX-2 staining intensity and stage, tumor size, lymph-node positivity or positive finding of carcinoma cells in ascites. In mucinous ovarian carcinoma, the only significant correlation was between epithelial COX-2 expression and high grades (grades 2-3) (P=0.0285). Stromal COX-2 expression in serous or mucinous carcinoma showed no correlation with any clinical parameters.

Table 13. Association of elevated epithelial COX-2 immunoreactivity with clinical parameters in serous (n= 442) versus mucinous (n=56) ovarian carcinomas.

Clinical features	**Serous COX-2/n (%)**	**P[1]**	**Mucinous COX-2/n (%)**	**P[1]**
Age:		**=0.0099**		NS[3]
≤ 57/≤ 54[2]	144/223 (65%)		13/29 (45%)	
> 57/>54 [2]	166/219 (76%)		9/27 (33%)	
Histological grade:		**<0.0001**		NS
1	96/166 (58%)		14/44 (32%)	
2	86/114 (75%)		7/11 (64%)	
3	125/156 (80%)		1/1 (100%)	
2-3	211/270 (78%)	**<0.0001[4]**	8/12 (67%)	**=0.0285[4]**
Stage:		NS		NS
I	63/90 (70%)		18/44 (41%)	
II	43/61 (70%)		1/2 (50%)	
III	159/237 (67%)		3/9 (33%)	
IV	43/52 (83%)		0/1 (0%)	
II-IV	245/350 (70%)	NS [5]	4/12 (33%)	NS [5]

[1] Chi-square test
[2] Mean age at diagnosis; 57 years in serous and 54 years in mucinous carcinomas
[3] Not significant
[4] Versus grade 1
[5] Versus stage I

2.3. Correlation of COX-2 expression with some biomarkers of ovarian carcinoma

We observed that elevated COX-2 expression occurred more frequently in serous tumors with altered (completely negative or increased) p53 staining than in tumors with normal (weak) p53 immunoreactivity (Table 14). In tumors with altered p53 expression COX-2 expression was also elevated in 81% (209/259) of cases and COX-2 was low in 19% (50/259) of cases ($p<0.0001$). In respect to SMAD4, elevated COX-2 expression also appeared more frequently in tumors with altered (negative or decreased) SMAD4 staining than in tumors with normal SMAD4 immunostaining ($P=0.0004$). Tumors with combined defective expression of p53 and SMAD4 more frequently expressed elevated levels of COX-2 than did tumors with only one of the tumor suppressor genes showing altered expression (Table 14). Elevated expression of COX-2 occurred more frequently in tumors with amplification of the oncogene *Her-2/neu* than in tumors with normal *Her-2/neu* status ($P=0.0479$; Table 14).

In mucinous carcinoma p53, SMAD4, or Her-2/neu alterations are not so frequent than in serous ovarian carcinoma. In mucinous tumors p53 was altered in 20% (10/50) of cases and in these tumors also COX-2 expression was elevated in 60% (6/10) of cases (not shown). There was no correlation between Her-2/neu amplification and elevated COX-2 expression in mucinous tumors. However, we observed correlation of stromal COX-2 expression with stromal inhibin-α expression ($p=0.0058$), and there appeared to be colocalization of these two signals. The expression of inhibin-α had no other associations with any clinical parameters like age, grade, or stage.

Table 14. Association of elevated epithelial COX-2 expression with immunostaining for p53 and SMAD4, and amplification of *Her-2/neu* in serous ovarian carcinoma.

Immunostaining	n	Elevated COX-2/n (%)	P[1]
P53:	437		<0.0001
Normal		96/178 (54%)	
Negative		59/67 (88%)	
Increased		150/192 (78%)	
SMAD4:	433		=0.0004
Normal		184/286 (64%)	
Negative or decreased		119/147 (81%)	
Normal p53 and normal SMAD4		76/143 (53%)	
Normal p53 and altered SMAD4		18/31 (58%)	NS[2,3]
Altered p53 and normal SMAD4		108/143 (76%)	=0.0001[3] NS[4]
Altered p53 and altered SMAD4		99/114 (87%)	<0.0001[3] =0.0013[4] =0.0264[5]
Normal HER-2/neu	323	163/254 (64%)	=0.0479
Amplified HER-2/neu		53/69 (77%)	

[1] Fisher exact test.
[2] Not significant.
[3] Versus normal p53 and normal SMAD4.
[4] Versus normal p53 and altered (negative or decreased) SMAD4.
[5] Versus altered (negative or increased) p53 and normal SMAD4.

2.4. Correlation of COX-2 expression with survival

Of 442 serous ovarian carcinoma patients 244, (55%), and of 56 mucinous ovarian cancer patients with a known COX-2 score, 7 (13%) died of ovarian cancer during follow-up. Elevated epithelial COX-2 expression was associated with decreased disease-specific survival among serous ovarian cancer patients (P=0.0011, Kaplan-Meier). The 5-year survival rate was 60% (95% CI; 51-68%) in patients with low COX-2 expression, and was 45% (95% CI; 39-51%) in patients with high COX-2 expression. When COX-2 expression was divided into three categories (score 0-1, score 2, and score 3) probability of survival was progressively lower as category number rose (P=0.0004). No correlation appeared between stromal COX-2 expression and survival among serous ovarian cancer

patients. For mucinous ovarian cancer patients, no significant correlation appeared between epithelial or stromal Cox-2 expression and survival (P=0.1417 and P=0.7189, respectively), although expression of inhibin-α correlated with better survival (p=0.0394; Kaplan-Meier).

Multivariate analysis to evaluate the independence of COX-2 expression as a prognostic factor in serous ovarian carcinoma showed that age (P=0.0134), grade (P<0.0001), stage (P<0.0001), and residual tumor size (P<0.0001) were independent prognostic factors, but inclusion of tumor size (P=0.8989) or COX-2 expression (P=0.7439) did not add significant independent prognostic information. COX-2 was also not an independent prognostic factor inside clinically meaningful subgroups (age, grade or stage) in serous ovarian carcinoma.

3. *HuR EXPRESSION IN SEROUS AND MUCINOUS OVARIAN CARCINOMA (III, IV)*

3.1. Expression of HuR protein in serous and mucinous ovarian carcinoma

Localization and frequency of HuR protein expression in serous and in mucinous ovarian carcinoma was similar. Nearly all serous and mucinous ovarian tumors had HuR expression in the nucleus or in both the nucleus and the cytoplasm. HuR protein was expressed only in the nucleus of epithelial cells in 47% (210/445) of serous and in 53% (30/57) of mucinous ovarian carcinoma samples. Cytoplasmic HuR expression was detectable in epithelial cancer cells in 52 % (233 of 445) of serous and in 47% (27 of 57) of mucinous ovarian carcinomas. In nonneoplastic stromal cells, HuR expression was observed mainly in the nucleus, and cytoplasm was positive in only 3% (15 of 439) of serous and in 7% (4 of 56) of mucinous ovarian carcinomas. For statistical analysis we have used two categories: ”only nuclear staining” and “cytoplasmic staining” (Table 15). Cytoplasmic HuR expression always appeared together with nuclear HuR expression, and no tumors showed only cytoplasmic HuR expression.

Table 15. Localization of HuR protein expression in serous and mucinous ovarian carcinomas.

HuR Staining	Serous Ovarian Carcinoma		Mucinous Ovarian Carcinoma	
	Epithelial (n=445) n (%)	Stromal (n=439) n (%)	Epithelial (n=57) n (%)	Stromal (n=56) n (%)
Negative	2 (0.4%)	6 (1.4%)	0 (0%)	0 (0%)
Only nuclear	210 (47%)	418 (95%)	30 (53%)	52 (93%)
Cytoplasmic	233 (52%)	15 (3%)	27 (47%)	4 (7%)

3.2. Association of HuR expression with COX-2 expression and clinical parameters

Elevated COX-2 expression correlated significantly with cytoplasmic HuR immunoreactivity in epithelial cancer cells both in serous (p=0.0045) and in mucinous (p=0.0162) carcinomas (Table 16). No correlation appeared between elevated stromal COX-2 expression with HuR protein expression in serous or mucinous tumors.

Table 16. Association of cytoplasmic HuR immunopositivity with elevated epithelial COX-2 expression in serous (n= 426) and mucinous (n=56) ovarian carcinoma.

	Serous		Mucinous	
HuR immunopositivity	**Elevated COX-2/n (%)**	**P-value[1]**	**Elevated COX-2/n (%)**	**P-value[1]**
Cytoplasm negative	131/205 (64%)	=0.0045[2]	7/29 (24%)	=0.0162[3]
Cytoplasm positive	169/221 (77%)		15/27 (56%)	

[1] Chi-square

[2] Cytoplasm-positive compared to cytoplasm-negative in serous tumors

[3] Cytoplasm-positive compared to cytoplasm-negative in mucinous tumors

In serous carcinoma, cytoplasmic HuR expression was associated with high grade (P<0.0001) and with residual tumor size over 1 cm (P=0.0028). No significant association existed between cytoplasmic HuR expression and age at diagnosis, stage, or tumor size in serous tumors. In mucinous ovarian carcinomas, no correlation appeared between cytoplasmic HuR expression and any clinical parameters (age, grade, stage). In addition, stromal cytoplasmic HuR expression showed no association with any clinical parameters in serous or in mucinous tumors.

Among the 445 serous ovarian carcinoma patients, cytoplasmic HuR immunoreactivity was associated with decreased disease-specific survival (P<0.0001, Kaplan-Meier). Five-year disease-specific survival in the cytoplasm-negative category was 60% (95% CI, 53–67), and in the cytoplasm-positive category 39% (95% CI, 32–46). Thus, cytoplasmic immunopositivity for HuR predicted poor outcome in serous ovarian carcinoma patients. No correlation existed between HuR expression and survival in mucinous ovarian carcinoma, which may be due to the few deaths among our mucinous ovarian cancer patients.

When multivariate survival analysis was made to evaluate the independence of cytoplasmic HuR expression as a prognostic factor, inclusion of cytoplasmic HuR expression added no significant independent prognostic information, when four independent covariates (age, grade, stage, and residual tumor size) were included in the analysis. However, in the multivariate analysis HuR expression was an independent prognostic factor among low-stage serous ovarian carcinomas. In mucinous carcinomas HuR was not an independent prognostic marker in any subgroup analysis.

3.3. Effect of HuR siRNA and LMB on COX-2 expression in ovarian cancer cells

In order to study the effect of HuR expression in ovarian cancer cells on ARE-containing mRNA molecules, we chose to utilize HuR-targeted siRNA molecules to inhibit HuR expression. In addition, we used LMB, which inhibits transport of the HuR-protein/COX-2 mRNA complexes from the nucleus to the cytoplasm, where the COX-2 protein translation happens (Brennan and Steiz 2001, Gallouzi and Steiz 2001, Kau and Silver 2003). OVCAR-3 cells transfected with HuR siRNA (200 nM), inhibited expression of HuR and IL-1β-induced COX-2 expression. β-actin siRNA, serving as a control, did not reduce expression of either HuR or COX-2. LMB inhibited IL-1β-induced COX-2 expression in OVCAR-3 cells in a concentration-dependent manner. All this suggests that in ovarian cancer cells HuR is necessary for COX-2 expression.

DISCUSSION

We investigated the expression and regulation of the inducible prostanoid synthesis enzyme COX-2 in female reproductive organs. COX-2 expression was studied in pregnant myometrium as a model of physiological state and in ovarian carcinoma as a model of pathological state. Regulation of COX-2 expression was studied in cultured myometrial smooth muscle cells and in cultured serous ovarian carcinoma cell lines.

1. COX-2 IN MYOMETRIAL CELLS IN VIVO AND IN VITRO

Myometrium is the main target organ of prostanoids during parturition. We observed that the onset of labor induced an over 15-fold induction of COX-2 mRNA expression in the myometrium. While it had been previously shown that expression of COX-2 transcript is elevated in amnion and chorion, induction of COX-2 at these sites had been only 2- to 4-fold (Hirst *et al.* 1995, Slater *et al.* 1995, Fuentes *et al.* 1996, Mijovic *et al.* 1997). These data indicate that although myometrial COX-2 expression is lower than that in the fetal membranes before the onset of labor, it increases dramatically during labor, and may thus play an important role in production of prostanoids during labor. According to previous studies in human myometrium, Slater *et al.* (1999) found expression of COX-2 mRNA to be elevated before the onset of labor, whereas Zuo *et al.* (1994) found a decrease, and Moore *et al.* (1999) found no change in expression level of COX-2 mRNA at the onset of labor. These discrepancies may depend on the collection of specimens or on different methods used, or both. In our material, all women in the in-labor group had received an oxytocin infusion to support their contractions. It is, however, unlikely that this oxytocin infusion can explain the induction of COX-2 expression in the in-labor samples, because we were unable to show any stimulation of COX-2 expression by oxytocin in cultured myometrial cells. We cannot, however, exclude the possibility that the sensitivity of cultured myometrial cells to oxytocin is different from that of myometrial cells *in vivo,* which could depend, for example, on the hormonal milieu in the *in vivo* condition. In contrast to oxytocin, treatment of myometrial cells with proinflammatory cytokines induced expression of COX-2. The highest, a 17-fold induction, was achieved by stimulation of myometrial cell lines with IL-1β. The effect of IL-1β was not dependent on pregnancy, while an identical pattern of COX-2 induction was observed in myometrial cells isolated from the fundal part of the uterus from a non-pregnant patient. Previously, cytokines, especially IL-1β, have been shown to stimulate the production of prostanoids in cultured human myometrial cells (Abel and Kelley

1979, Casey *et al.* 1984, Pollard and Mitchell 1996, Gramatopoulos and Hillhouse 1999). PGI_2 was the major prostanoid produced in myometrial smooth muscle cells, which is consistent with earlier data (Abel and Kelley 1979). Mechanistically this had been linked to activation of PLA_2, which liberates AA from membrane phospholipids. Our data thus provide an additional mechanism for stimulation of myometrial prostanoid production by induction of COX-2 expression. All this indicates that cytokines induce expression of COX-2, which leads to the subsequent increase in production of contractive PGs and relaxative PGI_2 in fetal membranes and also in myometrium.

In infection-induced premature labor (Romero *et al.* 1989) and also in normal labor in human (Romero *et al.* 1990), high levels of IL-1β have been detected. In animal models the increased release of IL-1β by mechanical stretch of myometrium has been shown to increase the expression of mRNA for COX-2 (Wu *et al.* 1999), oxytocin receptor (Ou *et al.* 1998), and connexin43 (Ou *et al.* 1997), all of which are important factors increasing the contractile activity in uterus. Also in human uterus mechanical stretch of myocytes results in increased expression of COX-2 mRNA and protein activity (Sooranna *et al.* 2004). Since cytokines may facilitate their labor-inducing properties through induction of COX-2, it is tempting to speculate that inhibition of COX-2 may be a useful strategy in preventing premature contractions. Indeed, non-selective inhibitors of the COX-enzyme, for instance indomethacin, have been used in suppression of preterm contractions, but their use is limited due to adverse effects such as closure of the ductus arteriosus, reduced amniotic fluid, and elevated risk for necrotizing enterocolitis. Some authors have speculated that the use of COX-2 selective inhibitors would be an effective means to reduce premature contractions with an acceptable side-effect profile (Sawdy *et al*. 1997). However, according to genetic disruption studies in mice, ductus arteriosus patency requires both Cox-1 and -2 enzymes (Loftin *et al.* 2001). There are no studies in which human fetal PG synthesis in ductus arteriosus has been studied. In addition, COX-2 enzyme may be important in normal renal function in fetus. This is supported with the case study which descibed a possible link between the use of the COX-2 selective inhibitor nimesulide and neonatal renal failure (Peruzzi *et al.* 1999). In another study, nimesulide showed a significant benefit in women at high risk for preterm delivery (Sawdy *et al.* 2004). In that study, no harmful side-effects were observed provided that fetal well being was monitored closely with ultrasound.

2. COX-2 EXPRESSION IN SEROUS AND MUCINOUS OVARIAN CARCINOMA

Our results provide evidence for COX-2 expression in serous (n=442) and mucinous (n=56) ovarian carcinomas. The COX-2 protein expression was observed mainly in the epithelial cancer cells, and also in the stromal compartment, but nonneoplastic ovarian surface epithelium was negative or weak for COX-2 immunostaining. All samples were taken in primary operations before the patients had received any chemotherapy, which was crucial because chemotherapy can induce COX-2 expression. The tissue microarray technique used in the immunohistochemical part of the study proved to be useful, since it enabled the simultaneous staining which made the protocol more consistent and reliable.

We found the amount and localization of COX-2 immunopositivity to vary significantly between serous and mucinous ovarian carcinomas. COX-2 expression in epithelial cancer cells was elevated significantly more often in serous (70%) than in mucinous (39%) tumors ($P<0.0001$), which is consistent with data published by Shigemasa *et al.* (2003) (34% vs. 17%) and Seo *et al.* (2004) (65% vs. 6%). In addition, there was a striking difference between the expression level in the stromal cells, in which COX-2 expression was found significantly more often in mucinous (62%) than in serous (7.9%) ovarian carcinomas ($p<0.0001$). There are no previous studies in which as high stromal COX-2 expression as we observed in mucinous tumors has been found in ovarian carcinoma, although elevated COX-2 expression has been detected in epithelial cells of ovarian carcinoma (Klimp *et al.* 2001, Matsumoto *et al.* 2001b, Denkert *et al.* 2002, Ferrandina *et al.* 2002a and b, Landen *et al.* 2003, Shigemasa *et al.* 2003, Ali-Fehmi *et al.* 2004). These studies have shown a relatively high variation in COX-2 positivity (42-89%), which may depend on the use of differing antibodies, scoring criteria, and patient selection. Two studies have failed to detect any COX-2 expression in ovarian carcinoma (Dore *et al.* 1998, Gupta *et al.* 2003), which may depend on a limited number of ovarian carcinoma specimens in these studies or be due to the use of insensitive assays.

In our study, elevated epithelial cancer cell expression of COX-2 in serous ovarian carcinoma was associated with reduced survival, and this is in accordance with previous results (Denkert *et al.* 2002, Ferrandina *et al.* 2002a and b, Ali-Fehmi *et al.* 2003, Seo *et al.* 2004, Raspollini *et al.* 2004). In the study of Denkert *et al.* (2002), COX-2 expression was an independent prognostic factor for poor survival, which is consistent with findings in esophageal carcinoma (Buskens *et al.* 2002). We were unable, however, to recognize COX-2 as an independent prognostic variable in the current

ovarian cancer material, but found instead a highly significant correlation of COX-2 expression with high histological grade, which was a strong independent covariate in the multivariate assay. Denkert *et al*. (2002) used a different grading system and included several histological subtypes in their material, which may explain the discordance in the findings between their and our material. In addition to survival and grade we observed epithelial COX-2 expression to associate with high age and large residual tumor size in serous carcinomas, but saw no association with stage, which is consistent with several other reports (Matsumoto *et al.* 2001, Denkert *et al.* 2002, Ferrandina *et al.* 2002a and b, Shigemasa *et al.* 2003, Li *et al.* 2004). Similarly to serous tumors, COX-2 expression correlated with high grade in mucinous tumors, but in contrast to serous tumors, we found no correlation with the survival of mucinous ovarian cancer patients. This may depend on the few deaths among our mucinous ovarian carcinoma patients.

Mucinous ovarian carcinoma is the first type of tumor, in which we have found high stromal expression of COX-2 without any evidence for tissue injury. Previously, our research group found strong stromal COX-2 expression at sites of erosion, ulceration, and around necrosis in digestive-tract (Buskens *et al.* 2002, Saukkonen *et al.* 2001), urothelial (Ristimäki *et al.* 2001), and lung (Wolff *et al.* 1998) carcinomas. In the studies by Ferrandina *et al.* (2002a and b) some stromal COX-2 expression was evident in lymphoid cells, but in other studies no stromal COX-2 immunopositivity appeared in ovarian carcinoma, although mucinous tumors were also included (Matsumoto *et al.* 2001, Shigemasa *et al.* 2003, Ali-Fehmi *et al.* 2004). In our study material, stromal COX-2 expression in serous or mucinous carcinoma showed no correlation with any clinical parameters, although in cervical carcinoma high stromal COX-2 expression correlated with better response to neoadjuvant treatment and to longer survival (Ferrandina *et al.* 2002c, Ferrandina *et al.* 2004). This might be explained by the low amount of stromal COX-2 expression in our serous ovarian carcinoma material and the relatively low number of deaths among mucinous carcinoma patients. The localization of COX-2 expression in the inhibin-α-positive stromal cells in proximity to cancer cells suggests that there may exist a cancer cell-derived paracrine factor that can regulate expression of both inhibin-α and COX-2 in the stromal cells. An alternative explanation is that expression of inhibin-α and of COX-2 is directly linked. This hypothesis is supported by the observation that prostaglandins can induce inhibin-α expression in human granulosa-luteal cells (Erämaa and Ritvos 1996). However, the role and significance of stromal COX-2 expression in ovarian carcinogenesis remains unknown.

3. *HuR EXPRESSION IN SEROUS AND MUCINOUS OVARIAN CARCINOMA*

Increase in nuclear HuR immunoreactivity has been observed in high-grade brain tumors (Nabors *et al.* 2001) and in colorectal carcinomas (Dixon *et al.* 2001). We were the first, however, to publish on cytoplasmic HuR expression as associated with poor outcome in the carcinoma patient (Erkinheimo *et al.* 2003). Thereafter, HuR has been the first mRNA stability protein expression of which has been linked to the reduced survival of carcinoma patients. We found that elevated cytoplasmic HuR expression was associated with decreased disease-specific survival ($P<0.0001$), high-grade ($P<0.0001$), and large residual tumor size ($P=0.0028$) in serous ovarian carcinomas. Furthermore, HuR expression was an independent prognostic factor among low-stage serous ovarian carcinomas when age, grade, and residual tumor size were included in the multivariate analysis. This may indicate that the carcinogenic role of HuR is more important in low-stage tumors. In contrast to serous tumors, mucinous ovarian carcinomas shared no correlation between HuR expression and clinical parameters, including survival. We also found that COX-2 immunoreactivity correlated with cytoplasmic HuR expression both in serous ($p=0.0045$) and in mucinous ($p=0.0162$) carcinomas, but only in the epithelial cancer cells.

After our publication (2003), Denkert *et al.* showed that cytoplasmic HuR expression is associated with elevated COX-2 expression, increased tumor grade, or mitotic activity in ovarian and breast carcinoma (Denkert *et al.* 2004 a and b), and lower survival in ovarian carcinoma (Denkert *et al.* 2004a). Overexpression of HuR has been shown to enhance COX-2 expression in colorectal cancer cells (Dixon *et al.* 2001) and inhibition of HuR to reduce COX-2 expression in breast carcinoma (Sengupta *et al.* 2003). We could show that IL-1β-induced COX-2 expression in OVCAR-3 serous ovarian carcinoma cells was inhibited by HuR siRNA and by LMB. Since LMB can inhibit nucleocytoplasmic transport of HuR protein (Kau *et al.* 2003) and HuR siRNAs were shown to reduce expression of HuR, our data indicate that there exists a direct link between HuR expression and COX-2 expression in ovarian carcinoma. All this indicates that cytoplasmic expression of HuR is predominantly a property of transformed epithelial cells in ovarian carcinoma, in which it can directly regulate COX-2 expression. Thus, induction of COX-2 expression may be part of the machinery that promotes HuR-induced carcinogenesis.

4. FUTURE PROSPECTS

The synthesis of PGs is induced during spontaneous labor, and the PGE_2 derivatives are used as effective drugs to induce labor in cases with maternal or fetal problems or prolonged pregnancy. In addition, nonselective inhibitors of the COX enzyme have been used in suppression of preterm contractions, although their use is limited due to fetal adverse effects. According to many studies on COX-2 expression in fetal membranes and myometrium, COX-2 activity is crucial in the synthesis of PGs during pregnancy and parturition. The COX-2 enzyme is responsible for the inducible synthesis of PGs which is needed during parturition. Thereafter, the inhibition of COX-2 activity would be an effective means to suppress preterm production of PGs and contractions. However, according to animal and human studies, COX-2 selective inhibitors may lead to renal and ductal side-effects as do nonselective inhibitors. Selective COX-2 inhibitors therefore cannot be recommended for routine use in prevention of premature labor, but randomized placebo-controlled trials are required to assess the usefulness of COX-2 selective inhibitors in this condition. These studies should be performed in centers that can provide sufficient ultrasound follow-up of the patient.

The main determinants of treatment options in ovarian carcinoma are stage
and grade. However, the biological behavior, response to available treatment, and outcome vary greatly inside the same stage and grade categories. Therefore it may be suggested that ovarian carcinoma constitutes a number of distinct subgroups whose optimal treatment should also differ. Thus far, none of the biological markers, including p53, has been strong enough for prognosis of ovarian carcinoma in the clinical use. It is therefore important to seek novel biological markers which could classify ovarian carcinomas more precisely. In our material, COX-2 expression was not an independent prognostic factor. COX-2 expression status alone is thus not a strong enough marker to classify ovarian tumors into subgroups. However, epidemiological and experimental data support the use of NSAIDs and selective COX-2 inhibitors as chemopreventive agents in a number of malignancies, including colorectal, breast, esophageal, and lung carcinomas. Evidence also exists that regular, long-term use of ASA or other NSAIDs is inversely associated with ovarian cancer incidence (Akhmedkhanov *et al.* 2001, Sorensen *et al.* 2003). Since we and others have observed that elevated COX-2 expression is associated with reduced survival in serous ovarian cancer, and COX-2 inhibitors can modulate tumor growth and metastasis, it is theoretically possible that COX-2 inhibitors would be beneficial in treatment of ovarian cancer. According to the Adenomatous Polyp Prevention on Vioxx (APPROVe) study, blood pressure was elevated in patients in the rofecoxib

group early in the course of the study and the incidence of myocardial infarction and thrombotic stroke was significantly increased after a year of treatment, results which are in accordance with those of the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (Merck, Bombardier *et al.* 2000). These results may reduce the enthusiasm to use selective inhibitors in chemoprevention, but may not have a great impact on the possibility of using COX-2 inhibitors in a neoadjuvant or adjuvant setting. However, clinical trials with selective COX-2 inhibitors are necessary in order to understand the role and observe the safety of these drugs in the treatment of ovarian carcinoma.

ACKNOWLEDGEMENTS

The present study was carried out at the Department of Obstetrics and Gynecology, at the Department of Pathology, Helsinki University Central Hospital and at the Molecular and Cancer Biology Research Program, Biomedicum Helsinki, University of Helsinki during the years 1997-2003. I wish to express my deep gratitude to Professor Olavi Ylikorkala, the Head of the Department of Obstetrics and Gynecology, for providing me excellent research facilities and especially for great expertise and support during my research and clinical work. Docent Maija Haukkamaa, the Administrative Head of the Department of Obstetrics and Gynecology, Professor Markku Seppälä, the former Head of the Department, and Professor Leif Andersson, the Head of the Department of Pathology, are warmly thanked for excellent research facilities.

I am the most grateful to my supervisor Docent Ari Ristimäki for providing me research facilities required. I admire his cabability to observe the meaningful scientific projects, and to work really hard in order to reach the goal. His knowledge in science has been helpful through my very first steps in the scientific world.

Professor Olli Carpen and Docent Johanna Mäenpää, the official reviewers of this thesis, are gratefully acknowledged for their thorough review and constructive comments about my manuscript.

Carol Norris is warmly acknowledged for that unforgettable 12 hours during which we went through the language revision at her home and for all those discussions after that.

I am especially greatful to Ralf Butzow for providing excellent microarray material and his knowledge about ovarian carcinoma. I am pleased with all those discussions which helped me to understand a little bit more about pathogenesis of ovarian carcinoma. Arto Leminen is acknowledged for collecting all the clinical material needed for microarrays.

My deepest thanks to Patrik Finne, Caj Haglund, Mira Heinonen, Jyrki Jalkanen, Heini Lassus, Henry Furneaux, Timothy Hla, Bastiaan van Rees, and Sibani Sengupta for fruitful collaboration.

I warmly thank all members of our research group in the 5th floor in Biomedicum. I am the most grateful to my dear colleagues “pikku-Kirsi” and Anna for all those unforgettable moments and discussions we have had in the laboratory and outside it. I also thank “iso-Kirsi”, Leena, and Johanna for a pleasant working atmosphere. My colleagues in the laboratory of the 4th floor in Biomedicum have made the writing atmosphere comfortable and fruitful. I want to thank for this atmosphere Erik Mandelin, Piia Vuorela, Katja-Anneli Wathen, Krisse Nokelainen, Riitta Koistinen, Laura Sarantaus, Pia Vahteristo, Susanna Lintula, Jakob Stenman, Jari Leinonen, Johanna Tapper, and all the others. This work would not have been made without the excellent technicians; Tuija in our own laboratory; Eira, Gynel, Kaija, and Marjatta at the Department of Obstetrics and Gynecology; Elina and Päivi at the Department of Pathology.

I wish warm thanks to Laila Selkinen and Leena Vaara for their humanity and irreplaceable help in many practical things during all these years.

Without all my colleagues at Helsinki University Central Hospital the scientific and clinical work would have been without joy and happiness. Your encouragement, support and interest in my work have helped me to complete this thesis. Especially warm thanks to Tarja Kalme with whom “vertaistukiryhmä” was grounded in order to share all things in joy and in sorrow.

My friends outside the laboratory have been irreplaceable during these years. My friend and colleague Leena Laitinen, thank you for the “therapeutic meetings and calls” during which so many clinical, scientific, and nonscientific problems were successfully ventilated. Thanks to all members and families of “Eksymä 360°” and MAST for bringing to me the whole new wold outside the medical world.

I wish to thank colleagues in Seinäjoki Central Hospital for support and patience during the completion of my thesis.

My deepest thanks to my dear parents Maija-Liisa and Pertti, and my brother Harri for caring and supporting me at all times. Mum and Dad, you have taught me to appreciate learning and education, although you have always shown that your love is not dependent on my achievements. Harri, you have shown with your own life that reputation is not the only valuable thing to seek during our life. Raili and Elis, thank you for all that encouragement and help I have got from you.

I owe a depth of gratitude to my parents-in-law, Terttu and Sauli, and to Sari, Erkki, and children for their support and help in our everyday life.

My special loving thanks to our children Suvi and Antero, who have been able to make me remember the most valuable treasures of my life. Suvi, in the future I promise to use more often that paper made computer made by you than this real one. Antero, after all this work has been finished I am sure you can enjoy fresh buns also at our own home. Finally I own my warmest thanks to my husband Antti for his never ending patiency and firm love and support during this work, which has been from time to time full of anger for computer problems and despair of overloaded work. Most of all, I want to thank Antti and children for bringing happiness and energy to my everyday life.

This study was financially supported by grants from the Emil Aaltonen Foundation, the Finnish Cultural Foundation, the Finnish Medical Foundation, the Research Funds of Helsinki University Central Hospital and Seinäjoki Central Hospital.

Nurmo, January 25th 2005

Tiina-Liisa Erkinheimo

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