Prostaglandins and Angiogenesis in Experimental Cancer

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To my family, especially my beloved children Moa, Ellen and Arvid

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Abstract:

Background and aim. Genes, proteins and pathways have been identified and suggested as potential targets in tumor angiogenesis, but current anti-angiogenic therapies have provided only modest benefits in survival of cancer patients. Therefore, further understanding of underlying mechanisms of tumor induced angiogenesis is mandatory in order to develop effective anti-angiogenic treatments in cancer disease. We have therefore focused on the role prostanoids may have to support tumor vasculature in progressive tumor growth of tumors.

Methods. Two fundamentally different tumor models were used. MCG-101 tumors induced increased systemic levels of PGE_2 and showed high sensitivity to COX inhibition, while K1735-M2 tumors did not produce PGE_2 and were thus insensitive to COX inhibition regarding tumor growth in syngenic wild type mice. EP_1- and EP_3-receptor knockout tumor-bearing mice were also used. COX-inhibition was provided by indomethacin in the drinking water to block prostanoid synthesis in tumor and host tissues. Intravital microscopy was performed using a dorsal skin fold chamber technique for studies of early tumor growth and associated angiogenesis. Immunohistochemical and microarray analyses were applied.

Results. Indomethacin reduced tumor growth and tumor related vascular area in wild type mice bearing MCG-101 tumors, but did not affect these parameters in K1735-M2 tumors. There was an unchanged relationship between the load of malignant cells and supportive vascular area among different tumor growth conditions. Unselective COX inhibition reduced tumor growth in EP_3, but not in EP_1 knockouts without significant alteration in tumor vascular density in EP_3 knockouts. Indomethacin treatment influenced expression of a large number of genes (5% of >40 000 probes) responsible for important steps in carcinogenesis, inflammation, angiogenesis, apoptosis, cell cycle activity and proliferation, cell adhesion, carbohydrate & fatty acid metabolism and proteolysis in tumors on wild type mice. Affected genes were widely and uniformly distributed on chromosomes over the entire genome. Variation of COX-2 staining in MCG-101 tumors was significantly reduced following indomethacin treatment. Effects of altered prostanoid metabolism were significantly related to EGF-R expression in tumor tissue and transcripts of KRas, PI3K, JAK1, STAT3 and c-jun were down-regulated by indomethacin, while STAT1 and ELK1 did not show any such decline.

Conclusion. Indomethacin treatment reduced tumor cell proliferation and increased tumor cell apoptosis in MCG-101 tumors with associated adaptive alterations in tumor vasculature. These effects were best predicted by alterations in EGF-R expression in tumor tissue with main downstream effects through KRas signaling.

Key words: angiogenesis, dorsal skin fold chamber, prostanoids, PGE_2, indomethacin

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I Axelsson H, Bagge U, Lundholm K, Svanberg E. A one-piece plexiglass access chamber for subcutaneous implantation in the dorsal skin fold of the mouse. Int J Microcirc Clin Exp. 1997 Nov-Dec;17(6):328-9.

II Axelsson H, Lönnroth C, Wang W, Svanberg E, Lundholm K. Cyclooxygenase inhibition in early onset of tumor growth and related angiogenesis evaluated in EP1 and EP3 knockout tumor-bearing mice. Angiogenesis. 2005;8(4):339-48.

III Axelsson H, Lönnroth C, Andersson M, Wang W, Lundholm K. Global Tumor RNA Expression in Early Establishment of Experimental Tumor Growth and Related Angiogenesis following Cox-Inhibition Evaluated by Microarray Analysis. Cancer Inform. 2007 May 1;3:125-39.

IV Axelsson H, Lönnroth C, Andersson M, Lundholm K. Mechanisms behind COX-1 and COX-2 inhibition of tumor growth in vivo. Manuscript.

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ABBREVIATIONS

AA	Arachidonic acid
ADAM	A disintegrin and metalloprotease
aFGF	Acidic fibroblast growth factor
AKT	Protein kinase B
ALK 5	Activin receptor-like kinase 5
AMH	Anti-Müllerian hormone
Ang	Angiopoetin
ANOVA	Analysis of variance
AP-1	Activating protein 1
Arf	ADP ribosylation factors
ATF	Activating transcription factor
ATP	Adenosine-5'-triphosphate
BAD	Bcl-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BCL-XL	B-cell lymphoma-extra large
Bcl-2	B Cell Lymphoma-2
bFGF	Basic fibroblast growth factor
BFL	Bcl-2 homolog isolated from a human fetal liver
BH	Bcl-2 homology region
BID	BH3 interacting domain death agonist
BIM	Bcl-2 interacting mediator of cell death
BMP	Bone morphogenetic proteins
BOK	Bcl-2-related ovarian killer protein
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
CDK	Cycline-dependent kinase
CD36	Cluster of differentiation 36
COX-1, -2, -3	Cyclooxygenase -1, -2, -3
CREB	cAMP response element-binding
CV	Coefficient of variation
DAF	Decay-accelerating factor
DAG	1,2-diacylglycerol
DNA, cDNA	Deoxyribonucleic acid, complementary DNA
DP receptor	D-prostanoid receptor
EDG-receptor	Endothelial differentiation gene receptor
EGF, EGF-R	Epidermal growth factor, EGF-receptor
EP_{1}-$_{4}$ receptor	E-prostanoid receptor 1-4
ErbB	Erythroblastic leukemia viral oncogene homolog
ERK	Extra-cellular signal-regulated kinase
FAS	Apoptosis Stimulating Fragment
FGF	Fibroblast growth factor basic FGF (bFGF, FGF-2)
Fos	FBJ osteosarcoma oncogene
FP receptor	F-prostanoid receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Grb2	Growth factor receptor-bound protein 2
HER	Human epidermal growth factor receptor
HIF-1	Hypoxia inducible factor 1

IAP	Inhibitors of apoptotic proteins
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL	Interleukin
INK 4	Inhibitor of CDK4
IP receptor	I-prostanoid receptor
IP_3	Inotiol 1,3,5-triphosphate
JAK	Janus kinase
JNK	Jun terminal kinase
Jun	Ju-nana (japanese for 17)
KIP	Kinase inhibitor protein
LOX	Lipooxygenase
MAbs	Monoclonal anti-EGF-R antibodies
MAPK	Mitogen activated protein kinase
MCG	Methylcholanthrene induced sarcoma
MCL-1	Myeloid cell leukemia
Mdm 2, 4	Murine double minute 2, 4
MEK	MAP Kinase/ERK Kinase
MMP	Matrix metalloproteinase
Myc	Myelocytomatosis related oncogene
MSG	Metastasis suppressor gene
NF-KB	Nuclear factor-KB
nm23	Non-metastatic 23
NRP	Neuropilin
NSAID	Non-steroidal anti-inflammatory drug
Par6	Partitioning-defective 6
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD-ECGF	Platelet derived endothelial cell growth factor
PDGF, PDGF-R	Platelet derived growth factor, PDGF-receptor
15-PGDH	15-hydroxyprostaglandin dehydrogenase
PGD_2, E_2, $F_{2\alpha}$, G_2, H_2, I_2	Prostaglandin D_2, E_2, $F_{2\alpha}$, G_2, H_2, I_2
PI3K	Phosphoinositide 3-kinases
PIP box	PCNA-interacting protein box.
PIP_2	Phosphatidylinositol 4,5-diphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PPAR	Peroxisome proliferator-activated receptors
PP2A	Protein phosphatase 2
pRB	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
PUMA	p53 up-regulated modulator of apoptosis
p 53, 21, 27	Protein 53, 21, 27
qRT-PCR	Quantitative real time polymerase chain reaction
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma

RFC	Replication factor C
Rho	Ras homolog gene family
RNA, mRNA	Ribonucleic acid, messenger RNA
SEM	Standard error of the mean
Shc	Src homology and collagen kinase
Smac	Second mitochondria-derived activator of caspases
SMAD	Sma and Mad related protein
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
Tie-receptor	Tyrosine kinase with immunoglobulin-like and EGF-like domains receptor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TP receptor	T-prostanoid receptor (thromboxane receptor)
TSP-1	Thrombospondin-1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TXA_2	Thromboxane A_2
VEGF, VEGF-R	Vascular endothelial growth factor, VEGF-receptor

INTRODUCTION

A malignant tumor is defined as a cell population characterized by neoplastic, unregulated growth with subsequent invasion into neighboring tissues and metastatic spread to distant locations in the body via lymph and blood circulations. Human malignant tumors are classified into different groups according to their cellular origin. Carcinomas arise from epithelial cells and are by far the most frequent malignant tumors in man. Other common malignant tumors are sarcomas with origins in mesenchymal cells, blastomas from embryonic tissue, hematopoetic neoplasms (lymphoma and leukemia) from hematopoetic cells, germ cell tumors from totipotent cells and neuroectodermal tumors, which originate from cells in the nervous system. Malignant tumors are the second most common cause of death in Western countries, next to cardiovascular diseases. The cancer incidence and prevalence vary among different human populations. Prostate-, breast-, colorectal-, lung-, and different form of skin cancer dominate in the Western world, while gastric cancer is common in Japan. Reported cancer incidences are below that of Western countries in developing countries, probably because of low expected survival and poor medical service including insufficient diagnostic means. Cancer affects individuals at all ages with higher risks for most types at increasing age. Hereditary factors dominate in 5-10 per cent of cancers, while the remainder is rather caused by acquired and sometimes unrecognized environmental factors. In Sweden, about 50 000 new cases of cancer are diagnosed yearly, and the overall risk to get cancer disease is one third across a lifespan.

Tumor formation is a complex process that involves a great number of pathophysiological events and multiple signal transduction pathways. Transformation from normal cells to cancer cells is probably a multi-step process, which generally occurs over extended period of time. Cancer cells may acquire properties that most normal cells do not possess or express, including ability to proliferate without high dependency on growth factor exposure, limitless replication, resistance to growth inhibition, reduced apoptosis and decreased sensitivity to immune surveillance as well as increased capacity to invade and metastasize based on induced angiogenesis.

Angiogenesis is the formation of new blood vessels from endothelium of existing preformed blood vessels, appearing in a number of physiological and pathological conditions. Physiological angiogenesis occurs in growing tissue e.g. in reproduction organs during the

menstrual cycle as well as in the growing fetus and child. Angiogenesis in pathological condition is seen during wound healing, chronic inflammation and ischemic conditions. Infantile hemangioma and diabetic retinopathy are other examples of disease conditions related to excessive angiogenesis. In 1945 Algire and Chalkley were the first to conclude that growth of a solid tumor is closely connected to the development of an intrinsic vascular network (1). In 1971 Judah Folkman presented his hypothesis that tumor growth is dependent on angiogenesis and that targeting blood supply, by inhibiting blood vessel formation, should lead to arrest of tumor growth with subsequent tumor shrinkage (2). This hypothesis is now regarded a hallmark of cancer treatment and a number of pro- and anti-angiogenic factors have been identified based on their ligands and intracellular signaling pathways (3). Without the capacity of stimulating angiogenesis, tumors cannot grow to a size larger than 1-2 mm^3. In larger tumors, blood vessels are essential for supporting the tumor with oxygen and nutrients and also for removal of waste products as CO_2 and other metabolites, whereas diffusion may be sufficient for required exchanges of such products in smaller tumors. A diffusion limit of oxygen has been estimated to be around 100µm. Growth of normal and neoplastic tissue is thus entirely dependent on angiogenesis for progression (2, 4, 5), and angiogenic processes are finely tuned and represent a balance between angiogenic stimulators and inhibitors. In addition to tumor cells, regulators of the angiogenetic process are also produced in tumor related endothelial cells, stroma cells, circulating endothelial progenitor cells, platelets and macrophages. Accordingly, tumor angiogenesis is a complex process dependent on both tumor- and other cells in a tumor microenvironment.

Many genes, proteins and pathways have been identified as potential targets for anti-angiogenic therapy. The VEGF/VEGFR signaling pathway is the most evaluated and considered highly important. Several strategies have been employed to inhibit this pathway, including antibodies to VEGF (bevacizumab/Avastin®; ranibizumab/Lucentis®) (6) and to the VEGF-receptor (IMC-1121B), and by blocking tyrosine kinase activity of the receptor by Small-Molecule Receptor Tyrosine Kinase Inhibitors (RTKIs) (sunitinib/Sutent®, sorafenib/Nexavar®) (7). However, there are additional possibilities of targeting angiogenesis, like mimicking endogenous inhibitors as thrombospondin-1 (ABT-510) and endostatin (8). Thalidomid is also known to attenuate angiogenesis by inhibition of endothelial cell proliferation, although the exact mechanism is still unclear (9). Angiogenesis can also be inhibited by preventing the degradation of extracellular matrix and basal membranes by MMP-2/MMP-9 inhibitors (MMI-166) (10). Inhibition of cellular adhesion

molecules, such as integrin$\alpha_v\beta_3$, also attenuates angiogenesis (11). Several established drugs have been found to have potential anti-angiogenic properties in addition to their primary mode of action. Such drugs include cetuximab (Erbitux®), which is an antibody to the EGF-receptor, the HER2-antibody trastuzumab (Herceptin®), Interferon-α (IntronA®) (12) and selective COX-2 inhibitors as celecoxib (Celebra®) (13). Anti-angiogenesis constitutes mainly a cytostatic therapy and it is assumed to provide greatest therapeutical effects when combined with cytotoxic chemotherapy or radiotherapy. However, current anti-angiogenic treatments have so far provided only modest survival benefits in cancer patients despite theoretically promising characteristics. The median survival was prolonged by 4.7 months in patients with metastatic colorectal cancer (20.3 vs. 15.6 months, $p<0.001$ (6)); 1.7 months in recurring and metastatic breast cancer (26.5 vs. 24.8 months, $p<0.14$ (14)); 2.0 months in advanced and metastatic non-small cell lung cancer patients (12.3 vs. 10.3 months, $p<0.003$ (15); and 2.0 months in patients with advanced or metastatic renal cell carcinoma (23.3 vs. 21.3 months, $p<0.34$ (16)). Such improvements refer to studies where standard chemotherapy was combined with bevacizumab (Avastin®) vs. chemotherapy alone. Future improvements will certainly be best provided by increasing our knowledge about underlying angiogenic and tumor mechanisms in future development of effective anti-angiogenic drugs. Studies in this field are thus mandatory to improve impacts on anti-angiogenesis in tumor treatments and its clinical applications.

The pathophysiology behind tumor development and growth cannot be entirely explained by alterations inside tumor cells. Therefore, it is frequently recognized how important tumor microenvironments are with stroma cells that profoundly may influence a variety of steps in the carcinogenic process, such as malignant transformation, tumor cell proliferation, invasion, angiogenesis and metastasis (17-24). Interactions between different cell types within tumor compartments, both via soluble factors and direct via cell to cell contacts are important. During recent years it has been recognized that prostaglandins are main mediators in such control and signaling activities. Therefore, one important issue in the present work was to further elucidate the roles of prostaglandins in regulation of tumor net growth and angiogenesis. A second aim was to understand angiogenesis and identify significant pathways behind tumor formation and growth with special emphasis on relationships to prostaglandins. These studies were therefore decided to be performed at in vivo experimental conditions to mimic as close as possible clinically relevant prerequisites.

AIMS OF THE PRESENT STUDY

Main aims of this thesis are:

1. To develop an in vivo, intravital chamber based, tumor model for studies of early tumor growth and angiogenesis in tumor-bearing mice.

2. To elucidate the role of prostaglandins in regulation of tumor establishment, angiogenesis and progressive tumor growth.

3. To survey angiogenic processes with special emphasis on connections between prostanoids and other signaling pathways in tumor tissue.

METHODOLOGICAL CONSIDERATIONS

Tumor models and animal groups

MCG-101 tumor

A methylcholanthrene induced sarcoma (MCG-101) was used in all experiments. This tumor model has been continuously transplanted in vivo at our laboratory for more than 25 years. The tumor was originally induced chemically as a sarcoma, while subsequent histological evaluation revealed that few tumor cells, if any, had characteristics of a sarcoma. Therefore, this tumor should rather be classified as a low or undifferentiated, rapidly growing, epithelial-like solid tumor. It has a reproducible and exponential growth pattern with a doubling time of 55-60 hours in vivo (25). It leads to 100% tumor take and does not give rise to visible metastases within the time period it kills the host. Tumors normally comprise 15 - 20% of the body weight of the tumor-bearing animals at the time of spontaneous death due to anorexia and cachexia. MCG-101 cells produce or may induce increased systemic levels of prostaglandin E_2, while COX-1/COX-2 inhibition by indomethacin and normalized systemic levels of PGE_2, reduced tumor growth, improved nutritional state and prolonged host survival (1, 2). Such effects by indomethacin were in part due to decreased tumor cell proliferation and increased apoptosis as well as attenuated angiogenesis (Paper II).

Experiments with MCG-101 tumors were performed in syngenic mice (C57 black mice), and gene knockouts (C57) deficient in prostaglandin E_1 and E_3 receptor subtype. Two different types of tumor preparations were used. Intravital chambers with implanted microscopic tumors (Paper II & III) were maintained in Mc Coy's 5 A medium (MP Biomedicals, Inc., Aurora, Ohio, USA) supplemented with fetal calf serum (FCS, 10%), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (292 µg/ml). Cells were split 1/5 once weekly with a medium change in between (Mc Coy´s 5A + 2% FCS, penicillin, streptomycin and L-glutamine as mentioned above). The viability of such tumor cells was >99% evaluated by trypan blue exclusion and microscopic examination before inoculation. Cells were trypsinized and suspended in Mc Coy´s 5A medium at a concentration of 1.15 x 10^5 cells/µl and 0.5 µl was inoculated into the intravital chamber as described. Solid tumors (Paper IV) were transplanted by tumor tissue ($3mm^3$) implantation subcutaneously on both sides of the back under light i.p. anesthesia (Ketalar®, Rompun®). Such tumor-bearing mice were killed after 10 days tumor growth. Tumors were dissected free for weight assessment.

K1735-M2 tumor

K1735-M2 tumors have different characteristics compared to MCG-101 tumors, such as slower growth rate (2, 4). Host animals did not develop anorexia or cachexia even in late stages of tumor growth beyond 20-25 days. Subcutaneous tumor progression is however associated with spontaneous appearance of lung metastases. K1735-M2 tumors do not produce or induce significant amounts of PGE_2 in vivo or in vitro. Therefore, COX-1/COX-2 inhibition did not affect tumor growth, host survival or nutritional state in this model. Experiments with K1735-M2 tumors were performed in syngenic mice (C3H/HeN mice) for intravital chamber experiments (Paper II), where tumor cells for inoculation were maintained in Mc Coy's 5 A (ICN) medium supplemented with 10% fetal calf serum (FCS) with a split ratio of 1/8 (K1735-M2) and a medium change (2% FCS) once weekly with L-glutamine, penicillin and streptomycin. The viability of the tumor cells was >99%. The cells were provided in suspension of 115 000 cells/µl and inoculated at 0.5 µl.

Animal groups

Intravital chamber experiments (Paper II & III)

Animals were housed in a temperature controlled room (24°C) with a 12 hour light/dark cycle. Mice were housed in separate cages during experiments to avoid interference with subcutaneously placed intravital chambers. All animals were allowed free access to ordinary rodent chow (ALAB AB, Stockholm, Sweden) and water ad libitum under all experimental conditions. Adult, weight stable, female mice, syngenic to the various tumors, were used in experiments. Animals were randomly assigned to treatment and control groups before implantation with tumor cell suspensions. Treatment groups received indomethacin (Confortid®, 5 mg/ml, Dumex-Alpharma) provided in the drinking water corresponding to 6 µg/ml water (1, 2, 4, 5). Appropriate dilution of indomethacin in the drinking water was calculated based on daily normal water consumptions of mice (3-4 ml water/mouse/day) corresponding to around 1 µg/g bw/day. Controls received ordinary drinking water. Indomethacin provision started two days before tumor cells were inoculated.

Solid tumor experiments (Paper IV)

Adult, age-matched, weight-stable (20-24g), female, wild type C57 black mice were used. Mice were randomly assigned to treatment and control groups before implantations and animals were treated with indomethacin in drinking water, as described above. All animals

were housed in plastic cages in a temperature controlled room (24°C) with a 12-hour light/dark cycle, and were provided free access to water and standard laboratory rodent chow.

EP_1- and EP_3-receptor knockout mice

Breeding parents of knockout mice were a kind gift from professor Narumiya, Department of Pharmacology, Faculty of Medicine, Kyoto University, Japan. All animals used in our experiments were bred in-house. Both EP_1 and EP_3 targeted structures were Neo-inserted form and the expected genomic defects were checked (6, 7). PCR analysis of genomic DNA was used for confirmation of disruption of genomic DNA isolated from our bred mice on white adipose and kidney tissue by QIA amp DNA 51306 (Qiagen).

Intravital chambers and microscopy

Intravital microscopy represents an experimental method for studies of angiogenesis and microcirculation in tumor- and host tissues. It is an in vivo method where living tissue is examined by microscopy by contrast to traditional immunohistochemistry microscopy, where tissues are fixed in formalin, sliced and stained before evaluation. Intravital microscopy allows repeated analyses of the same tissue over time (normally 2-3 weeks), making it possible to monitor time course events. Tissue samples can easily be excised and further examined at termination of experiments.

There are different chamber models available. Sandison presented the first model, implanted in rabbit ear in 1928 (26). Since then several modifications of material, surgical techniques, animal species and implantation sites have been presented. There are now a number of chamber models optimized for different research areas (27, 28) such as long-term chamber models, like the dorsal skin fold chambers (29), rabbit ear chambers (26, 30), cranial chambers (31, 32), femur chambers (33) and “body window” (to the kidney capsule) (34), where intravital microscopy can be performed for weeks or even months. There are also models based on acute preparations of the mesentery (35), omentum (36), cheek pouch (37), lymph nodes (38), liver (39-41) and mammary tissue (42, 43), where observations can be made only for hours.

The most commonly used chamber model is the dorsal skin fold chamber described by Algire 1943 (29). Since then similar models have been developed for the use in rats (44, 45) and

hamsters (46). These models are used in several research areas as microcirculation, ischemia/reperfusion, wound healing, tissue transplantation and tumor growth with subsequent formation of new blood vessels (angiogenesis) (47). Different types of tissue are possible to implant in the chamber depending on purpose. Material possible to implant include bone marrow (48), bones (49), pancreatic islands (50), ovarian follicles (51), vascular prosthesis (52), tissue-engineered scaffolds for bone (53), cartilage implants (54) as well as different types of tumor cells (47). Murine tumor cells implanted in syngenic immunocompetent mice as well as human tumors in immuno-incompetent mice have been used (47).

A great advantage of the dorsal skin fold chamber technique is that tumors can be directly and repeatedly observed over a period of 2-3 weeks. In this way tumor growth as well as associated tumor angiogenesis can be monitored. Multiple anatomical and functional parameters can be analyzed. However, there are also limitations. The observation time is limited to 2-3 weeks, which means that only rapidly growing tumors are favorable. The size of the observation window limits a maximum size of observed tumor and three-dimensional growth of a tumor may cause imaging problems. During the surgical procedure an open wound is created. This may induce granulation tissue and inflammatory signals when surgical procedures are not made gently. It is important that the chamber tissue is kept constantly wet to avoid drying. Bleeding should be avoided and carefully cleaned by saline. The skin should not be overstretched, since it may decrease blood flow and induce necrosis. The host tissue surrounding a tumor is used as internal control for the chamber quality. Users of this model should be aware of biological effects caused by organ specific microenvironments, since tumors implanted in the dorsal skin fold chamber are growing in subcutaneous tissue. Secretion of specific growth factors or cytokines may be limited.

We developed a modified chamber technique of dorsal skin fold in mice (Paper I). It had several advantages over previously described models. It is easy and cheep to manufacture, quick to install and has considerably lower size and weight reducing risks of overstretching chamber tissue. It is suitable for small animals with minimized discomfort for the animals. The visual field is improved, since there is no need for a fixation device (such as a spring washer) of the cover glass, which is kept in place by surface tension. The contour of the chamber is marked on a 20x30 mm sized 2-mm thick plexiglass plate. One 12-mm hole and four 1-mm holes are drilled at locations (Fig. 1). Excess plexiglass is adapted until final shape

of the chamber is attained. A slightly concave, paddle-shaped plate, made out of a 0.75-mm thick plexiglass tube, is joined to the straight bottom of the chamber by means of chloroform. This plate prevents the chamber from tilting sideways during experiments. Above measures can be changed allowing the chamber to be produced in a size suitable for larger animals.

Figure 1. a Intravital chamber with the front to the right. **b** Intravital chamber installed in mouse. **c** Intravital microscopy photo over the tumor and its neighboring tissue.

Before surgical procedures, mice were anaesthetized with i.p. injection of 0.15 ml from a 1 ml stock-solution composed of 0.4 ml Ketalar® (50 mg/ml; Parke-Davis), 0.05 ml Rompun® vet (20 mg/ml; Bayer), and 0.55 ml physiological saline. The dorsal skin of mice was shaved. Animals were kept at constant temperature of 36-37°C by heating pad during the procedure. An approximately 20 mm long midline incision was made in the dorsal skin just in front of the tail. Blunt dissection was used to free skin from underlying tissue. After being cleaned in alcohol the chamber was introduced into the skin fold with the tapering end forwards and positioned in the right place related to the vascular tree in transilluminating light. The

chamber was fixed to skin by 4-0 sutures using 1-mm holes in the periphery of the chamber. Sutures should not compromise blood vessels feeding the circulation in the chamber window. The midline incision, used for the installation of the chamber, was closed with two 4-0 stitches. The skin covering the right side of the central hole of the chamber was extirpated to expose subcutaneous tissue with its microvasculature of the contra lateral side. Tumor cells (5.75×10^4) were inoculated into the upper tissue layer of the chamber preparation by a Hamilton needle (10 µl). Small volumes of tumor cells (0.5 µl) were applied in order to avoid disseminated tumor growth within the chamber. The access chamber was closed by cover glass after inoculation kept in place by surface tension only. It can easily be removed and replaced giving full access to chamber tissues at any time. Mice were kept in separate cages so they could not inflict damage upon skin folds after insertion of the chamber.

There were few problems with infections, inflammations, edemas or hemorrhages in chamber tissue. Mice were in good condition during experiments and maintained body weight throughout the studies with observation time of 5 days, after which image problems may appear due to size. Without tumor cell implantation in chamber tissue, intravital studies can be made for 3-4 weeks without any significant problems of adverse tissue reactions. At the end of experiments chambers were gently removed from animals. The chambers are fully reusable after mechanical cleaning with dish brush, hot water and mild detergent followed by final disinfection with ethanol.

Microscopy

Observations of tumor growth and angiogenesis were made by intravital microscopy using Nikon Eclipse E400 microscope with Nikon Plan 4X/0,10 objective and Nikon Digital Camera DXM 1200. Photographic documentations were performed immediately after implantation of tumor cells at day 0 and at day 5 following tumor cell implantation. Digital pictures were kept in a computer for subsequent analysis. Image analyses were based on analysis of a digital photo across a specific area composed of tumor and its near surrounding tissues. This area was identical for day 0 and day 5 and the center of the photos corresponded to the central part of the tumor at visual inspection. The computer program Easy Image Analysis 2000, Tekno Optik AB was used for image analyses. A technique to quantify the area (mm^2) of tumor related blood vessels and the size of the tumor area in the same plane (mm^2) was applied. Tumor related vascular area was defined as the difference in vascular area between day 5 and day 0. This represents the appearance of net blood vessel formation around

the tumor during growth. Vascular density was the ratio between tumor vascular area and tumor area given in percent.

Immunohistochemistry (IHC)

IHC is a valuable technique utilized to localize and visualize protein expression in tissue sections. IHC has been used since 1950 to localize antigens in animal tissues and in 1970 this technique was used in plants. IHC may be used in conjunction with light or electron microscopy. Light microscopy usually provides sufficient resolution to describe distribution of antigens among tissues and cell types. Electron microscopy offers higher resolution and is particularly useful in determining distributions of antigens within a cell. Tissues for immunohistochemistry are fixed, embedded and then sectioned. Slides can either be generated from frozen or paraffin embedded sections. The ideal embedding medium should preserve both structure of tissue antigenicity. Two staining methods are used; the direct and the indirect method. One antibody, (the primary), is used in direct staining methods. The antibody binds to its specific epitope on investigated proteins and is usually prelabeled with a fluorochrome, which can be visualized by light microscopy. The indirect method is most commonly used with two different antibodies. The primary antibody is highly specific for the investigated epitope. The binding of primary antibodies is then detected by a secondary antibody, which forms complexes to the first antibody. The second antibody may be conjugated to a fluorochrome, gold particles or an enzyme such as phosphatase, which allows its visualization. This approach has the advantage that it introduces amplification and avoids initial conjugation of the fluorochrome to the primary antibody, which may decrease its affinity. Regardless of the procedures, it is essential to ensure that signals observed are due to the presence of the specific antigen. The tissue itself may give rise to signals by autofluorescence or the presence of endogenous peroxidase or phosphatase activity that have not been inactivated by the fixation and embedding procedures. Specificity of a primary antiserum is crucial and it may require affinity purification. A useful control is to confirm whether patterns of labeling are similar with crude and affinity purified antiserum. Tissue sections for analyses of specifically stained proteins (bFGF, TGFβ, NM23, p21, p27, p53, COX-2, EGF-R, BAX, Bcl-2, c-Jun, PCNA) as well as TUNEL and BrdU staining were in details as described separately in publications (Paper II & IV).

Solid tumor experiments for IHC (Paper IV)

After 10 days of tumor growth, mice were sacrificed and tumors were dissected free for weighing and studies with immunohistochemistry. Formalin-fixed and paraffin embedded tissue sections (4 µm) were deparaffinized and rehydrated according to standard procedures and rinsed twice in 5 mM Tris-buffered saline (TBS), pH 7.8. All further washes were done in TBS. Sections were either microwave-irradiated or enzyme treated. Specification of antigen retrieval (AR), antibodies, host species, final concentrations and suppliers are given in table 1, paper IV. Sections were mounted with Shandon Coverplates. Non-specific protein binding was initially blocked with TBS, containing 5% fat-free dry milk used for dilution of antibodies and normal IgG. Further non-specific binding was blocked with either normal goat IgG (sc-2028), rabbit IgG (sc-2027) (Santa Cruz) or normal mouse IgG_{2a} (X0943, Dako Cytomation), to match the type of secondary antibodies. This was followed by Dako Biotin Blocking System, X0590. Primary antibodies and corresponding concentrations of normal IgG for negative controls were incubated over night at + 4ºC. Secondary biotinylated antibodies were goat anti-rabbit (sc-2040, 1/400), goat anti-mouse (sc-2039, 1/200, Santa Cruz) or rabbit anti-goat (Dako E0466, 1/500). Streptavidin-alkaline phosphatase (RPN 1234, 1/150, Amersham Biosciences) was added following rinses. Dako Fast Red Substrate System (K699) was used followed by counter staining in hematoxylin for color development. Sections were mounted in Mount Quick Aqueous (Histolab Products AB, Sweden).

Intravital chamber experiments for IHC (Paper II)

At day 5 subcutaneous skin flaps were prepared from chambers containing the growing tumor following image analysis of the chambers. The tissue was fixed in phosphate-buffered 4% formalin at room temperature for 72 hours at + 4°C. Five µm thick sections were cut and mounted on Super Frost/Plus slides after tissue was embedded in paraffin blocks.

Image analysis

Immunohistochemically stained slides were studied in microscope and digital photos were recorded. Computer based image analyses (Easy Image Analysis 2000, Tekno Optik AB) were performed for quantification of expressed proteins as described (Paper II). Specifically stained protein area was the fraction (%) of each studied tumor area being a measure of the protein content in the tissue.

Microarray analysis

This allows measurement of the expression level of single genes in the whole genome within a particular tissue sample (55, 56). It represents a description of genome wide expression changes in health and disease. Microarray analyses can be used for diagnostic assessment and prognostic biomarkers, classification of diseases, monitoring response to therapy and evaluation of the biological processes in health and disease (57).

There are two major types of microarrays, as “single channel arrays”, which analyze one single sample at a time, and “multiple channel arrays”, which analyze two or more samples simultaneously. Two samples are labeled with two different dyes, which are simultaneously hybridized to the array in a competitive manner. This provides a ratio between the two samples (i.e. test and control samples) (57). All our experiments were based on two channel arrays (Paper III).

A DNA array is composed of a number of probes (nucleotide sequences) attached to an inert surface (microarray surface) (58). mRNA is extracted from the source of interest, reversed transcribed, labeled with a fluorescent dye (Cy3 green or Cy5 red) and hybridized to the array. An image is generated by using laser-induced fluorescent imaging (59). Fluorescent intensities for each gene are determined by use of a software program. The amount of fluorescence measured at each sequence specific location is directly proportional to the amount of mRNA with complementary sequence in the sample. The fluorescent intensities are used to generate a dataset, which has to be preprocessed before mathematical analysis. Data preprocessing includes background correction (adjustment for non-specific hybridization) (60), log transformation (improves the characteristics of the data distribution and allows the use of classical parametric statistics for analysis) (61, 62) and normalization (correct for systematic differences between genes and arrays) (63, 64).

Three major types of applications of DNA microarrays occurs: 1. Class comparison (finding differences in expression levels between predefined groups of samples, i.e. treated vs. untreated patients) (65). 2. Class prediction (identifying the class membership of a sample based on its gene expression profile) 3. Class discovery (analyzing a given set of gene expression profiles with the goal to discover subgroups that share common features). Each of these applications requires its own statistical strategy for data analysis. (57). Class comparisons were used in our experiments (Paper III).

RNA extraction and amplification

Tumors grown in intravital chambers were analyzed, where tumors treated with indomethacin were compared with untreated tumors. Pooling of tumors was made as described in paper III. RNA was extracted using Total RNA Isolation Microdissected Cryosections Kit (QIAGEN Sciences, Maryland, USA). Tissue disruption was done by aspiration with a syringe through 18 gauge needle 5 times in lysis buffer. Quality and quantity of RNA were checked in an Agilent 2100 BioAnalyzer with RNA 6000 Nano Assay kit (Agilent Technologies, Palo Alto, CA, USA). Concentrations of RNA were measured in a NanoDrop (ND-1000A) spectrophotometer (NanoDrop Technologies, Inc.). Isolated tumor weight ranged from 8.4 to 16 mg (Indo) and 7.2 to 20.1 mg (Ctrl) wet weight and total RNA ranged from 4.1 to 10.1 µg (both groups). RNA was amplified with BD Smart mRNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Unamplified total RNA for amplification reactions ranged from 425 ng to 946 ng with efficiency of 160 to 240 x amplification based on the assumption that 5% of the total RNA consisted of polyA+ mRNA. Amplified mRNA was checked for quality and quantity as described for total RNA.

cDNA Microarray profiling and data analysis

Expression array (Whole Mouse Genome Oligo Microarray, Agilent Technologies) containing 44290 features, including positive and negative control spots, was used. 400 ng of amplified mRNA fractions from indomethacin-treated animals (in experiment 1, pool of 200 ng 1A and 200 ng 1B= test) were labeled with Cyanine 3-dCTP (Amersham Biosciences) in cDNA synthesis reaction with Agilent Fluorescent Direct Label Kit. 400 ng of amplified mRNA fractions from untreated control mice (in experiment 1, pool of 200 ng 1C and 200 ng 1D= ctrl) were labeled with Cyanine 5-dCTP in parallel with the test-fraction. Hybridization was performed during 18 hours with test- versus control cDNA followed by post-hybridization washes according to “in situ Hybridization Kit Plus” (Agilent Technologies) instructions. Microarrays were dried with nitrogen gas in a laminar flow bench and images were quantified on Agilent G2565 AA microarray scanner and fluorescence intensities were extracted using the Feature Extraction software program (Agilent technologies). Dye-normalized, outlier- and background-subtracted values were further analyzed in a GeneSpring software program imported with the FE Plug-in (Agilent Technologies). Amplified mRNA from experiment 2 was analyzed in the same way as in experiment 1 as a replicate. Technical replicates of experiment 1 and 2 were performed in a second run.

Normal variation of gene expression in healthy, inbred mice was tested in muscle tissue from two individuals. PolyA+selected RNA was extracted and 400 ng from mouse 1 was labeled with Cyanine 3-dCTP and 400 ng from mouse 2 labeled with Cyanine 5-dCTP followed by hybridization to the same array targets with a ratio of 1.31±0.03 (M±SD) which confirmed validity and expected findings.

Quantitative real-time PCR

qRT-PCR combines PCR chemistry with fluorescent probe detection of amplified products in the same reaction vessel. This technology allows quantitative measurement of RNA concentrations and relies on real-time detection of amplified cDNA targets generated by successive rounds of PCR amplification. cDNA is detected on the basis of fluorescence, which increases proportionally with the PCR product. Quantification is determined by comparing the number of cycles required per sample to cross a certain threshold of fluorescence. This threshold is set in the linear phase of the reaction, such that the difference between samples in the number of cycles required to cross this threshold reflects the relative difference in starting amount of the target sequence. qRT-PCR reflects absolute value of the number of mRNA transcripts in the starting material, but more often is this method used to measure relative differences between different samples. Two different methods for detection are widely used. The DNA intercalating minor groove-binding fluorophore SYBR green only produces a strong signal when incorporated into double-stranded DNA. The dye selectively detects double-strand cDNA. The nested fluorescent probes, on the other hand, are designed to anneal to a specific sequence within cDNA. These probes contain a fluorescent label on one end and a quencher on the other. The fluorochrome is released from the quencher when the probe molecule binds to its target sequence with fluorescence directly proportional to the amount of specific product. Probes can be labeled with different fluorochromes, which makes it possible to measure several different products simultaneously in the same sample (multiplexing). The concentration of a reference gene, which is similarly expressed under the conditions tested, should be measured for every sample. Concentrations of experimental genes can then be expressed relative to the internal reference. Reference genes may be GAPDH, hprt, β-tubulin and β-actin.

qRT-PCR has high sensitivity and specificity and is a powerful tool for detecting and quantifying expression profiles of selected genes in tissue. The risk for release of amplified nucleic acids into the environment and contamination of subsequent analysis is negligent

compared with conventional PCR methods, since the nucleic acid amplification and detection steps are performed in the same closed vessel. The instrumentation requires considerably less hands-on and is much simpler to perform than conventional PCR methods. The procedure is completed in an hour or less, which is considerably faster than conventional PCR and detection methods. qRT-PCR has been available for more than 10 years, but there has been a dramatic increase in use over the last years (66). There are numbers of applications of this method in human medicine, as virology (67), bacteriology (68) in cancer research and clinical praxis. It is a commonly used validation tool for confirming gene expression results obtained from microarray analysis. Most of common cancers have been detected by measuring marker gene expressions. qRT-PCR can also be used for choosing drugs and monitoring therapeutic intervention in individual response to drugs (69, 70). There are also a great number of applications in veterinary- and plant medicine as well as in forensic science (66). The results of qRT-PCR depend critically on the correct use of calibration and reference materials. Sampling procedures are of great importance and are the largest, single source of error in the analyses. Another important step is extraction and the purification of nucleic acids (66).

RNA extraction, cDNA synthesis for quantitative real-time PCR

Total RNA was either isolated by the RNAzol method (code CS-101, CINNA/BIOTECX laboratories, Inc., Texas, USA) or extracted with RNeasy Micro Kit (cat. No. 74004, Qiagen) with the protocol for “Total RNA isolation from microdissected cryosections” (intravital chamber tumors). One microgram or 500 ng of total RNA from two experiments was reversed transcribed to cDNA with Advantage® RT-for-PCR Kit (ClonTech cat. No. 639506) according to kit protocol. Each sample was diluted to a final volume of 100 µl. Reactions were run in parallel with the reverse transcriptase being omitted in the control for DNA contamination.

Real-time PCR was performed in a LightCycler 1.5 with QuantiTect SYBR Green PCR Kit and QuantiTect Primer assays, as specified (table 2, paper IV). PCR conditions: 15 minutes, 95º initial activation; 3-step cycling with 15 sec, 94º denaturation; 20 sec, 55º annealing; 20 sec, 72º extension. Number of cycles was 45-50. Two microliters of cDNA fractions were used for each amplification. All samples were analyzed in duplicate and compared to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Control Amplimer Set, 639003, BD Biosciences), which was used as a housekeeping gene and amplified with LightCycler Fast Start DNA Master^{Plus} SYBR Green 1 (code 03515885001, Roche). PCR

conditions for GAPDH: 10 minutes, 95º initial activation; 3-step cycling with 10 sec, 95º denaturation; 6 sec, 60º annealing; 18 sec, 72º elongation for 40 cycles. Quantitative results were derived using the relative standard curve method, where the standard specimen was cDNA from MCG tumor tissue from an untreated control mouse. All PCR products had the expected size when analyzed with Agilent 2100 Bioanalyzer in DNA 1000 Chip. All reactions were confirmed using both positive and negative controls (one dilution of standard curve cDNA and water substituted for cDNA, respectively).

Statistics

Results are presented as mean ± SEM. Comparisons between several groups were either performed by factorial analysis of variance (ANOVA) followed by Fischer's post hoc test or by the Mann Whitney non-parametric method. Spearman rank coefficients were used in correlation analyses. Forward stepwise and conventional multivariate analyses were performed by standard linear regression methods. $P<0.05$ was regarded statistically significant in two-tailed tests.

Microarray data

The ratio between expressed transcripts in tumor tissue of MCG-101 inoculates from study versus control animals were calculated in the GeneSpring software program. Genes with p-values outside the 99% confidence limit ($p<0.01$) derived by t-testing were regarded to reflect significantly up- and down-regulated genes.

RESULTS

Differences between MCG-101 and K1735-M2 tumors

Tumor growth and vascularity

MCG-101-tumors had a significantly higher growth rate than 1735-M2-tumors; MCG-101 cells grew approximately 30 per cent more rapidly than K1735-M2 cells during initial 5 days observation ($p<0.001$). (Table 1, paper II). Both MCG-101 and K1735-M2 tumor cells stimulated growth of tumor vessels (Fig. 2, paper II). There was at least a two-fold increase in tumor related vascular area after five days of tumor growth, in both MCG-101 and K1735-M2 tumors growing in wild type mice ($p<0.0001$) (Fig. 3, paper II). There was a trend to increased tumor related vascular area ($p<0.12$) and vascular density ($p<0.17$) in MCG-101 tumors compared to K1735-M2 tumors. (Table 1, paper II) Both tumors displayed a highly significantly positive correlation between tumor area and tumor related vascular area ($p<0.0001$) (Fig. 5 A-C, paper II). Tumor tissue content of bFGF protein (basic fibroblast growth factor) did not differ between MCG-101 and K1735-M2-bearing mice at day 5 following tumor implantation.

Indomethacin treatment of tumor-bearing mice

Tumor growth and vascularity

Indomethacin reduced significantly tumor area ($p<0.02$) and tumor related vascular area ($p<0.04$) in wild type mice bearing MCG-101 tumors, but did not affect these parameters in K1735-M2 tumors. (Table 2, paper II)

Indomethacin treatment reduced cell proliferation in MCG-101 tumors ($p<0.02$), evaluated by BrdU incorporation to tumor cell DNA (Fig. 7, paper II), and increased tumor cell apoptosis ($p<0.02$) (Fig. 8, paper II). Tumor tissue area, stained for bFGF protein, did not differ significantly in MCG-101-bearing mice with or without indomethacin treatment (Fig. 6, paper II).

EP_1- and EP_3-receptor deficiency

Tumor growth and mortality

Tumor growth (tumor area) was significantly higher ($p<0.01$) in EP_3-knockouts compared to wild type mice, while there was no difference in EP_1-knockouts and wild types. There was a trend to increased tumor growth in EP_3-knockouts compared to EP_1-knockouts ($p<0.07$). (Fig.

9, paper II) Thus, tumor net growth was promoted in mice lacking EP_3-receptors, while EP_1-receptors in host tissue did not seem to influence tumor growth. Seven mice (5 %, out of 130 used in experiments in paper II), died initially due to the experimental procedures subsequently to implantation of the intravital chamber. The distribution of these mice was 2/58 C57 black mice (3%), 1/24 C3H/HeN mice (4%) and 4/24 EP_1-knockout mice (17%) and 0/24 EP_3-knockout mice (0 %). These mice died during the hours following implantation of the chamber and were excluded from further analyses.

Indomethacin treatment

Tumor area was significantly reduced in MCG-101 tumors in EP_3-knockouts on indomethacin treatment ($p<0.03$), but it was not altered in EP_1-knockouts. Indomethacin reduced tumor related vascular area and tumor vascular density with a trend to statistical significance in EP_1-knockout mice ($p<0.10$), but did not affect these parameters in EP_3-knockouts. (Table 2, paper II).

Tumor vessel growth

There was a numerical trend to increased tumor vessel formation (tumor related vascular area) in EP_3-knockout mice ($p<0.15$) and a numerical trend to decrease in EP_1-knockouts ($p<0.16$) compared to wild type mice. Tumor vessel growth was significantly increased in EP_3-knockouts compared to EP_1-knockouts ($p<0.02$), and a trend to decreased vascular density in EP_1-knockouts compared to wild type mice ($p<0.16$) and EP_3-knockouts ($p<0.07$). (Fig. 9, paper II) Thus growth of tumor vessels seemed to be increased by lack of EP_3-receptors and reduced by lack of EP_1-receptors in host tissue.

Gene expression in MCG-101 tumors

The whole genome, including 41 534 probes (genes) was analyzed comparing gene expression in tumors with and without indomethacin treatment. Indomethacin up-regulated 351 (0.8%) and down-regulated 1852 (4.5%) of these genes ($p<0.01$). 1066 of 2203 transcripts had unknown gene products or unknown biological function of the corresponding protein. Such genes were therefore excluded in further consideration (Fig. 2, paper III).

Indomethacin treatment and gene expression in MCG-101 tumors

Genes with significantly affected expression by indomethacin treatment were located on all chromosomes and were relatively uniformly spread over the entire genome (Fig. 3, paper III).

Indomethacin treatment affected a great number of genes, important in different aspects of the carcinogenic process including inflammation, angiogenesis, apoptosis, cell cycle, proliferation, cell adhesion, carbohydrate & fatty acid metabolism and proteolysis. Distribution, according to functional aspects, is shown in Table 1 and Appendix in paper III. Indomethacin treatment down-regulated mainly stimulatory genes.

The effect of indomethacin treatment on genes related to arachidonic acid metabolism are shown in Fig. 4, paper III. Phospholipase A_2, PGI_2-synthase, PGE-synthase, 15-PGDH, ThromboxaneA_2-synthase, EP_2, TPa TPb, TNFα, Bcl-2, PPARγ, bFGF and DAF were up-regulated and COX-2, LOX 12, IP, VEGF, aFGF, Raf, Akt and Mcl-1 were down-regulated. These alterations represent probably both direct effects by indomethacin as well as secondary compensatory mechanisms.

Specific protein staining

Specific protein staining in tumor tissue from indomethacin treated mice and control mice are shown in Table 3, paper IV. Protein expression of p53 ($p<0.01$) was significantly down-regulated while PCNA ($p<0.001$) and TGFβ3 ($p<0.03$) were significantly up-regulated by indomethacin treatment. The amount of COX-2 in tumor tissue was not significantly affected by indomethacin treatment (Fig. 1A / Table 3, paper IV).

Variation of COX-2 staining in MCG-101 tumors was significantly reduced following indomethacin treatment ($p<0.05$). (Fig. 1B, paper IV). There was a significantly positive correlation between tumor weight and coefficient of variation in COX-2 staining area (Fig. 3, paper IV).

Staining areas of BAX ($p<0.01$), TUNEL ($p<0.001$) and p53 ($p<0.01$) were positively correlated to COX-2 staining in tumors from control animals, while staining areas of c-Jun ($p<0.01$) and p27 ($p<0.05$) correlated to COX-2 staining in indomethacin treated animals, but not in control animals. Staining areas of Bcl-2 ($p<0.001$ / $p<0.01$), NM23 ($p<0.01$ / $p<0.01$) and p21 ($p<0.05$ / $p<0.01$) correlated to COX-2 staining in tumor tissue from both indomethacin treated and control mice (Table 4, paper IV).

EGF-R staining ($p<0.01$) was positively correlated to tumor weight, while c-Jun ($p<0.01$), NM 23 ($p<0.05$) and PCNA ($p<0.01$) correlated negatively in univariate analysis on

untreated, control mice (Table 5, paper IV). Forward stepwise regression analysis involving all evaluated protein factors showed that only EGF-R significantly predicted tumor growth in control animals. By contrast, indomethacin treatment changed the positive correlation between EGF-R and tumor weight into a negative correlation with additional predictive information by p21 and p27 in multivariate analyses (Table 6, paper IV). Transcript analyses confirmed that EGF-R and KRas pathways were down-regulated in vivo during indomethacin treatment, while cultured MCG-101 tumor cells did not seem to be dependent on EGF-R signaling, since these cells more or less stopped EGF-R transcription in vitro.

DISCUSSION

Tumor growth and progression

Carcinogenesis and cancer development are related to accumulation of genetic lesions, involving activation of proto-oncogenes and inactivation of tumor suppressor genes, bestowing cells with properties necessary for cancer development. However, autonomous properties of cancer cells are not sufficient for progression, since cancer development also demands involvement of adjacent, non-malignant cells including vascular endothelial and inflammatory cells. Such cells can be recruited either from various locations in the host, delivered to the tumor site by the blood stream, or by proliferative growth of neighboring tissues. Thus, tumor promotion and progression are the result of a complex interaction between cancer cells and surrounding non-malignant cells in tumor environments (3).

Self-sufficiency in growth signals

Normal cells require mitogenic growth signals to be transferred into a proliferative state, while tumor cells may lack dependency on exogenous growth stimulation, since they may produce own growth signals (71). Many oncogenes are mimicking normal growth signals and growth factor receptors are overexpressed and structurally changed in cancer cells making such cells hyperresponsive to growth signals (71, 72). In cancer cells there are also alterations in downstream cytoplasmatic circuitry that receives and processes growth signals with subsequent attenuation of normal homeostatic mechanism and cell proliferation (73).

Insensitivity to antigrowth signals

Multiple antiproliferative mediators operate within non-neoplastic tissue, securing cellular homeostasis. Such growth-inhibitory signals may be disrupted in a majority of human cancers, leading to progressive growth. Differentiation-inducing signals are usually blocked in cancer cells, impairing cellular differentiation and stimulating cell proliferation.

Evading apoptosis

DNA damage, oncogene activation and hypoxia activate different signaling systems that compromise programmed cell death including cellular, cytoplasmatic and nuclear membranes extrusion of cytosol and chromosome degradation, nucleus fragmentation and cell corpse engulfment by nearby cells (74). Apoptotic procedures are in part executed by intracellular

proteases termed caspases (75). Cancer cells appear to exhibit resistance toward apoptosis by altering components of the apoptotic machinery(76).

Limitless replicative potential

Non-neoplastic, mammalian cells carry intrinsic, cell-autonomous programs that restrict replicative potentials. There is a loss of telomeric DNA from the ends of the chromosomes during each cell cycle. Cells enter a state termed crisis when they normally undergone 60-70 cell divisions. This state is characterized by karyotypic disarray, associated with end-to-end fusion of chromosomes, with a lack of telomeric DNA protection with subsequent massive cell death (77, 78). Malignant cells maintain telomeres in part by upregulation of a telomerase enzyme, which adds hexanucleotide repeats onto the ends of the telomeric DNA (79).

Sustained angiogenesis

Cell survival and function depend on sufficient supply of oxygen and nutrients and removal of waste products including CO_2. Incipient neoplasia must therefore develop angiogenic ability for progression to a size larger than 1-2 mm^3 (2). This process is regulated by the balance of stimulating and inhibiting factors with currently around 50 known angiogenic factors.

Tissue invasion and metastasis

Tissue invasion and metastasis enable cancer cells to escape from a primary tumor mass in order to invade and colonize tissues at other locations, where oxygen, nutrients and space are not limiting. Metastases are a main cause of human cancer death (80). This process involves activation of extracellular proteases including changes in expression and function of cell to cell-adhesion molecules (as E-cadherin) and integrins (81, 82).

Inflammation and tumor growth

The link between inflammation and the development of cancer has been recognized since 1863, when Rudolf Virchow discovered leukocytes in neoplastic tissues (83). Since then, a number of cancers have been linked to inflammation, which is increasingly recognized an important component of tumorigenesis. The inflammatory process mediates several fundamental tumor properties, although mechanisms involved are not fully understood (84, 85). Epidemiological studies have demonstrated that chronic inflammation can be the origin of various types of cancer triggered by different conditions as microbial infections (Helicobacter pylori and gastric cancer/gastric lymphoma), autoimmune diseases

(inflammatory bowel diseases and colon cancer) and inflammatory conditions of unknown origin (chronic pancreatitis and pancreatic cancer; prostatitis and prostatic cancer). Various inflammatory cells and mediators, including prostaglandins, chemokines and cytokines, are present in the microenvironment of most tumors. Accordingly, treatment with anti-inflammatory drugs may decrease progression of malignant tumors with subsequently reduced mortality.

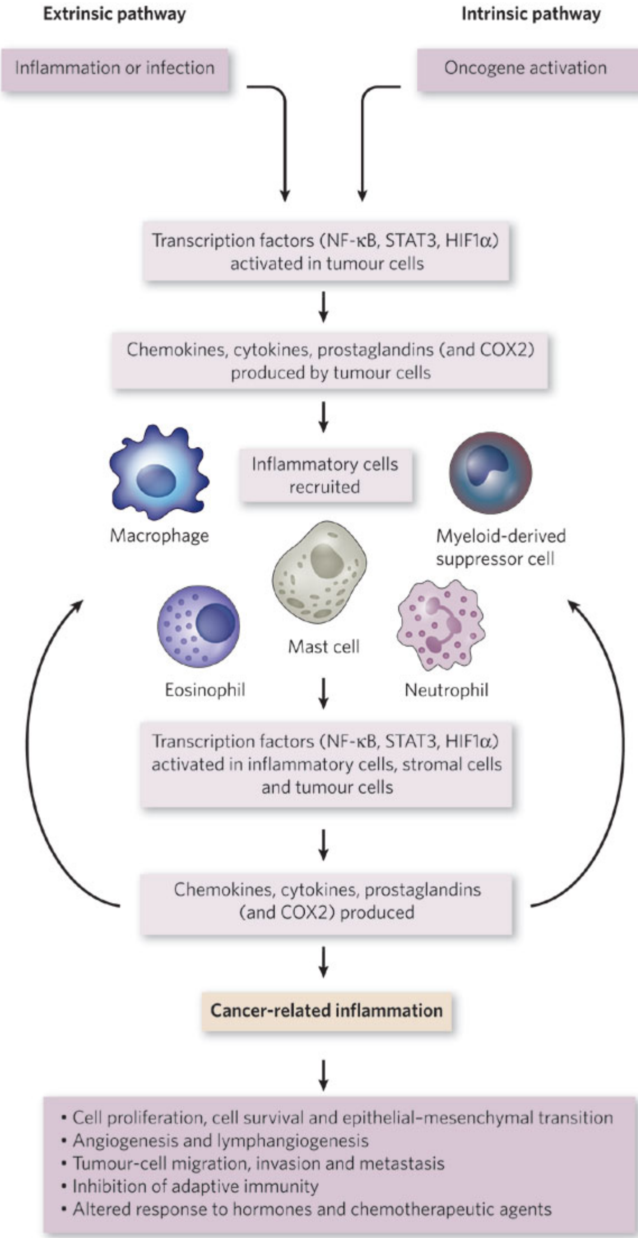

Figure 2. Upstream metabolic pathways connecting inflammation to cancer development and progression.
(Reproduced from fig. 1 Nature 454 doi10.1038/nature07205)

Cancer related inflammation may also create genetic events causing neoplasia, including activation of various types of oncogenes, chromosomal rearrangement and gene amplification as well as inactivation of tumor-suppressor genes. Cells transformed in this way display activated transcription factors (NF-KB, STAT3 and HIF1α), which may stimulate production of inflammatory mediators (chemokines, cytokines and prostaglandins), that may recruit and activate various types of inflammatory cells further (eosinophils, mast cells, neutrophils, macrophages and myeloid-derived suppressor cells). Thereby, a positive feed-back loop may be started generating cancer-related inflammatory microenvironment, which is in part a requirement for fundamental properties behind tumor development and progressive growth (Fig. 2).

Prostaglandin biosynthesis

Prostaglandins are 20-carbon fatty acid derivatives found in almost all tissues and organs in the body mediating a number of physiological and pathological functions. They are synthesized from different essential fatty acid precursors. Prostaglandins derived from arachidonic acid are termed series-2 prostaglandins or prostanoids and include prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 (TXA_2) (86). These prostaglandins share a common initial biosynthetic pathway, which begins with the hydrolysis of cell-membrane phospholipids with liberation of arachidonic acid into the cytoplasm (87). This step is mediated by membrane-bound phospholipase A_2, which is activated by diverse physiological and pathological stimuli (88). Arachidonic acid is converted by cyclooxygenase into unstable endoperoxide intermediate prostaglandin G_2 (PGG_2) which in turn is converted into oxygenated intermediate prostaglandin H_2 (PGH_2) (89). Phospholipase A_2 and cyclooxygenase are rate-limiting steps in prostaglandin biosynthesis. Three isoforms of cyclooxygenase have been identified; COX-1, COX-2 and COX-3. COX-1 is constitutively expressed and COX-2 is inducible by pathological stimuli (90-93). COX-3 is an isoform of COX-1 and is preferentially expressed in heart and brain (94). PGH_2 is in turn metabolized by cell-specific synthases (PGE-synthase, PGD-synthase, PGI-synthase, PGF-synthase and Tx-synthase) into series-2 prostaglandins (95). Prostaglandins are released outside cells immediately after being synthesized, where they interact with specific cell surface prostanoid receptors in autocrine or paracrine fashions (96). Alternatively, prostaglandins are transported by PG-transporters across cell membranes into cytoplasmatic compartments where effects are terminated by oxidizing and reducing enzymes (97, 98).

The biological action of the prostaglandins is mediated by specific prostanoid receptors located in cell membranes. These receptors belong to the Rhodopsin-type receptor family, which are characterized by their seven transmembrane domains coupled to different intracellular subunits of G proteins (99). There are five major types of prostanoid receptors as E-prostanoid receptor (EP receptor), D-prostanoid receptor (DP receptor), I-prostanoid receptor (IP receptor), F-prostanoid receptor (FP receptor) and T-prostanoid receptor (TP receptor). Each one of these major types consists of one or several subtypes with different structures and biological functions (96), which vary with the type of tissue and physiological condition. Functions and distribution of the receptors may vary among species (100) (Fig. 3).

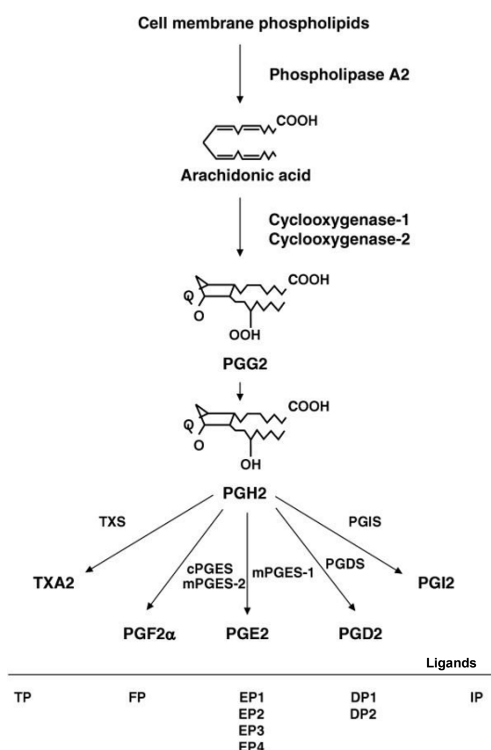

Figure 3. Biosynthetic pathways of prostanoid metablites.

PGE_2 is considered to be the most important among the serie-2 prostaglandins for physiological functions in malignant and non-malignant conditions. There are four different subtypes of EP receptors: EP_1, EP_2, EP_3 and EP_4. These receptors display overall sequence

identity of about 40% with putative transmembrane domains being most conserved (101). Biological signals are propagated through alteration in the intracellular calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) levels. Effects of PGE_2 are determined by the type and presence of EP receptors, which differ among cell types, organs and patophysiological conditions.

The EP_1 receptor has lowest affinity for PGE_2. It mediates signaling events by activation of phospholipase C and elevation of cytosolic Ca^{2+} concentration by activation of Ca^{2+} channels. This activates downstream kinases and transactivation of HER´s-2/Neu tyrosine kinase receptor with upregulation of vegetative endothelial growth factor-C (102). The EP_1 receptor also transactivates epidermal growth factor receptor, which promotes cell proliferation and invasion (103). The EP_2 receptor increases levels of cAMP and stimulates cellular growth by stimulating PKA and PI3K pathways (104). The EP_3 receptor is expressed in a wide range of tissues, mediating biological signals by inhibiting adenylate cyclase and thereby decreasing intracellular levels of cAMP. It is involved in acid-induced duodenal bicarbonate secretion and maintenance of mucosal integrity (105). It also participates in the regulation of tumor associated angiogenesis and tumor growth, and has been shown to activate the Ras signaling pathway (106, 107). Fever generation is regulated by the EP_3 receptors (108). In mice, there are three receptor isoforms of EP_3, generated by alternative splicing, differing in their C-terminal domain (109). Expression pattern of these isoforms differs between various cell types. It has been reported that EP_3 receptor isoforms differ in ability to down-regulate adenylate cyclase, but the biological significance of this finding is unclear (110). EP_4 receptors have high affinity for PGE_2 and rise intracellular levels of cAMP. It stimulates cell growth and proliferation like EP_2 receptors (104).

Altered expression of COX-2 and overproduction of prostaglandins are common in colorectal (111-114), breast (115), gastric (116), esophagus (117), pancreatic (118), bile duct (119), papillary thyroid (120), urinary tract (121), prostate (122), cervical (123) and lung cancer (124) as well as in malignant pheochromocytomas (125) and retinoblastoma (126). Elevated levels of COX-2 and PGE_2 are also seen in premalignant conditions such as Barrett´s esophagus (127) and colorectal adenomas (128). Thus, there is strong evidence that both COX enzymes are of importance for several cancer forms in man (129, 130).

It is well-recognized that non-steroidal anti-inflammatory drugs (NSAIDs), particularly indomethacin, attenuates tumor net growth (131), reduces tumor related cachexia, improves appetite and prolongs survival in tumor bearing mice (132-134) and in cancer patients (135, 136). There is also evidence from population based, case control and clinical trials that regular use of NSAIDs may reduce the relative risk to develop colorectal adenomas (137, 138) and colorectal cancer (113, 139, 140). Highly selective COX-2 inhibitors have retained anti-tumor effects, despite a lack of COX-1 inhibition, suggesting that COX-2 is the essential isoenzyme for tumor development (130, 141-143). However, in previous studies we have demonstrated that MCG-101 tumors are particularly sensitive to unselective cyclooxygenase inhibition compared to COX-2 selective drugs (144). Therefore we continued to use the unselective COX-inhibitor (indomethacin) in present studies. It is assumed that tumor reducing effects by indomethacin are mainly caused by blocking prostaglandin production (145) by competitive inhibition of substrate binding at the COX- isoenzyme.

A straight forward mean to attenuate tumor net growth may be to interfere with the prostanoid metabolism in tumor cells or neighboring endothelial cells, either by decreasing the formation of prostanoids or by blocking their corresponding receptors (90) (146, 147). However, the literature is not unequivocal in this respect and a large number of publications present results that seem to be divergent and even contradictory. Some studies report that NSAIDs seem to act directly on tumor cells (148, 149), while others put forward direct and main effects on the angiogenic process (150). Also, various tumor cell lines seem to exert different effects in similar experimental conditions (151). Surprisingly, it has been reported that COX-2 inhibition may increase tumor angiogenesis and metastatic potentials of tumor cells (152), although a majority of experimental studies report opposite results (153). One factor that may explain seemingly contradictory observations is variation in hypoxia in tumor tissue (148, 154). This is a factor that may impact unpredictably on both tumor and endothelial cells during experimental conditions. Most studies on NSAIDs and tumor related angiogenesis have usually been conducted on large biopsies from tumors where hypoxia is quite significant and variable. These conditions may involve adaptations to decreased tumor vascular supply in the presence of COX-inhibition. Therefore, it is important to evaluate effects of indomethacin in early onset of tumor growth when hypoxia is not significant. This phase may be differently dependent on angiogenesis compared to late stage tumor conditions. Our current experiments (Paper II) re-evaluated effects of unselective COX-inhibition for onset of tumor growth conducted in the chamber model based on intravital microscopy (Paper I); an experimental set

up where tumor cell hypoxia should be minimal. Our results demonstrated that indomethacin effects are observable immediately at onset of tumor growth. There were no significant differences in tumor vascular density among MCG-101 with high PGE_2-production and sensitivity to COX-inhibition in contrast to K1735-M2 tumors with minimal PGE_2-production and insensitivity to COX-inhibition. This fact demonstrates a rather constant relationship between the load of malignant cells and their supportive vascular area despite a two-fold difference in net tumor growth rate. This implies that prostanoids in endothelial cells were not of critical importance for establishment of tumor angiogenesis in contrast to findings in a number of previous reports. Indomethacin decreased tumor cell proliferation and increased tumor cell apoptosis; thus indomethacin seemed to primarily affect tumor cells with subsequently secondary effects on tumor angiogenesis. As expected angiogenesis was not a limiting step for tumor progression in early onset of tumor growth. Thus, a large number of reported alterations in expression of growth factor receptors and cell cycle control in endothelial cells may rather reflect adaptive changes secondary to primary alterations in tumor cells.

Tumor COX activities are not strictly dependent on tumor expression of COX-2, but may also be related to tumor tissue expression of prostaglandin E subtype receptors in addition to COX-1 expression (155). Earlier reports have demonstrated that tumor progression may also be dependent on other host prostanoid receptors and even to effects unrelated to COX-mediated mechanisms (156-158). Such effects may reside not only within tumor cells but also in surrounding stroma cells. Increased tumor growth and angiogenesis were seen in mice lacking EP_3-receptors. Deficiency of EP_1 receptor reduced tumor related angiogenesis, but not tumor growth. A simplistic interpretation of these results might be that host EP_3-receptors attenuate tumor growth and subsequent angiogenesis and that EP_1-receptors stimulate tumor angiogenesis. These results seem to disagree with a report by Amano et al in a sarcoma-180 model (106). However, available results demonstrate that early onset of tumor establishment is a complex interplay between tumor and host cells. One conclusion may be that host EP_1 receptors are preferentially involved in paracrinic loops with PGE_2 to support tumor angiogenesis, while host EP_3 receptors are more directly related to tumor cell proliferation and tumor growth (144). The explanation to discrepancy between present findings and the report by Amano et al remains unclear, but may relate to different models.

COX-2 is usually not evenly distributed among cells in malignant tumors, although it is often claimed that COX-2 is generally up-regulated in cancer cells. By contrast, immunohistochemical evaluations of tumor tissue usually demonstrate that COX-2 expression rather appears to be localized to certain areas within tumors, with increased expression also in neighboring stroma cells, as observed for RNA transcript of COX-2 in colon cancer tissue (159). Uneven appearance of COX-2 protein in tumor tissue implies tumor cell heterogeneity regarding prostanoid production (160, 161). Thus, it is difficult to confirm how cell signaling exerts effects among different cells in heterogeneous tumor compartments. In vitro co-cultivation of highly selected tumor and normal cells may not correctly reflect complex in vivo conditions among stroma, endothel and infiltrating inflammatory cells in proximity to proliferation of tumor cells in areas with hypoxia (162). Therefore, we evaluated co-variations between COX-2 protein and other proteins with importance for cell proliferation, apoptosis, cell adhesion, metastasis and angiogenesis (163, 164). These experiments focused on large established tumors with confirmed sensitivity to cyclooxygenase metabolites for progression. This approach was chosen in order to increase the power to detect long-term relevant relationships between estimates of protein staining and tumor growth in the present model highly dependent on tissue PGE_2. This was regarded important, since detection of alterations in protein staining is subjected to comparatively low sensitivity (165), particularly when compared to quantification of tissue content of RNA transcripts. However, transcription information is not always reflecting protein levels in cells, particularly in transformed rapidly proliferating cells with altered transporting and splicing of mRNA. Therefore, we preferred to remain with estimates of protein content by staining as major variables to allow evaluations of cellular distribution among cells, which should represent more definite information. Indomethacin provision to tumor-bearing animals altered p53, PCNA and TGFβ3 content in tumor tissue. Correlation analyses between COX-2 staining and other proteins confirmed significant positive relationships between COX-2 and BAX, TUNEL, Bcl-2, c-Jun, p21, p27, p53 and NM23. However, the total amount of COX-2 in tumor tissue did not directly correlate to tumor growth in our studies. The overall amount of COX-2 protein staining in tumor tissue was, as expected, not affected by indomethacin treatment, but variation in COX-2 staining within a tumor was significantly reduced by indomethacin treatment. Such results may suggest that indomethacin made tumors clinically more homogeneous.

Evaluations in paper III were based on intravital chamber- and microarray experiments in order to evaluate overall changes in RNA synthesis caused by indomethacin treatment to

further map important genetic areas behind tumor reducing effects by cyclooxygenase inhibition. Indomethacin altered expression of 2 203 genes out of 41 534 (5.3%). These genes were widely and relatively uniformly spread over the entire genome on all chromosomes. Indomethacin down-regulated five times as many genes as were up-regulated and affected genes were predominantly stimulatory in function. Indomethacin influenced the expression of a large number of genes responsible for important steps in the carcinogenic process including inflammation, angiogenesis, apoptosis, cell cycle, proliferation, cell adhesion, carbohydrate- and fatty acid metabolism and proteolysis. Malignant disease is also characterized by attenuation of cell mediated anti-tumor immune response, probably directed in part by PGE_2 based on reduced production of anti-tumor Th1 cytokines (TNFα, IFNγ and IL-2) (166) and increased production of Th2 cytokines (IL-4, IL-10 and IL-6) (167-169). TNFα was up-regulated, while genes coding for the other mentioned cytokines, were not changed by indomethacin in present experiments. Genes in control of fatty acid and protein metabolism were highly down-regulated by indomethacin, while genes for carbohydrate metabolism seemed to be both up- and down-regulated. Such alterations may contribute to reported beneficial overall host-metabolic effects by indomethacin attenuating catabolism caused by growing tumors in patients (132, 136).

Tumor angiogenesis

Malignant tumors may never turn from minimal residues into expanding tumors without angiogenesis. Such tumors may remain in a harmless condition called "tumor dormancy", a steady state where fully transformed and proliferating tumor cells do not grow, perhaps due to inabilities to induce angiogenesis. During the neoplastic process some tumors (~1/600) may switch to angiogenic phenotypes, where the net balance of positive and negative regulators are displaced and angiogenic stimulators as VEGF are produced and secreted from tumor cells (170, 171). This process is called "the angiogenic switch" and will cause formation of blood vessels. The angiogenic switch is a key step in early tumor progression, allowing exponential tumor growth and metastases. Oncogene-derived proteins as well as number of cellular stress factors, including hypoxia, low pH and nutrient deprivation, are important stimulators of angiogenesis (172).

Pro-angiogenic factors, produced by tumor cells, bind to endothelial cell receptors and induce angiogenesis. This process consists of highly regulated series of molecular and cellular

events. The process starts by the selection of endothelial "tip-cells", inside capillaries neighboring the tumor. Angiogenic stimuli cause major changes in the phenotype of tip-cells with properties of invasiveness and ability to migrate. It also activates secreted and cell surface proteases for partial destruction of adjacent basement membranes and extracellular matrix. Tip-cells start to migrate with directions regulated by VEGF gradients. Dissolution of extracellular matrix then allows the release of proangiogenic factors which, together with those produced by tumor cells, further stimulate angiogenesis. Endothelial cells proliferate and assemble in tubular structures behind migrating tip-cells. Maturation of newly formed blood vessels takes place when a sufficient amount of vascular tubes have been formed. The initial step of this process is fusion of newly formed capillaries. In this step, tip-cells stop migrating and make contact with other tip-cells or existing capillaries. A vessel lumen is formed when contact is made. Emerging blood flow contributes to stabilization of newly formed blood vessels by reducing hypoxia and thereby lowering VEGF levels. Capillaries are fused into large vessels including arteries and veins with junctional complexes. Newly formed blood vessels are covered by pericytes, basement membrane and smooth muscle cells during further maturation and stabilization. The walls of capillaries and fine blood vessels consist of a single layer of pericytes, whereas walls of arteries and veins are formed by several layers of smooth muscle cells separated from the endothelium by basement membrane (173, 174).

Maturation and stabilization of the vascular network are incomplete in tumor angiogenesis. This results in microvessels that are irregular and tortuous with partial endothelial linings and fragmentary basement membranes as well as increased microvascular permeability. Tumor vessels are different from normal vessels in several aspects with spreading without organization and changing diameters with loss of differentiation in arterioles, capillaries and venules.

Proangiogenic factors

Vascular endothelial growth factor (VEGF) was first discovered and cloned in 1989 by Napoleone Ferrara (175) and is a most potent angiogenic stimulating cytokine induced by hypoxia and several major growth factors expressed in tumors including EGF, TGF-α, -β, IGF-1, FGF and PDGF (176). Hormones, such as estrogen and thyroid-stimulating hormone and inflammatory cytokines as IL-1 and IL-6 are also known to induce VEGF. There are six known members of the VEGF family (VEGF-A, -B, -C, -D, -E and the placental growth factor) (177). Alternative splicing of the VEGF-A gene produces, at least four different

isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) (178, 179). $VEGF_{189}$ and $VEGF_{206}$ bind heparin with high affinity and are accumulated in extracellular matrix serving as a depot of VEGF, which can be released quickly through the cleavage of the heparin-binding domain by plasmin, releasing active VEGF-A (180). VEGF-A is mainly involved in angiogenesis, whereas VEGF-C and VEGF-D are involved in lymphangiogenesis.

The VEGF family activates endothelial cells by signaling through VEGF-receptors. There are three known VEGF-receptors (VEGFR-1, -2, -3) (177). VEGFR-1 and -2 are located on vascular endothelium and are up-regulated during angiogenesis, whereas VEGFR-3 is expressed on the endothelium of lymphatic vessels. Angiogenic effects are primarily exerted through the binding of VEGF-A to VEGFR-2, resulting in activation of a number of signal transduction pathways stimulating proliferation and migration of endothelial cells. VEGF is also a survival factor for endothelial cells; it prevents apoptosis by inducing expression of the antiapoptotic proteins Bcl-2 and A1 in endothelial cells (181-183). VEGF is known to regulate vascular permeability binding of VEGFR-1, which is important in inflammation and pathological conditions (184, 185). Lymphangiogenesis is preferably exerted through VEGF-C binding to VEGFR-3. Changes in the ratio of different types of VEGFR can be observed during tumor progression (186).

Fibroblast growth factors (FGF) acidic FGF (aFGF, FGF-1) and basic FGF (bFGF, FGF-2) constitute a family of heparin-binding proteins, known to induce angiogenesis (187). aFGF and bFGF bind to heparin sulphateproteoglucans in the extracellular matrix and induce differentiation of epiblast cells into endothelial cells for stimulation of proliferation. bFGF is also known to release urinary plasminogen activator and collagenases in endothelial cells and acts as a chemo-attractant.

Neuropilins (NRP) is a class of receptors located on some tumor and endothelial cells. The expression of these receptors is necessary for angiogenesis (188), and they are known to interact with VEGF, probably by acting as co-receptor to VEGFR. There are two different forms of neurophilins, NRP1, which is found in arteries, and NRP2, which is found in veins and lymphatic vessels (189-191).

Four types of angiopoetins are known (Ang-1, -2, -3, -4), where Ang-1 and -2 are best characterized. Both are exerting biological functions through binding to the Tie-2 receptor

(192). Ang-1 is expressed in tumor cells, pericytes and smooth muscle cells and promotes endothelial cell survival and sprouting (193). It is also known to stabilize newly formed vascular networks by recruiting and incorporating pericytes to immature vessel segments. It lowers vascular permeability and exhibits anti-inflammatory activity (194-197). Ang-2, on the other hand, is expressed on sites of vascular remodeling, causing loss of pericytes, which expose endothelial cells to angiogenic factors. This destabilization induces angiogenic response in the presence of VEGF, but Ang-2 contributes to vascular regression in the absence of VEGF (192).

The platelet derived growth factor (PDGF) family consists of four different types of PDGF (PDGF-A, -B, -C, -D), which exert biological functions by binding to one of two known PDGF-receptors (PDGFR-α and –β). PDGF-B plays a key role in maturation of newly formed blood vessels (198). It is expressed at high levels in tip-cells, which stimulates recruitment of pericytes and smooth muscle cells and the incorporation of these cells into vessel walls (194, 199, 200).

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric protein, known to play a central role in tissue response to hypoxia. Under normoxic conditions, one of the two subunits, HIF-1α, is rapidly degraded in a proteasome dependent pathway, while degradation is markedly diminished under hypoxic conditions, resulting in formation of stable HIF-1 heterodimers. p53 is a main regulator of the HIF-1α degrading process. HIF-1 induces activation of specific genes whose products act to decrease oxygen concentration in tissues, for example VEGF (201). Hypoxia independent up-regulation of HIF-1 has been described, occurring as a downstream event of growth factor signaling as EGF-R activations (202). Overexpression of HIF-1 in colorectal cancer has been demonstrated to correlate with VEGF expression and advanced tumor stage (203, 204).

Transforming growth factor β1 (TGF-β1) is activated during contact between endothelial cells and pericyte progenitors. This results in inhibition of endothelial cell proliferation and migration (205, 206), inhibition of VEGFR2 expression (207) and differentiation of pericyte progenitor cells into mature pericytes (193, 208).

Platelet derived endothelial cell growth factor (PD-ECGF) is a thymidine phosphorylase, acting as a powerful chemoattractant on endothelial cells, which exerts marked angiogenic

responses in tumor models (209, 210). Expression of this factor has been correlated to poor prognosis in gastric and pancreatic cancer (211, 212).

Chemokines are small (8-12 kDa) secreted proteins serving a wide array of receptor dependent immune functions (213). Chemokines displaying the ELR (Glu-Leu-Arg) amino acid motif have been shown to have direct angiogenic properties (ELR^+ chemokines; angiogenic chemokines). IL-8 is the most extensively studied angiogenic chemokine (214) and its effects are mediated by receptors CXCR1 and CXCR2 (215). In addition to a direct action on epithelial cells, release of secondary angiogenic factors are seen (216).

A multitude of additional secreted mediator molecules have been proven to have angiogenic effects. Interferon α, -β, -γ and tumor necrosis factor α (TNF α) have direct effects on endothelial cells, while hepatocyte growth factor, IGF-1 and IL-1 family members act indirectly on endothelial cells in secondary modulation of VEGF expression (217-219).

Antiangiogenic factors

Thrombospondin-1 (TSP-1) belong to a family of extracellular matrix proteins, which exerts action by binding to its cellular receptor CD36 (220), located on microvascular endothelium, starting a sequence of intracellular events finally resulting in endothelial cell apoptosis (221). TSP-1 can inhibit angiogenesis through interaction with pro-MMP2/9, MMP2/9 or induction of cell cycle arrest.

Endostatin is generated by cleavage of a 20-kDa fragment of collagen XVIII, a proteoglucan found in vessel walls and basement membranes. Endostatin is a powerful cytokine, inhibiting endothelial cell migration, inducing endothelial cell apoptosis and cell cycle arrest. The gene coding for collagen XVIII is located on chromosome 21. Individuals with Down´s syndrome, which holds an extra copy of this gene, are proven to have 1.6 - 2.0 –fold endostatin level in blood (222). Therefore, these individuals are most protected against cancer where the incidence of all malignant tumors is <0.1 the expected rate, except for testicular cancer and megakaryocytic leukemia (223). This correlation with high circulating levels of endostatin also extends to other angiogenesis-dependent diseases as retinal neovascularization in diabetes (224) and atherosclerosis (225).

Angiostatin is a 38-kDa plasminogen fragment. It functions as inhibitor of extracellular matrix enhanced and tPA catalyzed plasminogen activation, leading to reduced endothelial cell migration and invasion.

Seen together, main proangiogenic factors are vascular endothelial growth factor (VEGF) and fibroblast growth factor 1 and 2 (acidFGF & basicFGF). There is also evidence that COX-2 plays a role in tumor-associated angiogenesis (226, 227) with correlations between COX-2 and VEGF expression in tumor tissue (228), where PGE_2 is thought to be the mediator behind COX-2 activities and tumor angiogenesis (229). Both selective and nonselective COX-inhibitors may reduce tumor angiogenesis, by inhibiting production of proangiogenic factors and subsequent proliferation, migration and tube formation of endothelial cells (Paper II) (131, 153, 230-232). In our present analysis VEGF-A transcription was down-regulated by indomethacin whereas genes coding for VEGF-B & C were unaffected. AcidFGF showed a trend towards down-regulation, while basicFGF displayed a trend to up-regulation. Angiopoetin-4, PDGF-B, -C and the PDGFR-α and -β were down regulated by indomethacin treatment, while other genes coding for angiogenic proteins were mainly down-regulated. Thus, our results confirm that indomethacin affects tumor angiogenesis in addition to other processes related to tumor cell proliferation. As mentioned, VEGF is recognized as a very important factor for angiogenesis. However, our previous experiments with indomethacin treatment showed decreased mRNA expression of bFGF, while VEGF expression was unchanged after 10-14 days of indomethacin treatment (133). Therefore, we regarded bFGF as a highly significant factor in prostanoid related angiogenesis and used this factor as a marker for angiogenesis in our initial work (Paper II).

p53

This is a tumor suppressor protein with critical role in control of a number of biological functions, including cell cycle arrest, apoptosis, differentiation, replication, DNA repair, meiosis and mitosis (233, 234). In response to a wide variety of stress signals (ionizing and UV radiation, oncogene activation, DNA damage, metabolic stress, pH changes and hypoxia), p53 undergoes post-translational stabilization and acts as an important transcriptional regulator (233, 234). Activated p53 either stops cell cycle (by interfering with e.g. p21) or activates apoptosis by interfering with the Bcl-2/BAX-system, preventing multiplication of damaged cells and cancer formation (235). p53 also interferes with genes inhibiting angiogenesis, such as thrombospondin-1. p53 modulates the transcription of genes that govern

major defense against tumor growth by binding to specific response elements in DNA (236). Loss or change of p53 function, caused by decreased protein expression, inactivation or mutation of p53, is associated with increased cancer susceptibility. Malfunction of p53 is an universal hallmark of human cancer (233, 234) associated with unfavorable prognosis in some types of cancer (237). Cellular levels of p53 are the key to its activity and are tightly controlled in cells largely by covalent modifications. Numerous stress sensors that converge at p53 result in phosphorylation, acetylation, ubiquitylation and methylation of specific p53 residues, altering its stability, cellular location and activity (238-241). Under normal, unstressed conditions, regulation of p53 is under precise control by Mdm2, which prevents the interaction of p53 with basal transcription and targets p53 for ubiquitin-dependent degradation. Mdm2 is thereby acting as a critical negative regulator (242). p53 is also regulated by Mdm4, which is a structural homolog of Mdm2 (243). Degradation of p53 is blocked by Arf, which is a tumor suppressor interacting with Mdm2, thereby inhibiting its action as a negative regulator. Its degradation is blocked by CDK inhibitors, which have been shown to inhibit p53 expression of Mdm2 (244). There are two additional members of the p53 family, namely p63 and p73 (245, 246), which share a high level of sequence similarity in DNA binding domains among p53 family members. p63 as well as p73 can transactivate p53 responsive genes causing cell-cycle arrest and apoptosis. However, the different members are not entirely functionally redundant, but have specific biological functions (247). The p53 gene family members express multiple mRNA variants due to multiple splicing and alternative promoters with a number of different protein isoforms (247-252), which have various subcellular locations and biological functions with tissue-specific expression. This could explain tissue-specific regulation of transcriptional activity in responses to stress factors (253-255). Deregulation and abnormal isoform expression accounts for loss of tumor suppressor activity and may play a critical role early in tumor formation (251, 256, 257). Commonly, available p53 antibodies can not identify different p53 isoforms (251). This fact may explain difficulties to link p53 status to biological properties and drug sensitivity in human cancer determined by immunohistochemistry. In present studies, gene expression of p53 RNA and protein levels were significantly reduced by indomethacin treatment, but COX inhibition did not affect gene expression of Mdm2 and Mdm4. In untreated, control animals there was a positive correlation between the presence of p53 and COX-2 within tumors, whereas no such correlation was observed in indomethacin treated animals. Such results suggest that indomethacin reduced tumor growth was not mediated by p53, but rather reflects secondary and compensatory mechanisms.

Endothelial growth factor –receptor (EGF-R)

The ErbB family, also called HER family, is a group of tyrosine kinase receptors as ErbB1/HER1, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4 (258-261). ErbB/HER1 is also known as the endothelial growth factor receptor (EGF-R), which is a transmembrane glycoprotein composed of intracellular tyrosine kinase domain, a transmembrane lipophilic segment and extracellular ligand binding domain (262). Epidermal growth factor (EGF) and transforming growth factor α (TGFα) are the main endogenous ligands by which the receptor is conformed allowing dimerization either with another EGF-R (homodimerization) or with one of the other ErbB family members (heterodimerization) (263, 264). EGF-R function and activity are strictly regulated by these interactions (261, 265). EGF-R can also bind to other tyrosin kinase receptors expressed on the cell surface, such as insulin-like growth factor and platelet-derived growth factor receptors (266, 267). EGF-R is autophosphorylated by the tyrosine kinase domain after dimerization and phosphate is transferred from ATP to the intracellular part of the receptor (259, 268, 269). Autophosphorylation triggers a series of downstream, intracellular signal pathways, stimulating genes involved in cellular processes such as apoptosis, proliferation, invasion and angiogenesis (270-272). The complexity of receptor interactions, the cell type-dependent variability of receptor expression and the existence of several ligands, emphasize the enormous potential network of biological messages able to be mediated by EGF-R (259).

Several EGF-R mediated intracellular signaling pathways are known (Fig. 4). The association of Shc with EGF-R, which leads to the recruitment of the adaptor protein Grb2, is a critical step in the RAS-RAF-MEK-MAPK pathway (273). This, in turn activates RAS, resulting in activation of RAF, which phosphorylates and activates mitogen-activated protein kinases (MAPKs) (274, 275). MAPKs is a superfamily of protein serine-theronine kinases, including extra-cellular signal-regulated kinases (ERKs), c-Jun terminal kinases (JNKs) and p38-mitogen-activated protein kinases (276). The EGF-R can also interact directly with Phospholipase Cγ (PLCγ), inducing hydrolysis of PIP_2 to give IP_3, important for intracellular calcium release and DAG (277, 278), which is a cofactor in PKC activation of MAPK (279, 280). MAPKs can translocate into the nucleus and phosphorylate transcription factors for activation, which induce gene transcription leading to increased levels of inhibitors of apoptotic proteins (IAPs) and antiapoptotic Bcl-2 family members (281). The MAPKs ERK 1 / 2 positively regulate cell proliferation by activating major transcription factors as c-Myc (282, 283). EGF-R is also able to regulate STAT pathways through JAK–dependent or JAK-

independent mechanisms (284, 285). EGF-R induces phosphorylation of STAT1 and initiates formation of complexes between STAT1 and STAT3, causing STAT proteins to translocate into the nucleus with subsequent regulation of gene expression and cell survival (284, 286).

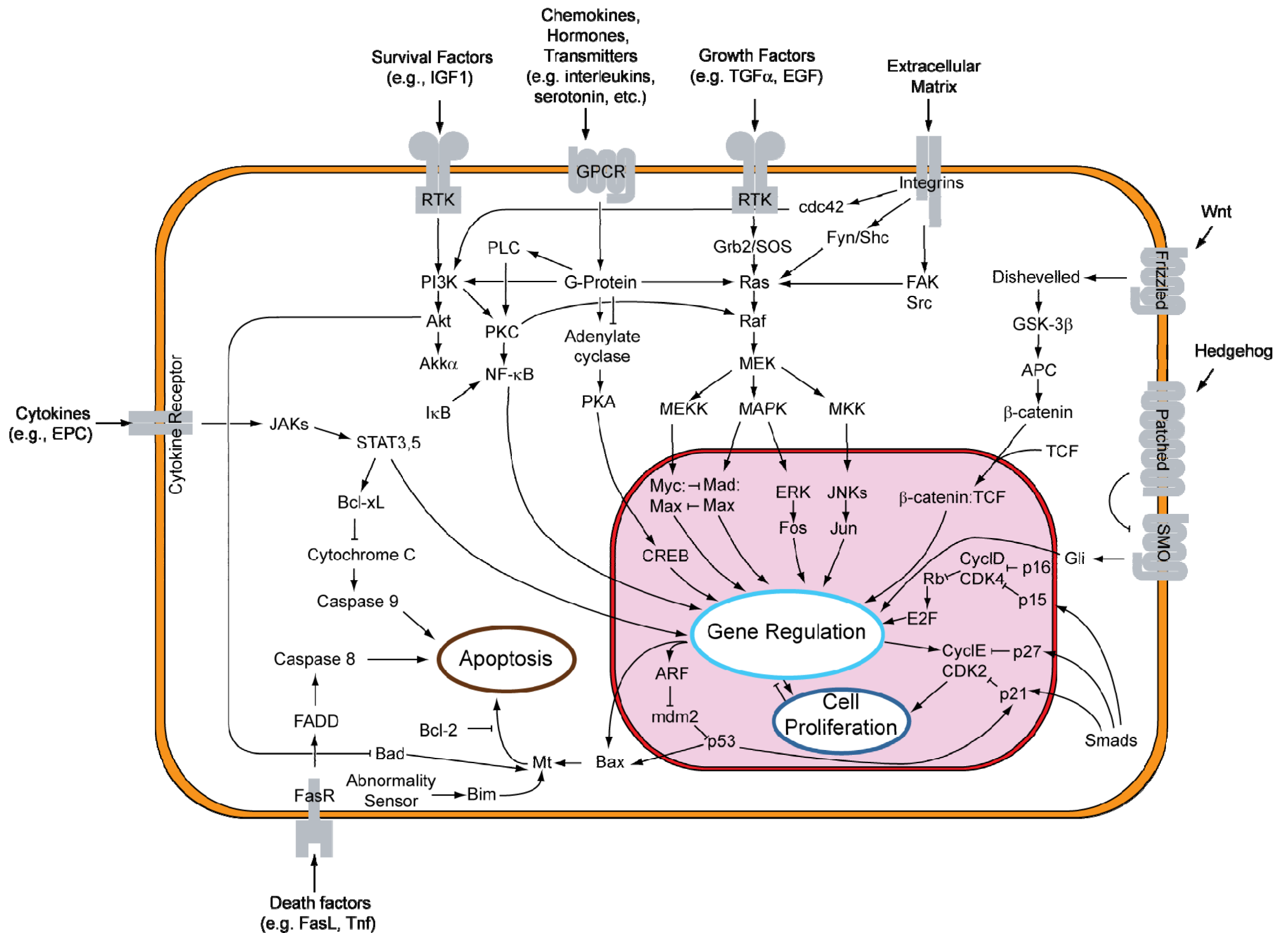

Figure 4. Signaling pathways for control of cell proliferation and apoptosis.

The intracellular domain of the EGF-R provides a docking site for PI3K (287). Activated PI3K generates PIP_3, which recruits and activates serine treonine kinase AKT by phosphorylation. PTEN dephosphorylates PIP_3, which results in reduced AKT phosphorylation, and is thereby a negative regulator of the PI3K-AKT pathway (288, 289). Activated AKT controls cell survival through phosphorylation of several downstream targets, such as apoptotic proteins, transcription factors and protein kinases. AKT phosphorylates and inactivates Bad, a pro-apoptotic member of the Bcl-2 family, and Caspase 9, an enzyme

included in the Fas-dependent death pathway (290). AKT activates some important transcription factors, like HIF-1α, NFkB and CREB, which cause increased transcription of anti-apoptotic genes (290-295). AKT also inactivates transcription factors of the Forkhead family and p53 resulting in decreased pro-apoptotic gene expression. AKT phosphorylates some protein kinases and inactivates Gsk-3β (a kinase involved in the regulation of the cellular metabolism) or mTOR (a kinase involved in cell survival) (296, 297).

Aberrant EGF-R signaling are associated with key features of cancer development and growth and can be initiated by several events such as receptor mutations and deletions; mutations in the downstream signaling pathways; altered ligand production or increased expression of the receptor caused by amplification of the receptor gene and increased gene transcription (298-302). Overexpression and enhanced activity of EGF-R has been found in most cancers and are associated with advanced tumor state, increased risk of metastases and poor prognosis including gastrointestinal tract, bladder, breast and lung cancer (303-307). EGF-R can be activated independently of ligands. This process is called receptor transactivation and is mediated by matrix metalloproteinases (MMPs) and disintigrin/metalloproteinases (ADAM) (308, 309). The EGF-R up-regulates the production of several pro-angiogenic growth factors, including VEGF and bFGF, stimulating angiogenesis (310, 311). The use of EGF-R inhibitors results in a concurrent down-regulation of tumor induced VEGF-mediated angiogenesis (312-314). Transfection of VEGF into cancer cells renders them significantly resistant to anti-EGF-R antibodies, demonstrating a functional link between the two pathways and a causal role of overexpression of VEGF in acquired resistance to treatment with anti-EGF-R antibodies in cancer patients (315, 316). In human cancer, decreased apoptosis is a key feature and EGF-R is effective in blocking apoptosis by death receptors, as the TNF receptor family, FAS and death receptor 4 and 5 (317, 318).

Blocking of EGF-R signaling can be targeted in several ways, providing monoclonal anti-EGF-R antibodies (MAbs), tyrosine kinase inhibitors, immunotoxin conjugates (to deliver toxins), antisense oligonucleotides or iRNA (that decreases the expression of RGF-R); and with drugs targeting transduction proteins downstream to the EGF-R signaling (319-323). The MAbs bind to the extracellular ligand binding region of the ErbB receptors, preventing endogenous, stimulatory ligands from binding and activation (324-326). The MAbs also recruit effector cells from the immune system, such as monocytes and macrophages, inducing antibody-dependent cell-mediated cytotoxicity, which might contribute to the therapeutic

effect (327, 328). Tyrosine kinase inhibitors competitively bind to the ATP binding region of the intracellular domain of the EGF-R, inhibiting signaling by blocking tyrosine kinase activity and following autophosphorylation (321, 324, 325). Two monoclonal anti-EGF-R antibodies (cetuximab, panitumumab) and two tyrosine kinase inhibitors (erlotinib, gefitinib) have been approved in several countries for treatment of metastatic non-small cell lung cancer, colorectal cancer, pancreatic cancer, squamous-cell carcinoma of the head and neck (329-333).

In our experiments EGF-R gene expression was significantly reduced by indomethacin treatment after 10 days of tumor growth (Paper IV) and there was a numerically but not clear cut reduction at 5 days based on both qRT-PCR and microarray analyses (Paper III & IV). EGF-R expression correlated to tumor growth in both univariate and multivariate analyses. Thus, our data indicated a connection between the prostanoid system and EGF-R signaling pathways. This is in agreement with several previous reports from cell culture experiments (334-344), where the EGF-R pathway was involved in prostaglandin forward and backward signaling within tumor cells. However, it is not yet clear how EGF-R may influence PGE_2 production and vice versa. Our results suggest that tumor cell clones, with increased COX-2 expression and increased PGE_2 production, may be sensitive to EGF-R inhibition particularly in combination with COX inhibitors. By contrast to in vivo conditions, cultured MCG-101 cells did not seem to be dependent at all on the EGF-R, since transcript levels were close to background during culture with and without indomethacin in the incubation medium. This may indicate that some factor(s) in fetal calf serum represents alternative upstream signaling to PI3K in cultured MCG-101 cells, since several downstream factors were reduced by indomethacin related to proliferation and increased apoptosis in cultured cells (345, 346). Present discrepant EGF-R results in vivo vs. in vitro conditions also imply a role of tumor stroma cells for in vivo communication as emphasized in our clinical studies (347). Genes of RAS and RAF in the RAS-RAF-MEK-MAPK pathway and AKT in the PI3K-AKT pathway were down-regulated by indomethacin treatment after five days of tumor growth. Genes coding for the STAT proteins were also affected by indomethacin treatment. These results indicate that COX-inhibition affects gene expression, not only at the EGF-receptor, but also in different downstream pathways. In conclusion, EGF-R pathways are important for tumor inhibition by indomethacin, which agrees with reported results in the literature. It is known that the EP_1 receptor can transactivate the EGF-R (103), that EP_2 receptors can stimulate the

PI3K-AKT signaling pathway (104) and that the EP_3 receptors may activate the Ras signaling (107).

B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (BAX)

Apoptosis or programmed cell death is a fundamental process that is essential for embryonic development and maintenance of adult tissue homeostasis. In apoptosis, damaged, infected and aged cells are eliminated without producing inflammatory response, compared to damage induced necrosis. Many human diseases, such as autoimmune disease, myocardial and cerebral ischemia, Parkinson´s disease and multiple sclerosis result from dysregulation of apoptosis (348-352). Resistance to apoptosis is a fundamental part in carcinogenesis and around half of human cancers contain mutations in p53, which is an important regulator of pro- and antiapoptotic proteins of the Bcl-2 family (353-355). Bcl-2 (B cell lymphoma 2) was the first human proto-oncogene discovered and has become the founding member of a family of anti- and pro-apoptotic proteins that share 1-4 homology domains (Bcl-2 homology regions BH1-BH4) (356-358). These domains are important for heterodimeric interactions among the members of the Bcl-2 family (244, 245). In addition to Bcl-2, four other anti-apoptotic Bcl-2 homologues have been identified (BCL-XL, BCL-W, BFL-1, MCL-1) (359, 360). Pro-apoptotic proteins can be divided into two classes according to function and number of BH domains possessed. The first class, called the BH3-only proteins, shares one single homology domain (the BH3 domain) and includes BAD, BID, BIM, NOXA and PUMA (353, 361-363). These proteins act upstream of cellular damage and are activated by many noxious stimuli, including DNA damage, growth-factor withdrawal and oncogene activation (364-368). A second class contains BH domain 1-3 and is known as multi-domain or effector proteins. This class includes BAX, BAK, and BOK (369-372). Over twenty different Bcl-2 family members have been identified and these proteins are essential for normal tissue development and homeostasis (368, 373, 374).

There are two main pathways for apoptosis; the extrinsic pathway that involves death receptors and the intrinsic, which involves mitochondria. The intrinsic apoptotic pathway is regulated by members of the Bcl-2 family and is most responsive to external or environmental cues and DNA damage as UV radiation and growth factor deprivation. The BH3-only proteins are activated in response to many types of stress or damage. This leads to activation of the pro-apoptotic proteins BAX and BAK at mitochondrion. Activated BAX and BAK homo-oligomerize and participate in the formation of pores in the outer mitochondrial membrane

through which pro-apoptotic proteins, including Smac and cytochrome C, escape from the mitochondrial intermembrane space into the cytosol (375, 376). These pro-apoptotic proteins activate caspases, which are proteases cleaving cellular proteins leading to the morphological characteristics of cell death. Expression of Bcl-2, or other anti-apoptotic proteins in the Bcl-2 family, blocks apoptosis by sequestering BH3-only proteins or activated, monomeric BAX or BAK, preventing activation and oligomerization of these pro-apoptotic proteins. Cells can survive permanent death signaling by a continuous expression of Bcl-2 and may use this strategy to avoid apoptosis. Thus, PGE_2 may reduce apoptotic rates by increasing levels of antiapoptotic proteins like Bcl-2 (377), although protein levels of these apoptotic factors were not significantly altered by indomethacin treatment in present studies.

Jun

The Jun protein is, together with Fos and ATF/CREB protein family members, a major component of the transcription factor complex AP-1, which regulates the expression of multiple genes essential for many physiological processes including cell proliferation, differentiation and apoptosis (378-385). Besides transcriptional regulation, AP-1 is also directly involved in DNA replication by stimulating DNA unwinding and binding of large T antigen to the origin of replication (386, 387). Induction of AP-1 occurs physiologically by growth factors and cytokines or aberrantly by tumor promoters, chemical carcinogens and oncoproteins acting upstream of AP-1 (378, 379, 388). It acts as a nuclear third messenger converting cytoplasmatic signals into long-term alterations in gene expression, a mechanism essential for gene regulation in response to many extracellular stimuli (382, 388). The expression and function of important cell cycle regulators is controlled by Jun proteins. For instance, expression of tumor suppressor proteins (p16, p21 and p53) is inhibited by c-Jun, and cyclin D1 is activated by c-Jun (382). AP-1 plays an important role in human oncogenesis and constitutive activation of endogenous AP-1 is required for tumor formation (381). For example, c-Jun is strongly activated in highly invasive breast cancer and expression of this protein correlates with development of distant metastases (389). However, relevant molecular events downstream of activated oncogenic transcription factor are largely unknown. The human c-Jun proto-oncogene is localized on chromosome 1p31-32, frequently involved in translocations and deletions in human malignancies (390). The v-Jun oncoprotein represents a mutated version of its cellular counterpart c-Jun. There are four major, structural alterations in v-Jun relative c-Jun. These differences result in enhanced DNA-binding affinity, reduced regulation of DNA binding activity, insensitivity to regulation by phosphorylation and loss of

docking domain for stress-activated, protein kinase JNK, which regulates the transforming potential of Jun. These differences account for increased oncogenic potential of v-Jun (391-400). The gene and protein expression of Jun was not affected by indomethacin treatment in our present experiments.

p21 and p27

The cell cycle comprises tightly regulated set of events that can lead to cell proliferation, senescence or apoptosis. Cells progress through the various phases of the cell cycle via interactions of different cyclins with respective CDK (cycline-dependent kinase) subunits. Following mitogenic stimuli, quiescent cells enter the cell cycle and up-regulate first D- and then E-type cyclins during G1-phase (401-403). D-type cyclins associate with CDK4 and CDK6 (404-406), while cycline E associates with CDK2 (407, 408). Once assembled, the cycline-CDK-complexes enter the nucleus, where they are phosphorylated by CDK-activating kinases (409-411). These activated complexes phosphorylate additional proteins including various members of the retinoblastoma family, such as pRB, p107 and p130. Phosphorylation of pRB prevents its binding to EF2 transcription factors, enabling expression of genes that regulate the entry into S phase (412). Inhibitors of cyclin-CDKs (CDK inhibitors) can modulate the cell cycle by preventing or limiting cyclin-CDKs from phosphorylating normal substrates. CDK inhibitors act as checkpoints during each step of the cell cycle, preventing replication of damaged DNA, which is either repaired or, if repair is not possible induce apoptosis. There are two classes CDK inhibitors. INK 4 class proteins including proteins p15, p16, p18 and p19 (413-417). These proteins specifically bind to CDK4 and CDK6 inhibiting association with cyclin D.

Kinase inhibitor proteins (KIPs) include p21, p27 and p57. In general, KIP class proteins inhibit cyclin-E-CDK and cyclin-A-CDK complexes. However, p21 has been found to interact also with cyclin-D-CDK complexes and is thereby a universal cyclin-CDK-inhibitor (409, 418-424). p21 and p27 is functional in the nucleus of cells (425), and block kinase activity of associated CDKs when bound to a cyclin. p21 is directly regulated by p53 and is one of the most potent and important effector molecules of p53 (426). p21 can also be regulated via p53 independent pathways (427). Cytoplasmatic localization inactivates p21 and is a common way of inactivation (428, 429). Regulation of p27 can be executed in several, independent ways. P27 mRNA levels are relatively constant throughout the cell cycle (430-432). Proteolysis and phosphorylation are also considered important mechanisms for

regulating p27 levels (433, 434). p27 is not inactivated when located in the cytoplasm unlike p21, but cytoplasmatic p27 have other functions (435, 436).

Gene expression and tumor protein content of p21 and p27 were not affected by indomethacin treatment, but p21 and p27 correlated to COX-2 in present experiments. p21 and p27 predicted tumor growth in indomethacin treated animals but not in untreated, control animals. These findings may imply increased activity of p21 and p27 in rapidly growing tumors, particularly in tumor areas with high COX-2 expression.

Proliferating cell nuclear antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) belongs to a family of DNA sliding clamps and form ring-shaped complexes, which encircle DNA able to slide freely in both directions. The PCNA ring tethers replicative polymerases firmly to DNA, making the sliding clamp an essential cofactor for DNA synthesis and coordinator of replication. Most factors involved in replication-linked processes interact with a particular face of PCNA through the same interaction domain, the so-called PIP (PCNA-interacting protein) box. The PIP box acts as a hydrophobic plug docking into a specific pocket of PCNA located on the C side of the protein (437, 438). PCNA is a homotrimer with possible binding of more than one PIP-box containing protein. Replication factor C (RFC) binds to PCNA and loads around DNA at specific sites, where DNA replication starts (439). RFC binds to the so-called C side of PCNA and loads it with this side positioned toward the 3´ end of the elongating DNA ensuring that replicative factors, which also bind to the C side of PCNA, are oriented in the right direction. RFC is dissociated after PCNA is bound to DNA, making it possible for other proteins to bind to PCNA. Replication is a stepwise reaction and factors bound to PCNA are switching through replication process in a predetermined sequence, where the PIP-box proteins are functioning one after another. Switching of PCNA partners is triggered by affinity-driven competition, phosphorylation, proteolysis and modification of PCNA by ubiquitin and SUMO (437, 440-448). PCNA is crucially regulated by p21 (449), which is a potent inhibitor of cell-division cycle kinases (see above). Binding of p21 to PCNA inhibits replication by blocking the surface required for binding of replicative polymerases (438, 450, 451). p21 has a PIP box and is an effective competitor of many PIP box proteins (451, 452). In fact the affinity of p21 for PCNA is higher than those of any other PIP box proteins (437). PCNA degradation and reduced DNA synthesis are triggered by PCNA dephosphorylation. The nuclear form of EGF-R, triggered by binding of its ligand EGF, phosphorylates PCNA and degradation is thereby

prevented (453). PCNA is also crucial for the balance of cell death and survival (454-456). p53 and its negative regulator Mdm2 contain PIP boxes and interactions with PCNA results in accumulation of p53 (457). PCNA levels in tumor tissue were elevated by indomethacin treatment and there was a negative correlation between PCNA and tumor net growth, which may in part reflect effector mechanisms to attenuate tumor cell division by indomethacin.

Transforming growth factor-β (TGF-β)

TGF-β family cytokines have been found to play diverse roles in control of different cellular and physiological processes including cell growth and differentiation, apoptosis, adhesion, migration, angiogenesis, immune response and development of multi-organ systems. Dysfunction or deregulation of TGF-β signaling has been associated with different human diseases, such as fibrosis, inflammation and tumorigenesis (458-470). Until now more than 30 factors belonging to the TGF-β family have been discovered and these are divided into two subfamilies. The first includes TGF-β, myostatin, activin, inhibin and Nodal and the other consists of AMH, BMP´s and many growth and differentiation factors (461, 471). There are three different types of TGF-β (TGF-β1, TGF-β2 and TGF-β3), which are synthesized as inactive precursors and bound to the extracellular matrix (472). Latent TGF-β can be activated either by enzymatic proteolysis (executed by plasmin, integrin or thrombin) or by conformational change (473, 474). Activated TGF-β transduces its signal by bringing together two types of serine/threonine kinase receptors, TβRI (ALK5) and TβRII. Upon TGF-β binding, TβRI is phosphorylated and activated by constitutively active TβRII. Activated TβRI then phosphorylates the R-Smads, Smad 2 and 3 proteins. The R-Smads form complexes with Smad 4 (Co-Smad) entering the nucleus for transcription regulation of specific target genes (475). This signal transduction pathway is called "the canonical Smad-mediated signaling pathway" and is most important for TGF-β. However, TGF-β can also regulate some physiological processes independent of Smad proteins, including MAPK, PI3K, PP2A, Par6 and Rho GTPases (476). TGF-β signal transduction is finely tuned at different levels, including ligand activation, receptor complex formation, R-Smads activation and translocation and transcription in the nucleus. Many proteins are involved in the regulation of the TGF-β family signaling and the I-Smads (Smad6 & Smad7) have been identified as key regulators. I-Smads are transcriptionally induced by TGF-β family cytokines and regulate these signaling pathways negatively, forming a negative feed back loop (477-479). The transcription of I-Smads, and TGF-β, is also regulated by inflammatory cytokines, EGF and UV irradiation, although exact mechanisms remain elusive (480, 481). There are indications

that other signaling pathways may be involved in the transcriptional regulation of I-Smads (482). TGF-β was up-regulated by indomethacin treatment, which may indicate in part how prostanoids attenuate tumor growth. TGF-β is also a potent immune modulator with effects favoring Th_2-responses.

NM23

Metastasis is the spread of malignant tumor cells from the primary tumor to secondary organs. Tumor metastases are the major contributor to cancer-related morbidity and mortality and remain a huge clinical challenge despite recent improvements in surgical and oncological treatment of cancer. Metastasis is a complex, multi-stage process where progression relies upon the completion of previous stages. Tumor cells obtain invasive and motile phenotypes in order to leave a primary tumor. There are changes in adhesion between tumor cells and extracellular matrix, to facilitate invasion of tumor cells through stroma. Vascular endothelium is disrupted during intravasation. Once in blood, tumor cells must survive the hard environment including sheer forces and immune surveillance. Surviving tumor cells are passively delivered to distant capillary beds adhering to vessel walls or stuck in small capillaries due to physical size. After attached to vascular endothelial cells, tumor cells invade through the capillary wall, adjust to foreign microenvironments and may develop metastatic colonies. However, only a fraction of shed tumor cells survive these processes. Most cells die or are forced into a dormant state at foreign sites, which is characterized by lack of tumor progression caused either by simultaneous blockade of growth or imbalance between proliferation and apoptosis (483, 484). This state may last for several years. One important cause of tumor dormancy may be a lack of vascularization (485). Molecular signals that underlie each step of the metastatic process are not completely elucidated, but general mechanisms have been unveiled (486-488).

Metastasis suppressor genes (MSGs) are defined by their ability to inhibit overt metastases in secondary organs without affecting the growth of the primary tumor. Over 20 different MSGs are confirmed and most of these factors have been identified by reduced expression in metastatic cancer cells compared to non-metastatic cells (489). Proteins encoded by this class of genes are involved in a wide range of signaling pathways and biochemical activities. They are suppressing metastases by inhibiting almost all of the different steps of the metastatic cascade, including reduced angiogenesis and forcing tumor cells into dormant states (490). NM23 was identified in 1988 and was the first discovered MSG (491). It is one of most

important MSGs and has been shown to arrest growth of micrometastatic lesions and suppress metastases in several models including melanoma, breast, colon, prostate and oral squamous cell carcinomas (492-501). The molecular mechanism of NM23-mediated tumor dormancy is not known, but it has been elucidated to reduce ERK1/2 activation and thereby restraining cell proliferation (502-505). NM23 also suppress tumor cell motility and invasion by inhibiting expression of the EDG2 receptor (506, 507). Indomethacin treatment had no significant influence on tumor NM23 levels, but NM23 showed a positive correlation to COX-2 protein in tumor tissue and a negative correlation to tumor net growth suggesting some kind of counter regulatory relationships between NM23 and prostanoids in tumor tissue.

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