Surgery and (anti) angiogenesis

door Elisabeth Atie te Velde

Surgery and (anti) angiogenesis

Chirurgie in combinatie met (anti) angiogenese
(met een samenvatting in het Nederlands)

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Elisabeth Atie te Velde

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Promotores

Inne H.M. Borel Rinkes MD, PhD, Professor of Surgical Oncology
University Medical Center Utrecht

Emile E. Voest MD, PhD, Professor of Medical Oncology
University Medical Center Utrecht

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Chapter 1

Introduction

Angiogenesis

Angiogenesis, the formation of blood vessels from preexisting vessels, occurs during embryonic as well as during some processes in adult life.[1] This is different from vasculogenesis, which is the development of blood vessels from in-situ differentiating endothelial cells. Increased scientific attention for angiogenesis over the past years has yielded many promising new therapeutic approaches.[2] Angiogenesis has been shown to play a pivotal role in pathological conditions, such as primary and metastatic tumor growth. Induction of angiogenesis precedes the formation of macroscopic malignant tumors, and increased vessel density correlates with the invasive properties of tumors. On the other hand, angiogenesis is likely to be essential for a variety of physiological processes as well, including healing of anastomoses and wounds, liver regeneration, and embryonic development. In this respect, cancer has frequently been referred to as a never healing wound.

Vascular structures undergo a so-called "angiogenic switch" when they change from benign to malignant tumor vessels. This is characterized by upregulation of pro-angiogenic regulators and/or downregulation of anti-angiogenic regulators. Hence, the balance is tilted towards vessel proliferation, resulting in an angiogenic phenotype. In one of the current most sophisticated technical experiments, St Croix et al. compared expression patterns of endothelial cells derived from blood vessels of normal and malignant colorectal tissues. They showed that tumor endothelium is distinct from physiological endothelium.[3] As shown in figure 1, immunohistochemistry with a monoclonal antibody against CD31 displays an example of differential expression of vascular phenotypes. In the tumor vessels of a murine colorectal liver metastasis, as well as in liver endothelium directly surrounding the tumor, CD31 is upregulated as compared to the normal liver sinusoids. With another recently developed tool to study tumor vessels, in vivo microscopy, Jain's group found that tortuousness and increased vascular permeability are characteristics of these new tumor vessels in vivo.[4]
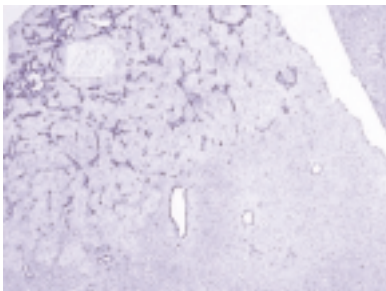

Figure 1. Example of the "angiogenic switch". (see color appendix)
Immunohistochemistry with a monoclonal antibody against CD31 displays a differential expression of vascular phenotypes of normal and tumor vasculature. In the tumor vessels of a murine colorectal liver metastasis, as well as in liver endothelium directly surrounding the tumor, CD31 is upregulated as compared to the normal liver sinusoids.

Angiogenesis is a complicated multi-step process and depends on different tissue components. Endothelial cell survival, migration, alignment, adhesion, proliferation, and subsequent formation of new capillary-like sprouts are all required. In addition, interaction of endothelial cells with basement membrane[5], extracellular matrix (ECM)[6], matrix metalloproteases (MMP's)[7], tumor cells, inflammation, and growth factors play an important role in angiogenesis. A pivotal growth factor is Vascular Endothelial Growth Factor (VEGF) that induces angiogenesis and tumor growth.[8] VEGF is upregulated via Hypoxia Inducible Factor-1alpha (HIF-1α).[9] HIF-1a is induced by hypoxia[10] and is involved in cancer progression in different organs[11-15].

MMP's are enzymes that can degrade virtually any component of the ECM. They are important for cell migration, invasion, proliferation, apoptosis and regulation of remodeling of ECM, embryonic growth, wound healing as well as angiogenesis.[7] In addition, ECM degradation is a prerequisite for tumor growth and metastasis. During tumor growth the regulation of MMP's is disturbed, and most of the times the balance between active MMP's and their inhibitors is in favor of proteolysis. Over-expression of MMP's in tumors has often been associated with increased invasiveness and grade of malignancy, corresponding with unfavorable prognosis.[16;17]

Anti-angiogenesis

Anti-angiogenic therapy is considered one of the most promising new developments in cancer treatment. By preventing tumor growth through inhibition of its vascular supply, a state of tumor dormancy is induced.[2] Small tumors and micrometastases (< 2 mm^3) are diffusion limited for their metabolism. For growth beyond this diffusion-limited volume, newly formed tumor vasculature is necessary. Tumor formation involves cell proliferation, metastasis, adhesion, transendothelial migration, and matrix degradation. All these mechanisms might be potential targets for anti-tumor agents. For example, reducing tumor angiogenesis can lead to an inhibition of hematogenous metastases. Promising results to inhibit tumor growth via anti-angiogenic treatment have been achieved in animal models and many clinical trials have been initiated since.[18]

The discovery of naturally occurring endogenous angiogenesis inhibitors was based on the following observations. After the removal of a primary tumor its metastases appeared to develop new vessels and displayed an increased growth rate. Therefore, the primary tumor was suspected to inhibit its remote metastases. A circulating angiogenesis inhibitor responsible for this control of distant tumor growth was discovered in the presence of the primary tumor. In this way, angiostatin, a 38-kDa internal proteolytically generated fragment of plasminogen was discovered in 1994 in the serum and urine of tumor bearing mice.[19;20] Indeed, serum and urine from tumor-bearing mice, but not from controls, specifically inhibited endothelial cell proliferation. Our group was able to generate this angiostatin from human plasma, and has demon-

strated in mice that its anti-tumor effects may be maximized by using an osmotic pump for continuous administration.[21] An other naturally occurring angiogenesis inhibitor, Endostatin, the 20kDa C-terminal fragment of collagen XVIII was originally isolated from a hemangioendothelioma cell line by the group of Dr. Judah Folkman.[22] Continuous administration of endostatin was also shown to be most effective in mice.[23] Since the discovery of angiostatin and endostatin, a multitude of anti-angiogenic agents have been identified or developed. Antibodies against growth factors (e.g. VEGF[24]) or their receptors, inhibitors of MMP's, inhibitors of cell adhesion or proliferation are now all promising drugs under investigation in pre-clinical studies and clinical trials.

Potential advantages of anti-angiogenic therapy include the fact that -in contrast to conventional chemotherapy- development of resistance has as yet not been observed[25], indicating that long-term treatment with these drugs should be effective. And, although the effects of anti-angiogenic therapy on physiological angiogenesis are unknown, serious toxicity was generally assumed not to be anticipated, because of the almost complete lack of angiogenesis in the healthy adult.[20;22]

Anti-angiogenesis and surgery

By blocking the formation of novel tumor-associated vessels, anti-angiogenic therapy has been shown to be capable of inducing and maintaining a state of dormancy. Therefore, the clinical application of such therapy will most likely involve long-term and (neo) adjuvant treatment schedules to maintain tumor dormancy. This implies that, in the clinical situation, patients will have to undergo treatment for prolonged periods of time, preferably in the (neo) adjuvant setting when patients have minimal residual tumor burden. Consequently, patients will be likely to undergo extensive surgery for primary or metastatic disease during anti-angiogenic therapy. Physiological processes such as wound healing, healing of intestinal anastomoses, and liver regeneration might be angiogenesis-dependent as well. Therefore, one could hypothesize that patients undergoing surgery and with subsequent wound healing might encounter serious problems when treated with anti-angiogenic drugs. This would increase the likelihood of serious complications during the peri-operative course. Knowledge about the effects of anti-angiogenic therapy on physiological angiogenesis is, therefore, essential.

In addition to wound healing, other interactions between surgery and anti-angiogenic therapy can be of importance. Surgery causes mobilization of microscopic tumor deposits, potentially leading to hematogenous metastases, circumstances during which angiogenesis and anti-angiogenesis might play an important role. Even in no-touch colorectal surgery with mesenteric vessel ligation, 12.5% of patients still have tumor spill in the circulation[26], which can eventually lead to tumor metastases. Furthermore, surgery may cause selective tissue hypoxia, but it is not known whether this hypoxia leads to subsequent tumor angiogenesis. No studies are currently available that address these interactions between surgery and (anti-) angiogenesis.

Conventional phase I/II trials are unlikely to resolve these important issues, since they are typically done in patients with advanced cancer stages who have bulky disease and a limited life span. It is unlikely that such patients will be exposed to any kind of (major) surgery while undergoing angiogenesis-inhibition. Some indications however, can be extrapolated from the early data now available from trials. Phase II trials with endostatin were initiated in November 2001. In the clinical trials so far, endostatin has shown no dose-limiting toxicity or significant side effects. Until recently, clinical trials with broad-spectrum MMP inhibitors have yielded disappointing results, and included the observation of considerable side effects on connective tissue.[27]

Wound healing

Wound repair is a complex combination of events. Considering regular peri-operative care and aseptic surgical conditions, the healing of patients' wounds depends mostly on the clinical condition of the individual patient and on his/her inflammatory response. Irrespective of the wounded tissue type, the continuous process from surgical injury to a healed wound can be divided into three phases: inflammation, proliferation and maturation.[28] Directly following wounding, the inflammation phase starts. Intravascular platelet aggregation, formation of a fibrin clot, growth factor release, in combination with increased vascular permeability cause migration of attracted inflammatory cells. After the arrival of fibroblasts, the proliferation phase starts. Endothelial cells are important in this phase, as they contribute to angiogenesis. Secondary wound healing in unsutured wounds, as opposed to primary wound healing in sutured wounds, involves the migration of cells towards the opposite wound edges. The migration of cells, angiogenesis and formation of granulation tissue are all dependent on the ECM remodeling during the maturation phase. Plasmin and other components of the plasminogen activation system play an important role in tissue repair by regulating ECM remodeling. Fibrin acts as a provisional matrix for the formation of granulation tissue and re-epithelialization. Without a correct balance between degradation and production of new ECM, impaired wound healing is likely to occur. For example, ulcers result from excessive degradation and hypertrophic scars from excessive formation of ECM.

The parameters to differentiate between correct or impaired wound healing are diverse. Healing is measured by mechanical, histological, and biochemical parameters, the latter being mostly determination of hydroxyproline content in lysed tissue samples. Furthermore, different experimental models of wound healing exist to investigate differences between the aforementioned parameters. A form of wound healing that can be life threatening if impaired, is the healing of colonic anastomoses,

since anastomotic leakage in the week following surgery comprises a disastrous event, leading to high morbidity and mortality[29]. In contrast to the healing of cutaneous wounds, the healing of colonic anastomoses is considered more dependent on angiogenesis, and less dependent on diffusion of oxygen through preexisting vasculature[30-32]. Thus, we expected this model to be more sensitive to angiogenesis-directed interventions. Hence, the healing of colonic anastomoses could serve as a model for "physiologically critical" angiogenesis. Putative adverse effects of anti-angiogenic agents might be more apparent in this model compared to cutaneous wound healing. Parameters to determine the healing of colonic anastomoses are mechanical (bursting strength, bursting pressure, or bursting wall tension), histological examination and measuring of the hydroxyproline content. The hydroxyproline content is an indirect reflection of ECM quality, since it only determines collagen quantity. Besides, the strength and quality of the ECM is dependent on the balance between MMP's and their inhibitors (TIMP's) instead of on collagen quantity only. Angiogenesis in colonic tissue has been studied using microscopy[32] or intravascular fluorescent, radioactive or radiopaque labeled circulating substances. Dynamic flow studies have been done with laser doppler velocity measurements. The use of in vivo microscopy can be very useful in determining real-time intra-colonic blood flow and angiogenesis and could replace most of the aforementioned experiments. However, its application and feasibility are still limited, mostly due to technical restrictions. At this moment, the determination of the mechanical bursting pressure is the most feasible and reliable parameter.[33] Genetically altered mice in whom specific components involved in wound healing are absent i.e. knock-outs, or over-expressed, are being recently developed to determine the exact contribution of that particular component after wounding.

Anti-angiogenesis and wound healing

Cutaneous wound healing has been investigated in several knockout mice deficient for components that regulate the formation and/or degradation of the ECM. Wound healing in plasminogen knockout mice showed a decrease in migration of keratinocytes to cover the wound, leading to a delay in re-epithelialization.[34] When these mice were additionally treated with a MMP-inhibitor, no wound healing was observed at all, indicating an important role for MMP's.[35] Accordingly, MMP-3-deficient mice lack wound contraction and consequently have delayed closure of the wound.[36] In mice deficient for plasminogen activator inhibitor-1 (PAI-1), the rate of wound closure was found to be accelerated.[37] In mice lacking fibrinogen the re-epithelialization of keratinocytes was inappropriately organized, leading to wound healing defects.[38] Plasmin and other components of the plasminogen activation system play an important role in tissue repair by regulating extracellular matrix remodeling, including fibrin degradation. Consequently, other proteins that are important

regulators of the ECM might also be players in the wound healing process. TAFI is a procarboxypeptidase that is synthesized in the liver and secreted into the circulation. Increased levels of TAFI have been associated with an increased risk for venous thrombosis. Activated TAFI can down regulate fibrinolysis and it has been hypothesized that it forms a link between coagulation and fibrinolysis. However, no physiological function of TAFI in vivo has been described so far. In addition to the contribution of MMP's and ECM proteins, VEGF is an important angiogenic growth factor in skin wound healing, and defects in VEGF in mice might be associated with wound healing disorders[39].

Studies on the effects of anti-angiogenic interventions on wound healing are scarce and incomprehensive and have provided contradictory results. Endostatin does not appear to impair cutaneous wound healing[40], but does affect the maturation of the vessels in the granulation tissue[41]. Data concerning short-term administration of two mildly angio-suppressive agents seem to suggest possible adverse effects during the early phase of intestinal wound healing.[42;43] No data exist on angiostatin in wound healing assays.

Colorectal carcinoma and liver metastases

In the USA, the combined mortality rate for colon carcinoma for men and women is 47.700 deaths annually (American Cancer Society, estimate for 2000), and hereby represents the second most probable cause of cancer deaths for both sexes. The prevalence of colorectal cancer is among the highest of malignancies in the Western countries.[44] In the Netherlands, annually, 4400 patients die of colon cancer, while 8600 new patients are registered (CBS). In as many as 50-80% of these patients the disease will metastasise to the liver[45] which is the sole site of initial colorectal tumor recurrence in approximately 30%[46]. Once colorectal liver metastases have developed, the natural course of the disease is associated with poor survival.[47] Surgical resection of the liver metastases offers the only hope of cure for these patients[48], but is, unfortunately, restricted to few selected ones, many of whom will still relapse within the liver or develop extrahepatic metastases after resection[49]. The majority of patients with hepatic metastases are not eligible for surgical resection because of a tumor size beyond resection limits, unfavorable location near intrahepatic blood vessels precluding a margin-negative resection, presence of too many tumors, or inadequate hepatic function prior or following resection. Radiofrequency ablation (RFA) is a relatively new surgical technique that is used to locally destroy (secondary colorectal) hepatic malignancies.[50-55] It is restricted to those patients not eligible for standard surgical resection, with metastases confined to the liver. However, these patients have cancer in an advanced stage and are likely to have distant micrometastases.

Through the use of adjuvant chemotherapy survival rates of patients with colorectal carcinoma have improved. As a consequence, adjuvant chemotherapy has

become standard practice in high-risk patients following surgery. Studies of chemo-therapy prior or post liver resection for secondary malignant disease, however, have produced contradictory results.[56;57] Nevertheless, responses in novel schemes including oxaliplatin were recently shown to increase resectability rates of previously unresec-table colorectal liver metastases.[58] Clearly, these results indicate that there is room for improvement, particularly regarding peri-operative systemic treatment. A search for alternative treatment modalities for colorectal liver metastases is, therefore, warranted.

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Chapter 2

Outline and objectives of this thesis

This thesis addresses three different aspects concerning the combination of surgery and angiogenesis or anti-angiogenic therapy.

In the first part, the safety of the administration of anti-angiogenic therapy when combined with surgery is investigated. The second part deals with the interaction between surgery and angiogenesis. Studies on the optimal administration of anti-angiogenic agents with respect to maximal anti-tumoral efficacy are described in the last chapters.

The following important topics are addressed: Is physiological angiogenesis affected by anti-angiogenic therapy as well? Can we safely combine antiangiogenic therapy with surgery? Is angiogenesis influenced by surgical procedures? Is optimal anti-tumoral efficacy of anti-angiogenic agents to be improved, i.e. for example by combining treatment strategies, or prophylaxis prior to surgery? What is the best timing of administration?

Serious toxicity of anti-angiogenic agents is generally assumed not to be problematic, because of the almost complete lack of angiogenesis in the adult. However, the effects of anti-angiogenic therapy on physiological angiogenesis are largely unknown, and the differences between physiological and pathological angiogenesis are unclear. Surgeons will encounter an increasing number of patients undergoing such therapy. Hence, knowledge about the effects of anti-angiogenic therapy on physiological angiogenesis in wound healing is essential. Until now, studies on effects of anti-angiogenic interventions on wound healing are scarce and incomprehensive and have provided contradictory results. A form of wound healing that can be particularly life threatening if impaired, is the healing of colonic anastomoses. Anastomotic leakage in the week following surgery comprises a disastrous event, leading to high morbidity and mortality.

To test the hypothesis that anti-angiogenic therapy affects physiological angiogenesis, the effects of anti-angiogenic treatment with angiostatin on the healing of colonic anastomoses is investigated in *chapter 3*. Here, direct measurements of endpoints of colonic healing are clinical appearance of the mice, and the mechanical strength and histology of the resected colon. Consequently, in an attempt to elucidate the underlying mechanisms of the observed effects of angiostatin treatment, the anastomotic tissues were subjected to immunohistochemical staining with an extensive panel of markers *(chapter 4)*. Cutaneous wound healing was studied in *chapter 5*. Cutaneous wound healing has been investigated in several knockout mice deficient for components that regulate the formation and/or degradation of the ECM. However, the physiological role in vivo of Thrombin-activatable fibrinolysis inhibitor (TAFI), a protein that is able to attenuate plasmin-mediated fibrin degradation, is currently unknown. In a knock-out mouse lacking TAFI tissue repair and regulation of the ECM remodeling were studied.

In the second part of this thesis, the interaction between surgery and angioge-
nesis is addressed. There is evidence that surgery causes selective tissue hypoxia, but
it is not known whether this hypoxia leads to subsequent tumor angiogenesis. No
studies are currently available that address this interaction between surgery and
(anti-)angiogenesis.

We hypothesized that clamping of the vascular inflow of the liver unfavorably
affects the prognosis of cancer patients, by inducing temporary hypoxia. This
hypothesis was tested in *chapter 6*, where levels of Hypoxia Inducible Factor-1alpha
(HIF1α), Vascular Endothelial Growth Factor (VEGF) were measured in livers of mice
and patients. In addition, tumor recurrence and survival was investigated in patients
after vascular clamping of the liver.

Surgical treatment is the only curative option for patients with colorectal liver
metastases. Studies on the treatment of patients pre- or post- liver resection for
secondary malignant disease with conventional chemotherapy, have produced contra-
dictory results. Occasionally, an increase in resectability rates of previously unresec-
table colorectal liver metastases was described. Nevertheless, a search for alternative
treatment modalities for colorectal liver metastases is warranted. This is investigated
in the latter part of this thesis.

Combining conventional chemotherapy with antiangiogenic compounds might lead
to improved anti tumor efficacy by targeting both tumor cell and endothelial cell
compartments. Strategies combining these two targets have not, however, been
investigated thoroughly in models of early widespread metastatic colorectal liver
disease. In *chapter 7* the efficacy of combinations of conventional chemotherapy and
strong angiogenesis inhibitors was assessed in a murine model of early metastatic
colorectal cancer. The effects of the combination therapies were compared with those
of conventional and antiangiogenic agents alone.

Surgery causes mobilization of microscopic tumor deposits, potentially leading to
hematogenous metastases, circumstances during which angiogenesis and anti-angio-
genesis might play an important role. We hypothesized that a significant part of the
in vivo anti-tumor efficacy of endostatin could be achieved during that initial phase of
the metastasizing process of tumor cells. In *chapter 8* we have investigated the
influence of endostatin on the early spatiotemporal fate of murine colon carcinoma
cells metastasizing to the liver.

Chapter 3

Adverse effects of the antiangiogenic agent angiostatin
on the healing of experimental colonic anastomoses
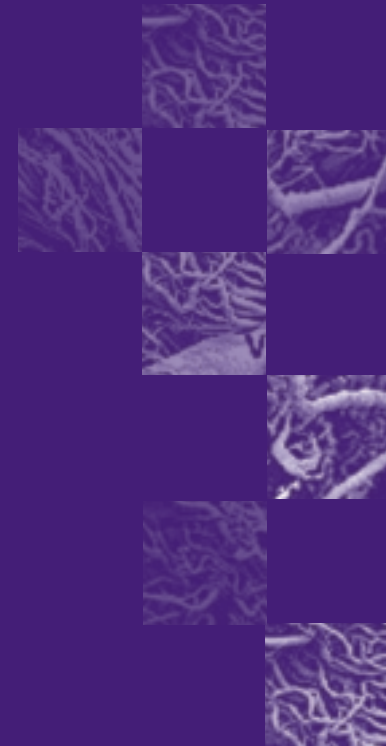
Elisabeth A. te Velde[1,2]**, Emile E. Voest**[2]**, Joost M. van Gorp**[3]**,
André Verheem**[1]**, Jeroen Hagendoorn**[1,2]**, Martijn F. Gebbink**[2]**,
and Inne H. M. Borel Rinkes**[1]

Department of Surgery[1], Laboratory of Medical Oncology[2],
University Medical Center Utrecht and
Department of Pathology[3], Diakonessen Hospital, Utrecht, The Netherlands

Abstract

Background: Antiangiogenic cancer therapy is likely to be administered long term for sustained suppression of tumor outgrowth. Surgeons will encounter more patients undergoing such therapy. Therefore, it is essential to know the effects of anti-angiogenic agents on physiological angiogenesis, as occurs during the healing of colonic anastomoses.

Methods: Angiostatin was generated from human plasma and administered continuously. In 38 mice, the right colon was anastomosed after transection: group 1 (n = 13), anastomotic healing under angiostatin treatment from surgery until death (day 7); group 2 (n = 13), phosphate-buffered saline controls. For healing on discontinuation of treatment, group 3 (n = 6) received angiostatin treatment preceding surgery during 4 days; group 4 (n = 6) included controls. On day 7, all mice were inspected for signs of anastomotic leakage. Bursting pressure measurements were performed to test anastomotic strength. Neovascularization was assessed semi-quantitatively by immunohistochemistry.

Results: Mice treated with angiostatin postoperatively showed significantly more signs of leakage, more adhesions, and peritonitis. One mouse died on day 5. Five mice had paralytical ileus. The bursting pressure in group 1 was 135+/-20 mm Hg, versus 175 ± 12 mm Hg in group 2 (mean ± SEM). Significantly fewer new vessels were found surrounding the anastomosis in the treated group (6.6 ± .9) versus controls (16 ± 1.6). All controls, as well as those animals treated with angiostatin only until surgery (group 3), displayed normal healing and showed no signs of peritonitis or ileus.

Conclusions: Angiostatin impairs anastomotic healing in mice. However, on discontinuation of antiangiogenic therapy, normal anastomotic healing is promptly restored.

Introduction

Antiangiogenic therapy is a highly promising new strategy in the treatment of cancer. Because the growth and metastasizing capacity of a tumor depend on the formation of new blood vessels, many antiangiogenic agents have been developed that are directed against the endothelial cells of the tumor vasculature.[1] Potential advantages of antiangiogenic therapy include that the development of drug resistance seems unlikely[2] and that serious toxicity is not anticipated[3,4].

Angiostatin, a fragment of plasminogen, is considered to be one of the most potent inhibitors of angiogenesis.[5] Experimental models have shown that, during long-term antiangiogenic therapy with angiostatin, tumors remain in a state of dormancy.[3] However, when antiangiogenic therapy is discontinued, the tumor or its metastases resume their outgrowth.[2] Accordingly, angiostatin recently proved to be more potent when administered continuously, instead of twice daily.[6] From a clinical point of view, antiangiogenic therapy should preferably be administered during a prolonged period of time, e.g., in an adjuvant or neoadjuvant setting, to maintain remission after surgery. As a consequence, surgeons may increasingly be confronted with patients undergoing antiangiogenic therapy. It is, therefore, of great importance to investigate any adverse effects that antiangiogenic therapy might have on physiological angiogenesis, as occurs during wound healing.

A form of wound healing that can be lifethreatening if impaired is the healing of intestinal anastomoses, because anastomotic leakage in the week after surgery is a disastrous event that leads to high morbidity and mortality.[7] Until now, the scarce studies regarding the effects of antiangiogenic strategies on intestinal anastomotic healing have not been comprehensive and have provided contradictory results. Recent data concerning short-term administration of two mildly angiosuppressive agents suggest possible adverse effects during the early phase of wound healing.[8,9] In contrast, endostatin does not seem to impair cutaneous wound healing.[10] The influence of strong and continuous inhibition of angiogenesis—as would be needed for sustained tumor suppression—on intestinal healing has never been studied. Moreover, data on the temporal aspects of the relationship between angiosuppressive treatment and its discontinuation are currently lacking. Besides, the effects of angiostatin on physiological angiogenesis are unknown. Such knowledge is of the utmost importance for the design of clinical studies and administration schedules with antiangiogenic agents.

This study was undertaken to evaluate the effects of strong antiangiogenic treatment with angiostatin, with maximally suppressive conditions, on the healing of colonic anastomoses. First, we evaluated anastomotic healing under angiostatin treatment by treatment from day 0 to day 7 after surgery. In a second set of experiments, we investigated the effects of discontinuation of angiostatin therapy directly preceding colonic surgery.

Our data provide strong evidence that angiostatin impairs anastomotic healing in mice. However, on discontinuation of antiangiogenic therapy, normal anastomotic healing is promptly restored.

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Materials and methods

Generation and Purification of Angiostatin

Human angiostatin was generated as described by O'Reilly et al,[5] with minor modifications.[6] Outdated human plasma, heated (37°C) and filtered, was diluted 1/2 with distilled water, supplemented by 3 mM of EDTA, and applied to a lysine-Sepharose™ column (Pharmacia, Uppsala, Sweden). All column purifications were performed at room temperature. After washing the column with .5 M of phosphate buffer, plasminogen was eluted with .2 M of ε-aminocaproic acid, pH 7.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed one band of apparent molecular weight (Mr) 92.000, corresponding to plasminogen. The eluant was dialyzed against distilled water (MWCO, 6– 8000 Spectra/Por; Spectrum Laboratories, Inc., Rancho Dominguez, CA; 4 › 10[7] dilution; 4°C), buffered with 20 mM of Tris 7.6, and followed by proteolytic digestion with .8 U/mg plasminogen porcine pancreatic elastase (Calbiochem, San Diego, CA) (shaker overnight at 37°C; 120 rpm). The solution was applied to the same column, which had been equilibrated with a salt solution. After the column was washed, angiostatin was eluted with .2 M of ε-amino-caproic acid while the flow-through was collected and treated as recently cleaved plasminogen, to collect angiostatin fragments with low lysine affinity. The angiostatin was dialyzed against distilled water (MWCO, 6– 8000 Spectra/Por; 4 › 10[7] dilution; 4°C) and freeze dried. After the angiostatin and its flow-through were combined, sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed three distinct bands of approximately Mr 40.000, Mr 42.000, and Mr 45.000, according to the triplet described by O'Reilly et al.[5] In all experiments (unless stated otherwise), the administration of angiostatin was as follows. The mice received a dorsal osmotic pump (Alzet™ pump, type 2001; Alza, Palo Alto, CA) subcutaneously (SC) for continuous administration of angiostatin in a dose of 100 mg/kg/day plus a single bolus loading dose of 2.5 mg/ 200 mL SC. This dose of angiostatin proved to be the optimal dose to elicit a maximal antitumor effect in the murine SC and liver metastasis models.[6] All controls received an identical pump filled with phosphate-buffered saline (PBS). The bioactivity of the angiostatin was confirmed by the mouse cornea neovascularization assay, as described elsewhere (data not shown).[6] Briefly, a corneal micro-pocket was created by a keratotomy. A micropellet (.4 x .4 x .2 mm) containing approximately 100 ng of basic fibroblast growth factor (Life Technologies, Inc., Rock-ville, MD) was inserted. The mice were divided into a treated group (which received angiostatin) and a control group (which received PBS). The corneas were examined daily by use of a microscope to determine the outgrowth of newly formed vessels from the limbus toward the pellet. When the vessels in the eyes of the controls had reached the pellet, the experiment was terminated. According to the formula .2 x π x maximal vessel length x clock hours, the surface area of neo-vascularization of both groups was determined and compared.[11] At a dose of 100 mg/kg/day, continuous administration of human angiostatin consistently caused virtually complete (~93%) inhibition of angiogenesis.

Animals

The animals studied were BALB/c male mice, aged 12 weeks and weighing 25 to 30 g, purchased from the General Animal Laboratory of the University Medical Center Utrecht. They were allowed food and water ad libitum. All experiments were executed according to the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht.

Figure 1

A. *In 38 Balb/C mice the right colon was lifted from the abdomen, transected and microscopically anastomosed.*

B . *During the same procedure an osmotic pump was implanted for continuous administration of angiostatin or PBS.*

C. *After sacrifice, the ana-stomosis was resected and ligated on both sides. PBS was volumetrically infused at 60 ml/hr, until rupture of the intestinal segment.*

Surgical Procedures

In all mice, the right colon was anastomosed after transection. The mice were anesthetized intraperitoneally with fentanyl citrate/fluanisone (.3 mg per mouse; Janssen- Cilag, Brussels, Belgium) and midazolam chloride (12.5 mg per mouse; Roche, Brussels, Belgium). The mice were shaved and placed on a heated surgical microscopy table. A midline laparotomy was performed under aseptic conditions. The cecum was identified and lifted from the abdominal area. The right colon was transected 1 cm distal to the cecum with microscopic scissors, taking care not to damage the mesenteric vessels. The colon was anastomosed by single-layer 8-O nonabsorbable (Prolene™; Ethicon, Brussels, Belgium) inverted running sutures. The abdominal wall was closed by two-layer 5-O absorbable (Vicryl™, Ethicon) running sutures. Next, the osmotic pump for administration of angiostatin or its solvent was implanted SC through a 1-cm dorsolateral incision.

Study Design

For each individual experiment, groups of three or four mice underwent transection of the right colon with anastomosis. They were randomly assigned to the angiostatin- treated group or the control group.

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Anastomotic Healing Under Angiostatin Treatment The following groups were identified: group 1 (n = 13), continuous angiostatin treatment from surgery until death (day 7); and group 2 (n = 13), PBS controls. These two groups received the osmotic pump during the surgical procedure of colonic anastomosis.

Anastomotic Healing After Discontinuation of Angiostatin Treatment In group 3 (n = 6), angiostatin was administered for 4 days before surgery. Group 4 (n = 6) included PBS controls. In the last two groups, pumps were implanted 4 days before surgery and were removed during the surgical procedure of colonic anastomosis, to determine whether any adverse effect on the healing of those anastomoses would persist on discontinuation of the angiostatin therapy.

Outcome Measures

The observers were blinded to the treatment in all outcome measures.

Clinical Performance Two independent observers recorded the postoperative clinical condition of the mice daily, according to a pre-determined clinical assessment score, as listed in Table 1. For calculation of the percentage of postoperative weight loss, body weights on day 7 after surgery were compared with those on day 0, i.e., before surgery.

Macroscopic Intra-Abdominal Analysis On day 7, the abdomen was reopened and inspected for anastomotic dehiscence, local peritonitis, ileus, and the formation of adhesions. Peritonitis was defined as macroscopic signs of inflammation, fibrin deposition, and hypervascularity. Mechanical ileus was defined as obstruction of the intestine caused by the suture, with distention proximal to the anastomosis but not distal to the anastomosis, and was considered a failure of the surgical procedure. Paralytical ileus was defined as distention of atone intestine and colon, with paling and swelling of the colonic wall, in conjunction with the absence of mechanical ileus. In addition, intestinal diameters 2 cm distal and proximal from the anastomotic line were measured with calipers to quantitatively confirm these macroscopic findings. Adhesions involving the suture line were counted, because the presence of adhesions surrounding the suture line is indicative of an inflammatory healing process, e.g., as caused by intestinal spill. All surrounding structures adhesive to the anastomosis (colon, intestine, omentum, and pelvic fatty tissue) were considered separate adhesions and were counted as such. Bacterial growth of the abdominal cavity was determined by cotton swab, seeded on agar and Schaedler plates, and incubated.

Mechanical Analysis To test the anastomotic strength, 3 cm of colon, including the anastomotic line, was resected, while care was taken not do disturb the anastomosis and adhesions. The resected segment was ligated on both sides and, ex vivo, was connected via a cannula to a volume-directed infusion pump filled with PBS. A side arm of the cannula was connected to a pressure transducer, which in turn was connected to a recorder. The intestinal segment was gradually filled with PBS at a constant rate of 60 ml/hour while the intraluminal pressure was monitored until burst occurred, as indicated as an abrupt loss of pressure. The bursting pressure was documented, and the site of bursting was noted as being either at the anastomotic line or outside the anastomotic line.[12]

Table 1. Postoperative clinical scores

Score	Category	Description
0	Dead	
1	Sick	Lies in corner, curled up, fur raised and grayish, high respiratory frequency, eyes shut all the time, sticky feces (does not wash itself)
2	Reasonable	Low reactivity, eyes opened but not clear, color of fur not homogenously white
3	Fair	Stands up straight, clear opened eyes, eats if disturbed in sleep, clear white fur
4	As before surgery	

Immunohistochemistry Anastomotic lines were fixed in 4% formaldehyde and embedded in paraffin. Serial sections of 4-μm thickness from each block were mounted on poly-L lysine–coated slides, and hematoxylin and eosin–stained sections were made of every 10th section to identify the newly formed granulation tissue surrounding the suture and adjacent to the normal colon tissue (as judged by two independent observers). Generally, this granulation tissue was easily discernible from the surrounding tissue. The selected adjacent unstained slides containing granulation tissue were deparaffinized, rehydrated, and incubated with hydrogen peroxide for 10 minutes. The slides were pre-treated with pepsin and preincubated with normal goat serum for 15 minutes. The tissue was incubated with a polyclonal primary antibody against factor VIII/von Willebrand factor (DAKO, Carpinteris, CA) (1:500 in PBS/ bovine serum albumin) for 1 hour. Incubation with a biotinylated goat anti-polyvalent secondary antibody and large-volume streptavidin peroxidase (Lab Vision, Fremont, CA) was followed by 3,3'-diaminobenzide tetra-hydrochloride, as chromogen. Sections were counter-stained with hematoxylin and dehydrated. Negative controls were prepared by substituting the primary anti-body for PBS and were negative in all cases.

Morphometric analysis was performed with a LEICA-Q- Prodit (Leica Microsystems BV, Rijswijk, The Netherlands). The number of factor VIII–positive newly formed vessels was counted in granulation tissue surrounding the anastomotic line. The morphometric area of the granulation tissue and the relative area of the newly formed vessels were determined in all slides (according to the following formula: area vessels/area granulation tissue).

Statistical Analysis

Independent *t* tests (equal variances not assumed) were performed to determine statistical differences between the treated and nontreated groups, and Fisher's exact test was performed for the presence of peritonitis. For the difference in clinical scores over time, repeated-measurements analysis of variance was performed. Results are presented as mean ± SEM. Data were considered significant when P < .05.

Results

Anastomotic Healing Under Angiostatin Treatment

The clinical scores showed that the postoperative recovery of the mice treated with angiostatin was worse than that of control mice (P = .001). As shown in Figure 2, the control mice had almost completely recovered from surgery by day 7. In Table 2 the data on the clinical analysis and the intra-abdominal macroscopy are summarized. One angiostatin-treated mouse died on day 5. The clinical scores of this particular animal were 1 to 2 on days 1 to 4, indicating a particularly poor postoperative recovery. In accordance with these findings, the angiostatin-treated group on day 7 had lost more weight than the controls: 8.3 ± 2% as compared with 3.6 ± 1%. No mechanical ileus was observed, indicating anastomotic patency. Five mice that had been treated with angiostatin developed paralytical ileus. The intestinal diameter 2 cm distal to the anastomosis was significantly wider in the angiostatin-treated group, whereas the proximal diameter did not differ between groups. Six angiostatin-treated mice had clear macroscopic signs of local peritonitis, versus none in the control group (P = .02). In three cases, this was confirmed microbiologically with objective bacterial growth. In the controls, neither peritonitis nor ileus was found. The number of adhesions was significantly higher in the angiostatin-treated group. In all mice, all the other organs had normal macroscopic appearance. In 7 of 12 angiostatin-treated mice and in 1 of 13 controls, the rupture of the intestinal segment during measurement of the bursting pressure occurred on the anastomotic line instead of in adjacent colon tissue. As a consequence, the anastomosis was judged to be weaker than the
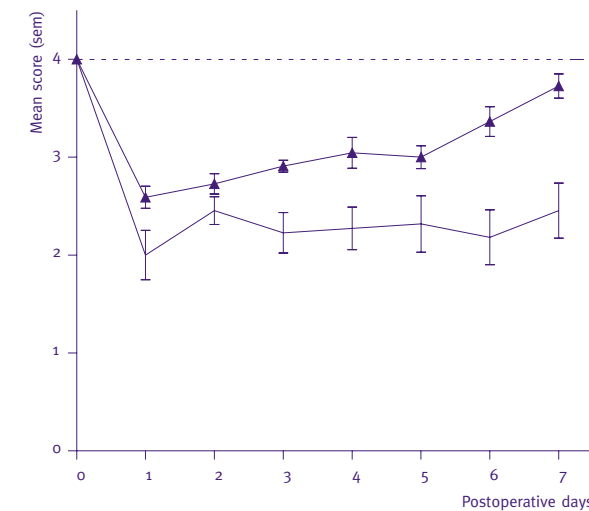

Figure 2.

Clinical appearance after colonic anastomosis on day 0. (—) Scores of mice treated continuously with angiostatin; (▲) control group (phosphate-buffered saline).

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surrounding tissue in these animals. In the controls, all but one mouse had an anastomotic line that had become at least as strong as the surrounding tissue 1 week after surgery, with bursting occurring in the normal intestinal tissue. The bursting pressure in the angiostatin-treated group was 135 ± 20 mm Hg, versus 175 ± 12 mm Hg in the controls. On immunohistochemical analysis, 16 ± 2 newly formed vessels were counted in the granulation tissue adjacent to the suture in the controls. In contrast, the granulation tissue of the mice that were treated with angiostatin contained significantly fewer new vessels (6.6 ± 1; P = .001). In addition, the area of granulation tissue occupied by new vessels was significantly decreased in the mice that were treated with angiostatin (.5 ± .1% vs. 2.2 ± .7%; P = .005) (Figure 3). The administration of angiostatin itself, in animals without an intestinal anastomosis, did not affect mouse body weight (as shown in our previous experiments with angiostatin[6]), intestinal diameter, or histology (data not shown).

Table 2. Anastomotic healing under angiostatin treatment

Variable	Angiostatin	PBS	P value
N	13	13	
Mortality	1	0	NS
Weight loss % (mean ± SEM)	8.3 ± 2	3.6 ± 1	NS
Parallytical ileus	5	0	NS
Diameter 2 cm distal (cm)	4.7 ± .3	3.1 ± .1	<.001
Local peritonitis	6	0	.02
Bacterial growth	3	0	
Adhesions	3.25 ± .3	1.08 ± .14	<.001

PBS, phosphate-buffered saline; NS, not significant. Data were pooled from seven independent experiments with identical design; angiostatin was administered from the time of operation until the day of termination of the experiment (day 7 after surgery).

Anastomotic Healing on Discontinuation of Angiostatin Treatment

The clinical scores of the angiostatin-treated mice (group 3) were comparable to those of the controls (group 4) during postoperative recovery (P = .93). None of the mice died. Weight loss in the angiostatin-treated group was 2.5 ± .3% vs. 3.0 ± .4% (P = .4) (Table 3). None of the mice showed signs of peritonitis, ileus, or intestinal distention. The number of adhesions did not differ between the two groups (P = .08). The intestinal diameter 2 cm distal to the anastomosis was 3.1 ± .06 cm in the angiostatin-treated group, compared with 3.1 ± .05 in the control group (P = .8). Again, no macroscopic abnormalities were found in the other organs. Mechanical analysis
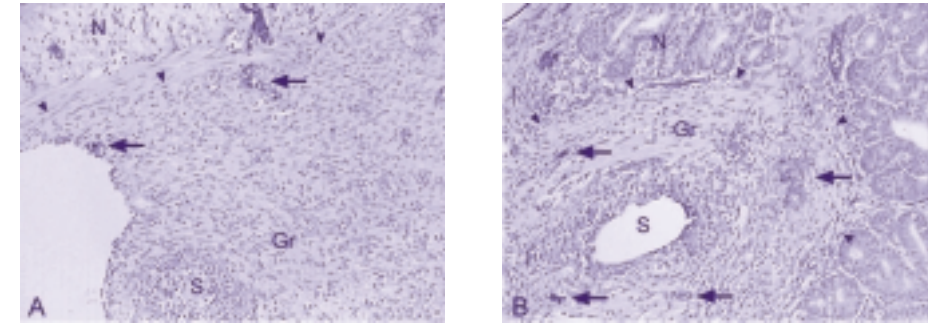

Figure 3. (see color appendix)
Anastomotic healing on day 7 after surgery. (A) Group 1; (B) group 2. A decreased number of newly formed vessels alongside the anastomosis in the granulation tissue were found in the angiostatin-treated mice (6.6 ± 1 vs. 16 ± 2). Also, the relative area of the newly formed vessels in the granulation tissue was less in those mice (.5 ± .1% vs. 2.2 ± .7%). Factor VIII immuno-histochemistry visualized the vessels (brown staining; some representative examples indicated by arrows). S, suture; Gr, granulation tissue (delineated by arrowheads); N, normal adjacent intestinal tissue.

revealed that all mice in groups 3 and 4 had bursting pressures above 130 mm Hg (control animals, 164.5 ± 7 mm Hg versus angiostatin-treated animals, 151.6 ± 6 mm Hg; not significant). Also, the anastomotic lines of these mice had become at least as strong as the adjacent intestinal tissue.

Table 3. Anastomotic healing directly after discontinuation of angiostatin administration

Variable	Angiostatin	before PBS	P value
N	6	6	
Mortality	0	0	NS
Weight loss % (mean SEM)	2.5 ± .3	3.0 ± .4	.4
Parallytical ileus	0	0	NS
Diameter 2 cm distal (cm)	3.1 ± .06	3.1 ± .05	.8
Local peritonitis	0	0	NS
Adhesions	1.5 ± .2	1.0 ± 0	.08

Data was pooled from four independent experiments with identical design; angiostatin was administered from four days before surgery until the day of the surgical procedure. PBS, phosphate-buffered saline; NS, not significant.

Discussion

Our data provide strong evidence that angiostatin impaired anastomotic healing in mice when administered continuously during the phase of postoperative repair. Until now, it has been generally assumed that antiangiogenic agents specifically affect activated, proliferating, and migrating endothelial cells and thus would lack any major side effects during treatment.[3,4] In concordance with this assumption, endostatin, another strong inhibitor of angiogenesis, was recently reported to have no adverse effects on physiological angiogenesis in cutaneous wound healing,[10] whereas no data exist on angiostatin in wound healing assays. But, in contrast to the healing of cutaneous wounds, the healing of colonic anastomoses is considered more dependent on angiogenesis and less dependent on diffusion of oxygen through pre-existing vasculature.[13,14] Thus, our model might be more sensitive to angiogenesis-directed interventions. This healing of colonic anastomoses could therefore serve as a model for physiologically critical angiogenesis. Putative adverse effects of antiangiogenic agents might be more apparent in this model compared with cutaneous wound healing. Indeed, in two recent studies, the short-term administration of two mildly angiosuppressive agents seemed to have adverse effects during the early phase of intestinal healing.[8,9] However, the adverse effects of suramin on rats' anastomotic healing did not include any clinical signs of dehiscence of the anastomotic sites, such as abscess formation, and were not exclusively restricted to the intestine. Even at low doses, suramin was toxic to other organs in all animals and caused intra-abdominal bleeding and splenomegaly.[9] In contrast, in this study, angiostatin did not impose toxicity on any organ besides the anastomosis (data not shown).

Furthermore, the other two studies mentioned do not provide temporal data. We have examined the kinetics of the adverse effects of angiostatin on the healing of colonic anastomoses. This was undertaken by administering the agent before surgery and discontinuing the treatment on the same day as the surgical procedure. Administration of angiostatin immediately before surgery had no negative effect on the healing of the anastomoses. These results in mice suggest that surgical patients who are treated with angiostatin can safely be operated on as soon as the treatment is discontinued.

Recently, angiostatin was shown by our group to be effective against experimental colorectal liver metastases.[6] Assuming that antiangiogenic treatment is used perioperatively in patients undergoing colonic resection, it should preferentially be discontinued as briefly as possible, in an attempt to keep any metastatic tumor cell deposits in a state of dormancy. This is based on the findings of recent studies that showed that, after resection of the primary tumor, the levels of circulating endogenous antiangiogenic agents produced by the primary tumor diminish, leading to accelerated metastatic outgrowth.[6,15] Considering the short half-life of angiostatin (4 to 6 hours), one might speculate that brief discontinuation of angiostatin treatment and resuming its administration shortly after anastomotic healing could circumvent the risk of anastomotic leakage while still effectively suppressing metastatic outgrowth.

Although the exact working mechanism of angiostatin has not been elucidated, it was recently shown to have a direct effect on endothelial cells.[16] Our immunohistochemical evaluation showed a reduction in the number of newly formed vessels in the granulation tissue surrounding the anastomotic line. On the basis of these data, we speculate that the formation of new vessels at the site of the anastomosis is impaired by angiostatin. This in turn may lead to a decrease in delivery of oxygen and nutrients to the healing colon. The adverse effects of angiostatin on physiological angiogenesis suggest mutual underlying mechanisms for tumor-induced and physiological angiogenesis. This assumption is supported by the knowledge that many other factors involved in angiogenesis, such as vascular endothelial growth factor, do indeed affect both physiological and pathologic angiogenesis.[17] It could well be that the antitumor effect and the effects on physiological angiogenesis of angiostatin are both mediated through a direct effect on endothelial cells.

We conclude from these results in mice that the healing of experimental colonic anastomoses is impaired by angiostatin when it is administered during the postoperative repair. However, on discontinuation of angiostatin therapy, normal anastomotic healing is promptly restored.

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Chapter 4

Histological Analysis of Defective Colonic Healing as a Result of Angiostatin Treatment

Elisabeth A. te Velde[1], Benno Kusters[2], Cathy Maass[2], Rob de Waal[2] and Inne H.M. Borel Rinkes[1]

Department of Surgery[1], University Medical Center Utrecht, Department of Pathology[2], University Medical Center Nijmegen, The Netherlands.

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Abstract

Antiangiogenic therapy is a highly promising new strategy in the treatment of cancer. One of the first angiogenesis inhibitors described was angiostatin, a 38-kDa internal proteolytically generated fragment of plasminogen. In a previous study we found that angiostatin affected physiological angiogenesis as well as tumor angiogenesis. It impaired healing when administered during repair of experimental colonic anastomoses, as reflected by a decrease in mechanical strength. On histology, we observed a decrease in factor VIII stained vessel amount and -volume in angiostatin treated colonic anastomoses. The exact working mechanism of angiostatin has not been elucidated. Based on the available studies on proposed working mechanisms of angiostatin, we have attempted to address histological differences in physiological angiogenesis between the tissues of colonic anastomoses of mice with impaired healing and control mice. After angiostatin treatment there was more inflammatory tissue as a result of impaired healing. Furthermore, we found fewer vessels in the granulation tissue after angiostatin treatment. However, especially with respect to ECM, endothelial cell apoptosis, proliferation, or neutrophil influx, no gross differences were discerned one week following surgery, using histology and immunohistochemistry techniques.

Introduction

Antiangiogenic therapy is a highly promising new strategy in the treatment of cancer. Since the growth and metastasizing capacity of a tumor depend on the formation of new blood vessels, many antiangiogenic agents have been developed that are directed against the endothelial cells of the tumor vasculature. One of the first angiogenesis inhibitors described was angiostatin, a 38-kDa internal proteolytically generated fragment of plasminogen. It was discovered in the serum and urine of tumor bearing mice.[1] Gelatinase A (matrix metalloproteinase-2, MMP-2), produced directly by those tumor cells, was found to be responsible for the production of the angiostatin.[2] Other MMP's (matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9)) were also shown to produce angiostatin.[3] Its anti-tumor activity has been proven in many experimental models as a single agent[4,5] as well as in combination with conventional therapies[6].

Still many questions concerning the working mechanism of angiostatin in anti-tumor efficacy remain to be elucidated.[7] Thus far, those studies have resulted in the identification of angiostatin-specific receptors, its role in cell migration through the extracellular matrix and its effects on endothelial cell death or apoptosis. The earliest studies showed that the antiangiogenic properties of angiostatin are caused by blocking endothelial cell migration and proliferation.[8-9] One of the identified receptors through which angiostatin was shown to act was ATP-synthase. Angiostatin was demonstrated to suppresses endothelial-surface ATP metabolism.[10,11] Another predominant receptor for angiostatin was found to be $\alpha_v\beta_3$.[12] In addition, a third receptor was identified in a yeast two-hybrid screen using a placenta cDNA library investigating the angiostatin-binding peptides, resulting in the identification of angiomotin.[13] Angiostatin inhibited cell motility and migration by interfering with angiomotin activity in endothelial cells. It is localized to the lamellipodia at the leading edge of migrating endothelial cells. In vivo, angiomotin is expressed in the endothelial cells of capillaries as well as larger vessels of the human placenta. All the aforementioned receptors could act independently or in combination with each other.

Neutrophils were shown to express both mRNAs for the angiostatin receptors ATP synthase and angiomotin. As shown by Benelli et al. angiostatin directly inhibited neutrophil migration and neutrophil-mediated angiogenesis, suggesting that angiostatin might inhibit inflammation as well as angiogenesis.[14]

In addition to inhibition of tumor angiogenesis, we showed in a previous study that angiostatin also influenced physiological angiogenesis in vivo. We evaluated the effects of angiostatin treatment during anastomotic healing.[15] Even though serious toxicity was not anticipated5,[16] we found that angiostatin impaired healing when administered during tissue repair, as reflected by a decrease in mechanical strength. On histology, we observed a decrease in factor VIII stained vessel amount and -volume in angiostatin treated colonic anastomosis.[15] Since no other reports on angiostatin and physiological angiogenesis have been published so far, the working

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mechanism of angiostatin under those circumstances are not known.

The purpose of the present study was to attempt to further investigate colonic anastomoses for effects of angiostatin treatment in physiological angiogenesis by use of histology. Specimens of the two groups of mice (angiostatin treated vs. controls) were compared for additional immunohistochemical and histological markers. The selection of the markers was based on the presumed working mechanisms of angiostatin in tumor angiogenesis, as suggested by literature.

Materials and methods

The mouse tissue under investigation was derived from a previous study.[15] In short, groups of 3-4 Balb-c male mice underwent transection of the right colon with anastomosis. Human angiostatin was generated as described[8] using minor modifications.[4,15] The mice had been randomly assigned to angiostatin treatment (n=13), or the PBS-control group (n=13). Angiostatin-treatment consisted of continuous administration from surgery until sacrifice (7 days). Based on macroscopic, mechanical as well as histological parameters, the anastomosis had been judged to be weaker in animals receiving angiostatin. In the controls, all but one mouse had an anastomotic line that had become at least as strong as the surrounding tissue one week after surgery.

The resected anastomotic lines were divided in two. One half was fixed in 4% formaldehyde and imbedded in paraffin, whereas the other half was snap-frozen in liquid nitrogen. Pending on antigen retrieval capacity of the epitopes frozen or formalin fixed material was used. Serial sections of 4-µm thickness from each block were mounted on poly-l-lysine-coated slides, and hematoxylin & eosin-stained sections were made of every tenth section in order to identify the newly formed granulation tissue surrounding the suture and adjacent to the normal colon tissue. Generally, this granulation tissue was easily discernable from the surrounding tissue. Differences in inflammation were assessed with H&E staining, anti-NIMP-R14, and FA11. NIMP-R14 is a 25–30 kDa epitope mainly present on neutrophils. Rat monoclonal antibody FA/11 (rat anti-mouse FA-11) identifies macrosialin, a heavily glycosylated transmembrane sialoglycoprotein of 87-115 kDa, highly and specifically expressed by mouse tissue macrophages. The ECM was investigated using polyclonal anti-Laminin antibody (Dako, Glostrup, Denmark), serving as matrix for endothelial migration. Conventional matrix staining for reticulin-elements (Laguesse) and Azan for complete collagenous matrix staining were used in order to determine differences in matrix components reflecting a mechanical tissue instability. Anti-murine CD31 (MEC 13.3; Pharmingen) and anti-murine CD34 (Hycult Biotechnology, Uden, The Netherlands) identified the endothelial cells. In addition we stained MMP2 by rabbit anti-MMP-2 (pAB809; Chemicon, California), and Vascular Endothelial growth factor receptor flk-1 by rabbit anti-flk-1 (Santa Cruz Biotechnology, Santa Cruz, California). An antibody against NG2-chondroitin-sulfateproteoglycan (pAB5320; Chemicon, California) was used to study pericytes in pathological microvasculature and NG2-positive cells that may bind, internalize, and co-immunoprecipitate with angiostatin. In addition, proliferation of endothelial cells was assessed using double-staining of Ki67 by rabbit anti-mouse Ki-67 (Dianova, Hamburg, Germany) and anti-CD31. Endothelial cell apoptosis was investigated using rabbit anti-activated-caspase-3 (Pharmingen). For architectural analysis of the specimen, 4 µm sections of formalin fixed and paraffin embedded material were stained. Depending on the antibody, either frozen of paraffin embedded material was used for staining. Frozen 4 µm sections were fixed in acetone for 10 min, dried and incubated with primary Ab overnight at 4 °C temperature in

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phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA). After washing with PBS, bound Abs were detected with a peroxidase-conjugated secondary Ab (Vector, Burlingame, California) using the Vectastain elite ABC kit (Vector, Burlingame, California). Sections were counterstained with hematoxylin. Immunohistochemistry for formalin fixed and paraffin embedded material was carried out using a similar protocol preceded by an antigen retrieval using microwave or proteinase-K digestion after deparaffination.

Results and discussion

The colonic anastomoses healed worse when treatment with angiostatin was administered, and apart from more inflammatory tissue, a lower number of vessels in the granulation tissue were discerned at histology (figure 1, CD31), in concordance with factor VIII stainings in our previous work[15]. Angiostatin affects tumor angiogenesis, by causing a decreased number of tumor vessels o.a.[4,5] Therefore, the demonstrated difference in number of vessels is comparable between tumor- and physiological angiogenesis.
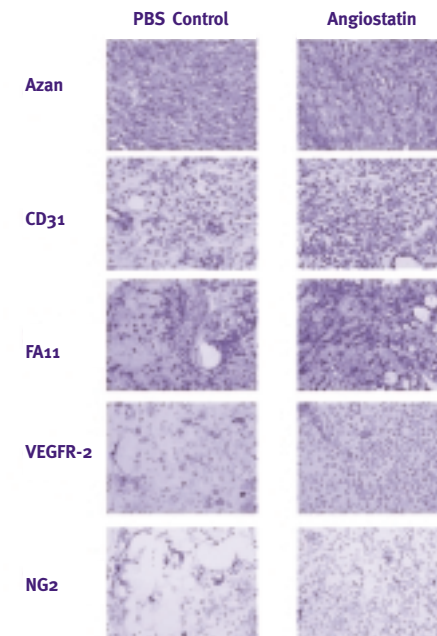

Figure 1 Comparison of granulation tissue in colonic wound healing in control mice and angiostatin treated mice. (see color appendix)
Azan staining shows comparable collagen deposition (blue matrix components) in both groups, ruling out a lack of collagen deposition in angiostatin treated mice as reason for mechanical instability of the wound tissue. CD31 staining highlights the vessels, There is an overall lower vessel density in angiostatin treated mice, but no difference in vascular phenotype: capillaries stain positive for VEGFR-2, characteristic for angiogenic blood vessels. Pericytes are positive for NG2 in both groups, neither arguing for a functional angiostatin depletion, nor showing a lack in normal microcapillary maturation. FA11 staining demonstrates the abundance of macrophages (besides granulocytes, not shown) in the granulation tissue, more prominent in the angiostatin treated group.

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This reduction of vessels in the granulation tissue of angiostatin-treated mice could be a result of less endothelial cell production, or conversely, result from an increase in endothelial cell destruction. Therefore, we investigated endothelial cell proliferation (ki67) and endothelial cell apoptosis (caspase-3). Both ki67 and caspase-3 stainings were similar in the tissues of mice that were treated with angiostatin (and suffering from impaired healing), and the controls. Therefore, the observed differences in angiogenesis could not be explained by immunohistochemical decrease in endothelial cell proliferation or increase in endothelial cell apoptosis. This was, however, only investigated on day seven following colonic anastomosis. We could speculate angiostatin to affect either apoptosis or proliferation in the week following the surgical procedure, resulting in fewer vessels later, at sacrifice. Indeed, in both groups the vasculature in the granulation tissue expressed VEGFR-2 (figure 1), thus representing an angiogenic phenotype[17]. In concordance, it has been speculated that the in vivo target of angiostatin is not the mature endothelial cell, but the circulating endothelial progenitor cells (EPCs) that contribute to the neovascularization.[18] In that study, EPCs were isolated from human subjects and was demonstrated that, in contrast to that of mature endothelial cells, the growth of EPCs is exquisitely sensitive to angiostatin. These results suggest that angiostatin works through inhibition of the contribution of EPCs to angiogenesis in an early phase, and not by altering the growth of mature endothelial cells later. If angiostatin indeed is most effective in early angiogenesis, then this could be similar for physiological and tumor angiogenesis.

In order to examine the gross morphological background of the low mechanical resistance present in the angiostatin treated mice, we investigated the ECM-formation in the granulation tissue. The reticulin and azan staining (figure 1) demonstrated matrix deposition in angiostatin treated mice that was comparable to the controls. Staining for laminin revealed that this matrix component was expressed in the granulation tissue (not shown) of both groups, especially located in basement membranes of micro capillaries. Therefore, it seems that the architectural pattern of the ECM deposition does not predict mechanical resistance. In a study on the interaction between angiostatin and the ECM, matrix angiostatin functioned as a non-competitive inhibitor of the ECM-enhanced, t-PA-catalyzed plasminogen activation.[19] Angiostatin in the cellular microenvironment could inhibit matrix-enhanced plasminogen activation, resulting in reduced invasive activity. This study suggested a biochemical mechanism whereby angiostatin-mediated regulation of plasmin formation could influence cellular migration and invasion. However, investigating this mechanism requires functional assays instead of immunohistochemical staining procedures.

More inflammatory tissue was present in the mice that were treated with angiostatin. H&E staining already showed a prominent granulocyte influx at this site, confirmed by NIMP-R14 and FA11 staining (figure 1). Therefore, we could not substantiate the findings that angiostatin directly inhibits neutrophil migration and neutrophil-mediated angiogenesis[14]. The increase in inflammation with recruitment of neutrophils

was most likely a direct effect of the impaired healing, with subsequent leakage and local peritonitis, as was found more frequently in the angiostatin treated mice. We speculate that proteases derived by the more prominent granulocyte influx into the granulation tissue in angiostatin treated animals may have caused a weakness of ECM-fibrils in the tissue.

NG2 was located in vascular structures and abundantly stained pericytal cells in both groups (figure 1). NG2 -proteoglycan was known to be expressed by pericytes in pathological microvasculature.[20] In addition, NG2 is up-regulated in the adult brain during neovascularization in granulating tissue of healing wounds[21,22], which support a role for NG2 during vascular proliferation and morphogenesis. On the other hand, NG2-positive cells bind, internalize, and co-immunoprecipitate with angiostatin.[23] Immature vessels contain NG2-positive pericytes, suggesting a role for this cell population in modulating endothelial cell proliferation by blocking the inhibitory effects of angiostatin during vessel development. In this study, however, we could not observe immunohistochemical differences in NG2-positive cells between the angiostatin treated mice and their controls.

In conclusion, adverse effects of angiostatin on experimental colonic healing coincide with fewer vessels and increased inflammatory tissue one week after surgery. Especially with respect to ECM, endothelial cell apoptosis, proliferation, or neutrophil influx, no gross differences were discerned one week following surgery, using histology and immunohistochemistry techniques.

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Chapter 5

Impaired healing of cutaneous wounds and colonic anastomoses in mice lacking thrombin-activatable fibrinolysis inhibitor
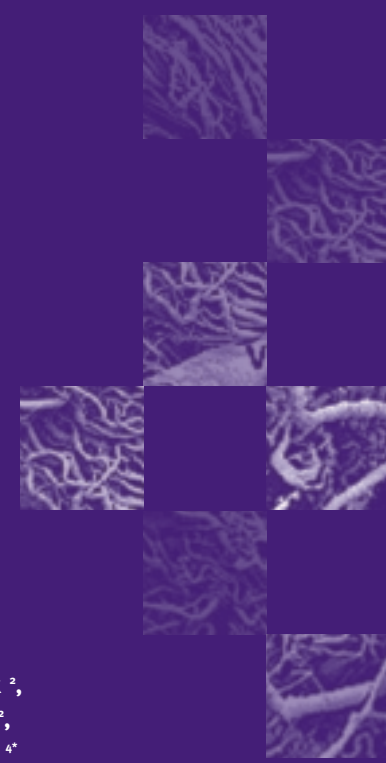
Elisabeth A. te Velde [1,2*]**, Gerry T. M. Wagenaar** [5*]**, Arie Reijerkerk** [2]**,
Merone Roose-Girma** [4]**, Inne H.M. Borel Rinkes** [1]**, Emile E. Voest** [2]**,
Bonno N. Bouma** [3]**, Martijn F. B.G. Gebbink** [2*]**, Joost C.M. Meijers** [4*]
equal contribution

Department of Surgery [1], Medical Oncology [2], and Haematology [3],
University Medical Center Utrecht, Utrecht,
The Department of Vascular Medicine [4], Academic Medical Center,
University of Amsterdam, Amsterdam,
Institute for Biomembranes [5], Utrecht University, Utrecht, The Netherlands.

Abstract

Plasmin and other components of the plasminogen activation system play an important role in tissue repair by regulating extracellular matrix remodeling, including fibrin degradation. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a procarboxy-peptidase that, after activation, can attenuate plasmin-mediated fibrin degradation by removing C-terminal lysine residues from fibrin. These C-terminal lysines play a role in the binding and activation of plasminogen. To test the hypothesis that TAFI is an important determinant in the control of tissue repair we investigated the effect of TAFI deficiency on the healing of cutaneous wounds and colonic anastomoses. Histological examination revealed inappropriate organization of skin wound closure in the TAFI knockout mice, including an altered pattern of epithelial migration. The time required to overall heal the cutaneous wounds was slightly delayed in TAFI deficient mice. Healing of colonic anastomoses was also impaired, as was reflected by a decreased strength of the tissue at the site of the suture as well as by bleeding complications in 3 out of 14 animals. Together these abnormalities resulted in increased mortality in TAFI deficient mice after colonic anastomoses. Although our study shows that tissue repair, including re-epithelialization and scar formation can ultimately proceed in some TAFI deficient mice, TAFI appears to be important for appropriate organization of the healing process.

Introduction

Wound healing is a complex process involving formation of a provisional matrix, cell infiltration and subsequent tissue remodeling. Intravascular platelet aggregation, formation of a fibrin clot, growth factor release, in combination with increased vascular permeability cause migration of attracted inflammatory cells. Blood vessel disruption caused by tissue injury leads to extravasation of plasma proteins, platelet aggregation and activation of the coagulation system. Macrophages, as well as platelets, are activated and produce growth factors for the initiation of granulation tissue formation. After the arrival of fibroblasts, the proliferation phase starts. Endothelial cells are important in this phase, as they contribute to angiogenesis. Secondary wound healing in unsutured wounds, as opposed to primary wound healing in sutured wounds, involves the migration of epithelial cells towards their opposite wound edges while they cover the wound. The migration of cells, angiogenesis and formation of granulation tissue are all dependent on the extracellular matrix (ECM) remodeling during the maturation phase. Plasmin and other components of the plasminogen activation system play an important role in tissue repair by regulating ECM remodeling. Furthermore, activated platelets greatly facilitate the coagulation cascade and secrete several adhesive proteins, including fibrinogen, fibronectin and collagen type V. Fibrin acts as a provisional matrix for the formation of granulation tissue and re-epithelialization. Without a correct balance between degradation and production of new ECM, impaired wound healing is likely to occur. Ultimately, the provisional matrix is degraded as the wound contracts, leaving a collagen scar. Both the coagulation and fibrinolytic systems play a fundamental role in the formation and subsequent removal of a blood clot. The removal of the haemostatic plug during the process of wound healing occurs by the fibrinolytic system in which plasminogen is converted into plasmin, resulting in proteolysis of the fibrin clot into soluble fibrin degradation products.

Cutaneous wound healing has been investigated in several knockout mice deficient for components that regulate the formation and/or degradation of the provisional matrix. Wound healing in plasminogen knockout mice is impaired.[1] In these mice migration of keratinocytes is decreased, leading to a delay in re-epithelialisation. A double knockout model that is deficient in plasminogen and fibrinogen showed a correction of the healing time to normal, implicating that an essential role for plasminogen is fibrinolysis.[2] On the other hand, in a wound-healing model of the skin in mice lacking fibrinogen the re-epithelialization of keratinocytes was inappropriately organized, leading to wound healing defects.[3] In mice deficient for plasminogen activator inhibitor-1 (PAI-1), the rate of skin wound closure was found to be accelerated.[4] Another form of wound healing is the healing of colonic anastomoses. Since anastomotic leakage in the week following surgery comprises a disastrous event, leading to high morbidity and mortality[5] this form of wound healing can be life threatening if impaired. We have recently investigated colonic healing in a mouse

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model[6], and proved this model to be useful in investigating delicate effects of impaired healing.

TAFI is a procarboxypeptidase that is synthesized in the liver as a prepropeptide and secreted into the circulation as a 55 kDa glycoprotein, both in man and in mice.[7,8] Activated TAFI (TAFIa) can down regulate fibrinolysis by removing carboxy-terminal lysines from fibrin, which act as binding sites for plasminogen and tissue-type plasminogen activator. In vitro TAFIa is rapidly (t1/2 ~ 10 min) inactivated by thrombin and/or by temperature-dependent instability.[9-11] TAFI can be activated by thrombin, both in the presence or absence of its cofactor thrombomodulin, plasmin, trypsin and meizothrombin. In the absence of thrombomodulin, high levels of thrombin are required for the activation of TAFI. Based on in vitro and in vivo studies it has been hypothesized that thrombin-activatable fibrinolysis inhibitor (TAFI) forms a link between coagulation and fibrinolysis.[12,13] No physiological function of TAFI in vivo has been described so far, although increased levels of TAFI have been associated with an increased risk for venous thrombosis.[14-16]

To study the biological function of TAFI in blood coagulation, fibrinolysis and wound healing, we generated genetically modified mice in which the endogenous TAFI gene is disrupted. We here show that mice lacking TAFI have impaired healing of colonic anastomoses and cutaneous wounds.

Materials and methods

CNBr-activated Sepharose and Rediprime™II were from Amersham Pharmacia Biotech (Uppsala, Sweden), bovine serum albumin (BSA, fraction V), L-α-phosphatidyl-choline, dioleoyl (PC), L-α-phosphatidylserine, dioleoyl (PS), L-α-phosphatidylethano-lamine, dioleoyl (PE) and proteinase K were from Sigma (St Louis, MO, USA). Tissue-type plasminogen activator (tPA, 531,000 IU/mg) was obtained from Chromogenix (Mölndal, Sweden). Hippuryl-arginine and H-D-Phe-Pro-Arg-chloromethylketone (PPACK) were obtained from Bachem (Bubendorf, Switzerland) and potato carboxypeptidase inhibitor (CPI) from Calbiochem (La Jolla, CA, USA). Human thrombin was a generous gift from Dr. W. Kisiel (University of New Mexico, Albuquerque, NM, USA). Thrombomodulin was from American Diagnostica (Greenwich, CT, USA). RNAzol™ was from Campro Scientific (Veenendaal, The Netherlands). T4 DNA ligase, G418 and PCR primers were from Life Sciences (Paisley, UK). AmpliTaq Gold™ DNA polymerase and gancyclovir were obtained from Roche (Almere, The Netherlands). Peroxidase-conju-gated swine antibody against rabbit IgG (SWARPO) was from DAKO (Glostrup, Denmark). Immobilon™–P PVDF membrane was from Millipore (Bedford, MA, USA). ECL Renaissance Western blot chemiluminescence reagent plus was from NEN Life Science Products (Boston, MA, USA).

Animals

Mice were kept in a controlled dark/light cycle and had access to pellet food and water ad libitum. Animal welfare was in accordance with institutional guidelines of the University of Utrecht. Mice were killed by cardiac puncture under ether anaesthesia. Blood was collected into citrate (3.8% weight/volume, 9:1 volume/volume) containing tubes and centrifuged for 15 min at 3000 rpm in an Eppendorf centrifuge. Plasma was collected, frozen immediately in 100 µl aliquots in liquid nitrogen and stored at −70 °C until use. Organs were removed immediately after decapitation after ether anaesthesia, frozen in liquid nitrogen and stored at −70 °C until use for Northern blot analysis or fixed overnight in a freshly prepared formaldehyde solution (4% parafor-maldehyde (weight/volume) in PBS, pH (7.4) at 4 °C for histological examination.

Generation of the targeting vector for homologous recombination in ES cells A 200 kb genomic DNA clone (BacM-13908, nr 18412, Genome Systems, USA) was obtained after screening of a mouse (129/Ola) genomic library with PCR primers (forward primer: 5'-GAG GCA CGT GGA TTT CTA CAT CAT-3'; reversed primer: 5'-GCA GCG GTT GTT CTT GTG AG-3' corresponding to nt 716-830 of mouse TAFI cDNA (Genbank AF 164524). Restriction fragment analysis and Southern blotting with a 300 bp *Xho*I-*EcoR*1 mouse TAFI cDNA probe, containing part of exon 1 and 2, revealed a 4.0 kb *EcoR*1 DNA fragment that was cloned in pBR-GEM11 and was used for the generation of the mouse TAFI knockout construct, and a 2.5 kb *Sac*I DNA fragment that was used

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for the generation of a 500 bp *EcoR*I-*Sac*I DNA probe for Southern analysis. Please note that the 3' *Sac*I site in the 2.5 kb *Sac*I DNA fragment was derived from the cloning vector and was therefore absent in the genomic mouse TAFI DNA sequence. The targeting vector was constructed by inserting a 1.6 kb phosphoglycerate kinase (PGK) neomycin (neo) cassette in exon 2 in the opposite transcriptional orientation to the TAFI gene (Figure 1A). Two PCR fragments were generated in which unique restriction sites were added to facilitate cloning of the neo cassette in exon 2: PCR1 (forward primer: 5'-GTA ATA CGA CTC ACT ATA GGG C-3' (T7); reversed primer: 5'-CTT **ATC GAT** ACA GAA TCT TAC TAC AAC G-3', *Cla*I restriction site is bold) and PCR 2 (forward primer: 5' CCC **CTC GAG** GTT GAA CTT GCC TGG AGG-3', *Xho*I restriction site is bold; reversed primer: 5'-GCC TGA CTG CGT TAG CAA TTT AAC-3' (pBR-GEM11rev) and cloned via the TOPO vector into pBluescript-SK. Flanking the neo cassette the 2.4 kb *EcoR*1-*Xho*1 PCR 2 fragment and the 1.6 kb *Cla*1-*EcoR*1 PCR 1 fragment were included at the 5' and 3' ends, respectively. This approach resulted in the deletion of 11 nucleotides in exon 2. At the 5' end a 3.0 kb PGK thymidine kinase cassette was included in the opposite transcriptional orientation to the TAFI gene. The final 8.6 kb construct was cloned in pBluescript-SK, linearized with NotI and introduced into E14 embryonic stem (ES) cells by electroporation (800 V, 3 μF, 0.1 ms). Targeted ES cells were selected after culturing in the presence of 200 μg/ml G418 and 5 μM gancyclovir. Individual ES cell clones were screened for homologous recombination by Southern blot analysis using a 500 bp *EcoR*I-*Sac*I genomic fragment downstream of the targeting construct. Targeted ES cells of 2 out of 56 individual ES cell clones were microinjected into the blastocoele cavity of C57BL/6J blastocysts and were implanted into pseudopregnant females. Three chimeric males of both targeted ES cell lines thus generated were bred to C57BL/6J females to produce heterozygous TAFI± offspring. Genotypes of mice were obtained by Southern blot or PCR by using tail tip DNA. TAFI± mice were backcrossed with C57Bl/6J mice. For experiments mice with a mixed background (75% C57Bl/6J and 25% 129/Ola) were used.

Southern blot analysis of genomic DNA

Genomic DNA was isolated from ES cell clones or mouse tail tips. The DNA was digested with *Sac*I and homologous recombination was analysed by hybridization with a 500 bp *EcoR*I-*Sac*I genomic DNA fragment localized at the 3' end outside the targeting vector. The probe detects a 7.4 kb wild type and a 8.8 kb mutant fragment.

PCR analysis of genomic DNA

For the PCR reactions 1 μl of 250-fold diluted DNA in sterile water was used. Each sample was subjected to two separate PCR reactions. The reaction containing a neo-specific primer set (forward primer: 5'-ACA AGA TGG ATT GCA CGC AGG-3' corre-

sponding to nt 750-770 of PGK-Neo; reverse primer: 5'-GAA TGG GCA GGT AGC CGG ATC-3' corresponding to nt 1099-1199 of PGK-Neo) revealed the presence of a 350 bp neo-specific product in TAFI± and TAFI-/- samples. The PCR reaction containing primers annealing in exon 2 (forward primer) and intron 2 (reverse primer) of mouse TAFI (forward primer: 5'-TGG CCA GGT TTT ATC TGC -3' corresponding to nt 155-172 (Genbank AF 164524) and reverse primer 5'-CCA CTT GTC ATG AAT TAC-3' revealed the presence of a 1300 bp amplification product in TAFI+/+ and TAFI± samples.

Northern blot analysis

Total RNA was extracted from tissue homogenates using the RNAzol™ B method as described by the manufacturer. Total RNA samples (10-30 μg) were electrophoretically fractionated on denaturing 1% agarose gels, blotted onto nylon filters and UV-crosslinked. The probe was the 0.8 Kb *Hind*III-*Hind*III mouse TAFI cDNA fragment (Genbank AF 164524).[17] After hybridisation, the blots were washed and exposed to Kodak X-Omat Blue XB-1 film with intensifying screens.

Detection of TAFI in mouse plasma

Mouse TAFI protein was immunoprecipitated from fresh frozen citrated plasma using a polyclonal rabbit anti-human TAFI antibody[18] coupled to activated CNBr-Sepharose (4 mg antibody to 1 g Sepharose). 45 μl mouse plasma was added to 385 μl Tris buffer (50 mM Tris, 150 mM NaCl; pH 7.4), containing 100 μl polyclonal antibody coupled Sepharose (diluted 1:1 in Tris buffer) and PPACK (15 μM). Binding was allowed for 90 min at room temperature on a top over top shaker. Samples were washed 4 times in buffer (50 mM Tris, 500 mM NaCl, pH 7.4), eluted with 0.1 M glycine (pH 2.7) and neutralized in 0.1 M Tris, pH 9.0. Samples were subjected to SDS-PAGE under reducing conditions. Proteins were blotted on PVDF membranes, detected with a rabbit anti-human TAFI polyclonal antibody and SWARPO, and visualized with ECL followed by exposure to Kodak X-Omat Blue XB-1 film.

TAFI activity assay

TAFI activity was measured as described previously.[19] In this assay, plasma is treated with thrombin-thrombomodulin, and the carboxypeptidase activity generated is determined with the substrate hippuryl-arginine. The assay is performed both in the presence and absence of CPI, to be able to correct for the constitutively active carboxypeptidase N present in plasma.

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Clot lysis assay

The clot lysis assay was performed essentially as described previously.[18] Experiments were performed with plasmas of at least 5 mice per experimental group.

Cutaneous wound healing

Mice were anaesthetized intraperitoneally with fentanyl citrate/fluanisone (0.3 mg/mouse; Janssen-Cilag, Brussels, Belgium) and midazolam chloride (12.5 mg/mouse; Roche, Brussels, Belgium). The dorsal skin was shaved and disinfected by alcohol 80%. Excisional wounds were inflicted by a full thickness 3 mm disposable punch biopsy (Stiefel Laboratories Inc, Florida, USA). Full thickness 1-cm incisional wounds were created by surgical blade. The incisional as well as the excisional wounds were created lateral to the dorsal midline. In the first set of experiments, the incisional and excisional wounds were created on either side of the dorsal midline, 2 cm apart, in the same animal. In a second set of experiments, only excisional wounds were studied and all wounds were uniquely inflicted by 3-mm punch biopsies, one per animal. Surgery was carried out blinded to the genotypes of the mice. All wounds were left unsutured and undressed, in order to study secondary wound healing. The mice were housed individually for the duration of the study and allowed food and water ad libitum. Postoperatively, mice of each genotype were divided into three different groups, consisting of 4-5 mice per group. Wounds were examined and photographed on days 4, 7 and 15 to follow progression of healing of the wounds. On days 4, 7 and 15 respectively, one group of each genotype was sacrificed. At sacrifice, the wounded tissue was excised, formalin fixed, sectioned into half and completely embedded in paraffin. Per wound serial 4-μm slides, perpendicular to the original wound incisions, were stained with hematoxylin and eosin and analyzed blinded to the genotypes of the mice, by two independent observers. Complete reepithelialization was defined as 100% approximation of the keratinocyte layer covering the wound.

Colonic anastomosis

28 animals were subjected to surgery; 14 knockout and 14 wild-type animals. Surgery was performed blinded to the genotype in 7 independent experiments. The right colon was anastomosed following transection, as described previously.[6] Briefly, anesthetized mice were shaved and placed on a heated surgical microscopy table. A midline laparotomy was performed under aseptic conditions. The right colon was transected one cm distal to the coecum by microscopical scissors, taking care not to damage the mesenteric vessels. The colon was anastomosed by a single-layer 8.0 non-absorbable (Prolene™, Ethicon, Brussels, Belgium) inverted running suture.

Two independent observers recorded the postoperative clinical condition of the mice daily, according to a predetermined clinical assessment score as described in table 1. For calculation of the percentage of postoperative weight loss, body weights on day 7 postoperatively were compared to those on day 0, i.e. prior to surgery. All mice that had died prior to sacrifice on day 7 were subjected to autopsy by the veterinarian of the animal housing facility. On day 7 the abdomen was reopened and inspected for anastomotic dehiscence, local peritonitis, ileus, and the formation of adhesions. Peritonitis was defined as macroscopic signs of inflammation, fibrin deposition and hypervascularity. Mechanical ileus was defined as obstruction of the intestine caused by the suture, with distention proximal to the anastomosis but not distal to the anastomosis, and was considered a failure of the surgical procedure. Paralytical ileus was defined as distention of atone intestine and colon, with paling and swelling of the colonic wall, in conjunction with the absence of mechanical ileus. Adhesions involving the suture line were counted, because the presence of adhesions surrounding the suture line is indicative of an inflammatory process, e.g., as caused by intestinal spill. All surrounding structures adhesive to the anastomosis (colon, intestine, omemtum, and pelvic fatty tissue) were considered separate adhesions and were counted as such.

Table 1. Clinical appearance scoring system

Appearance			
0	=		normal
1	=		fur untreated
2	=		1 + red spots surrounding eyes
3	=		2 + abnormal posture
Behaviour			
0	=		normal
1	=		minor changes
2	=		decreased activity
3	=		immobility
Reactivity			
0	=		normal
1	=		increased or less
2	=		1 but increased
3	=		2 but even more increased
Body weight			
0	=		normal
1	=		<10% weight loss
2	=		10-20% weight loss
3	=		>20% weight loss

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To test the anastomotic strength, 3 cm of colon, including the anastomotic line was resected, while care was taken not to disturb the anastomosis and adhesions. The resected segment was ligated on both sides and, ex vivo, was connected via a cannula to a volume-directed infusion pump filled with PBS. A side arm of the canula was connected to a pressure transducer, which in turn was connected to a recorder. The intestinal segment was gradually filled with PBS at a constant rate of 60 ml/hour while the intraluminal pressure was monitored until burst occurred, as indicated as an abrupt loss of pressure. The bursting pressure was documented, and the site of the bursting was noted as being either at the anastomotic line or outside the anastomotic line.

Anastomotic lesions were fixed in 4% formaldehyde and imbedded in paraffin. Serial sections of 4-μm thickness from each block were mounted on poly-L-lysine-coated slides, and hematoxylin & eosin-stained sections were made of every tenth section in order to identify the newly formed granulation tissue surrounding the suture and adjacent to the normal colon tissue (as judged by two independent observers). Generally, this granulation tissue was easily discernable from the surrounding tissue. The selected adjacent unstained slides containing granulation tissue were analyzed.

All animal experiments were executed according to the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht.

Statistical analysis

Independent t tests (equal variances not assumed) were performed to determine statistical differences between the treated and non-treated groups for the wound healing experiments. Results are presented as mean ± SEM. Data was considered significant when $p < 0.05$.

Results

Targeted inactivation of the mouse TAFI gene was achieved by homologous recombination in embryonic stem cells using a targeting construct, including part of intron 1, exon 2 and part of intron 2, in which a neomycin cassette was introduced in exon 2 (Figure 1A). A thymidine kinase cassette was introduced at the 5' end of the targeting construct. Two independent, correctly targeted ES cell clones were obtained, which gave rise to chimeric mice after blastocyst injection. Germline transmission of the inactivated TAFI gene was detected by PCR analysis of tail tip DNA. Intercrossing of these heterozygous TAFI-deficient mice yielded homozygous TAFI-deficient offspring. The absence of TAFI mRNA in livers of TAFI-/- offspring was demonstrated by Northern blotting (Figure 1B). In addition, no TAFI protein could be detected by Western
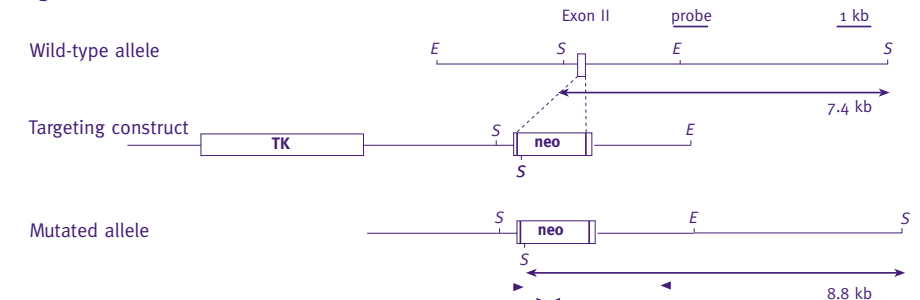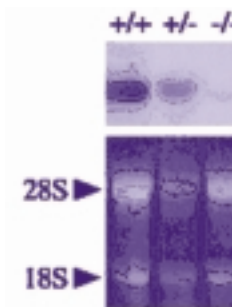
Figure 1.a
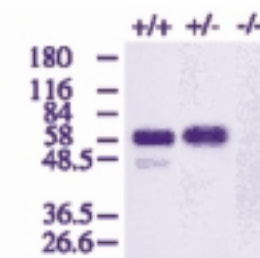

Figure 1.b   **Figure 1.c**

Figure 1. TAFI gene inactivation.
(A) Structure of the mouse TAFI gene, the targeting vector and the predicted disrupted gene. The probe used for Southern blotting as well as the detected fragments are indicated. The PCR primers used for genotyping are indicated with arrowheads. E, EcoRI; S, SacI. (B) Northern blot analysis of total liver RNA from PCR genotyped animals. The loading of the gels was confirmed by ethidium bromide staining of ribosomal RNA (lower part). (C) Western blot analysis of plasma from PCR genotyped animals.

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blotting of plasma of TAFI-/- mice (Figure 1C) or by ELISA (2.1 + 0.5 μg/ml in TAFI+/+, 1.7 + 0.4 μg/ml in TAFI±, and 0 μg/ml in TAFI-/- (mean + SD; n=14, 45 and 65, respectively; detection limit of assay ‹ 0.02 μg/ml)). TAFI deficient mice were fertile and produced offspring according to Mendelian ratios. TAFI-/- mice produced similar sizes and numbers of litter as TAFI+/+ mice. No signs of spontaneous bleeding or (developmental) abnormalities were observed in TAFI-/- mice. The oldest TAFI-/- mice are more than 1 year old and still appear healthy.

We evaluated the consequences of the absence of TAFI on clot lysis in vitro. In TAFI+/+ plasma (Figure 2A), the clot is protected from lysis, which can be reversed by the specific TAFI inhibitor CPI (potato carboxypeptidase inhibitor). In contrast, in plasma from TAFI-/- mice, the clot is lysed rapidly, and addition of CPI to the plasma did not result in enhanced lysis (Figure 2A). This was confirmed by the absence of TAFI activity in plasma of TAFI-/- mice (data not shown). TAFI deficiency could not protect the animals from LPS-induced disseminated intravascular coagulation in a lethal endotoxemia model (data not shown).
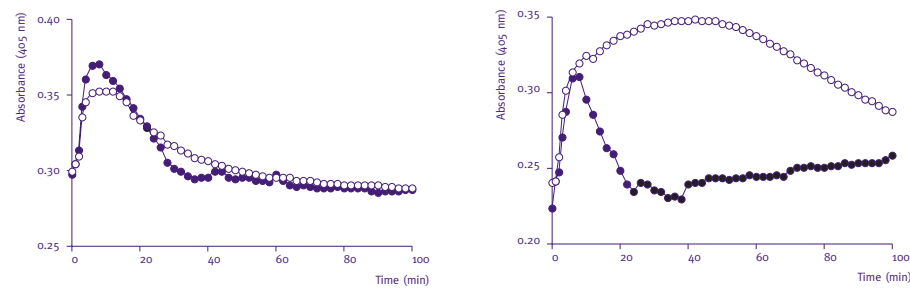

Figure 2. Clot lysis assay in plasma of wild-type and TAFI-deficient mice.
(A) Wild-type mice. (B) TAFI deficient mice. Plasma was mixed with potato carboxypeptidase inhibitor (closed circles) or buffer (open circles). Coagulation and fibrinolysis were initiated by addition of thrombin and tPA, respectively. Clotting and fibrinolysis were followed in time as the change in turbidity at 405 nm.

Cutaneous wound healing

To explore the role for TAFI in secondary cutaneous wound repair we have generated incisional and excisional dorsal skin wounds. Within two days both control and TAFI deficient mice developed a similar fibrotic scar over the wound area (Figure 3). Daily examination of the excisional wounds revealed no gross differences between the healing of control and TAFI deficient mice (Figure 3A-D). The incisional wounds of TAFI deficient mice showed a larger wound area as well as scar and appeared to be more red due to protruding tissue (Figure 3E-H).
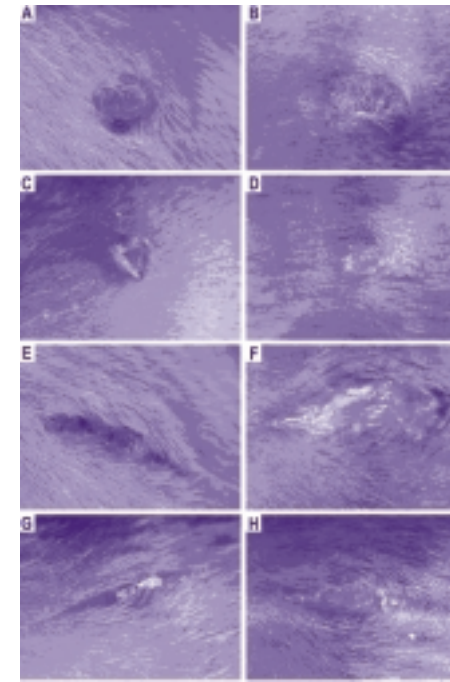

Figure 3. Macroscopical appearance of skin wounds. (see color appendix)
Representative examples of excisional (A-D) and incisional (E-H) wounds at 4 days (A,B,E,F) and 7 days (C,D,G,H) postoperatively. The left panels show the wildtype mice (A,C,E,G) and the right panel show the TAFI-/- mice (B,D,F,H). No gross differences were found in macroscopical appearance of excisional wounds on either days (A-D). However, following incisional wounds TAFI-/- mice (F,H) showed a subjective larger wound as well as scar and the wounds appeared to be more red due to protruding tissue, as compared to wildtype mice (E-G).

Histological examination of all wounds revealed that re-epithelialization in TAFI deficient mice was different from that in control mice (Figure 4). Although keratino-cytes seem to proliferate and migrate in both genotypes, the direction of migration in TAFI deficient mice (5/8 at day 4, 1/8 at day 7) was different from wild type animals. Keratinocytes migrated as usual between scar and granulation tissue from one wound edge towards the opposing edge in wild-type animals (Figure 4A,C). In TAFI deficient mice, however, keratinocytes followed an abnormal pattern in another direction, mostly down into the dermal layer (Figure 4B,D). Furthermore, the edges of these layers of keratinocytes were blunt and thickened (Figure 4B,D). Sinuses (Figure 4E) were formed and keratinocyte inclusions (Figure 4F) were observed in the TAFI deficient mice only. The aberrant epithelial cell migration resulted in prolonged wound healing in TAFI-/- mice (Figure 5). Complete re-epithelialization in control mice on day
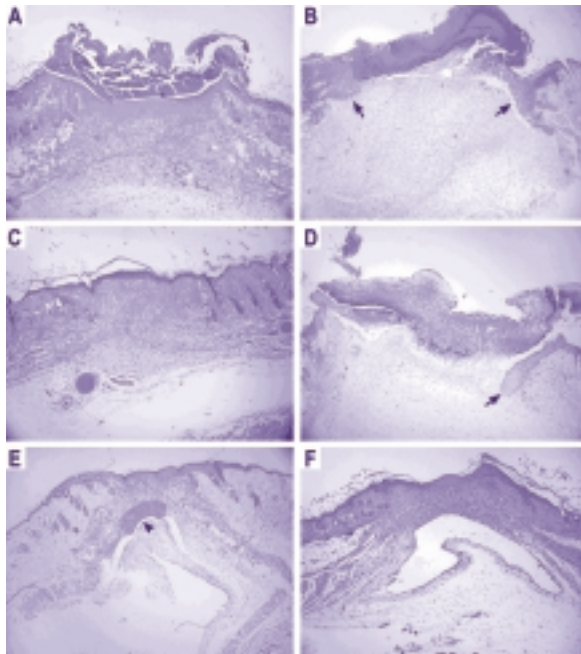
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Figure 4. Microscopic appearance of skin wounds. (see color appendix)
Cutaneous wounds of TAFI+/+ (A,C) and TAFI-/- (B,D,E,F) mice. Sections were prepared from isolated skin tissue and stained with H&E. The images are shown perpendicular to the longitudinal direction of the wounds. (A) A normal epithelial migration of a TAFI+/+ mouse on day 4 postoperatively. (B) A representative example of an altered pattern re-epithelialisation of a TAFI deficient mouse on the same day as the control, day 4 postoperatively. The keratinocytes seem to migrate into the dermal layer (as indicated by the arrow), with blunt edges of the keratinocytes' layer. (C) Normal healing of a TAFI+/+ mouse on day 7 postoperatively, with complete re-epithelialisation. (D) A TAFI-/- mouse on day 7; Non-complete healing, with the layer of keratinocytes ending blunt into the dermis (arrow). (E) A keratinocytes-inclusion in the dermal layer of a TAFI deficient mouse (arrowhead). (F) A wound sinus in a TAFI-/- mouse, paved with keratinocytes.

4 and 7 occurred in 89% and 100% respectively, whereas 38% and 63% of the wounds in the TAFI deficient mice were closed at these time points, respectively. All mice had completed re-epithelialization by day 15 (using Fisher Exact-test comparing wild-type and knockout animals on day 4, p=0.043, and on day 7, p=0.082). Cohesive provisional matrix had developed normally in the wounds and the formation of abundant and highly vascularized granulation tissue closed the wound gap in both genotypes.
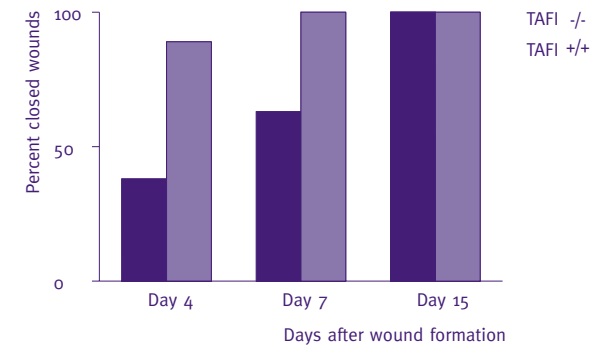

Figure 5. Quantification of skin wound healing.
The histological slides were H&E stained and analysed for re-epithelialisation. The aberrant epithelial cell migration resulted in prolonged wound healing in TAFI-/- mice. On day 4 postoperatively complete re-epithelialisation in the wild types was 89% vs. 38% (p=0.043) in the TAFI deficient mice. On day 7 postoperatively 100 % of control vs. 63% (p=0.082) in the TAFI deficient mice had healed. On day 15, all mice in both genotypes had complete re-epithelialization.

Healing of colonic anastomosis

We have examined the consequences of TAFI defiency on colonic anastomotic healing, a sensitive model of wound healing that, if impaired, can have life threatening consequences.[5]

The clinical scores showed that the postoperative recovery of the TAFI-/- mice was worse than control mice, as shown in Figure 6. In accordance with these findings, the TAFI-/- mice on day 7 had lost more weight than the controls, 8.2 ± 1.6% as compared to 4.2 ± 0.5% in the wild-type controls (p=0.015). In table 2 data on the clinical analysis as well as the intra-abdominal microscopy are summarized. Four of the knockout mice died, one each on days 1 and 5, and two on day 7. Autopsy revealed peritonitis caused by impaired anastomotic healing in two cases (died on day 5 and day 7). One mouse had a mesenterial thrombosis on day 7 and consequently widespread intestinal and colonic ischemia. The mouse autopsied on the first day postoperatively showed severe hemorrhage of the inner layer of the dorsal skin, at the sites of inflicted wounds by littermates' bites. In all the dead knockout mice, we found bleeding and ecchymoses at places of mechanical trauma, such as the injection site in the subcutaneous layer of the abdominal wall. Those signs of bleeding were not found in dead wild-type littermates, nor in sacrificed wild-type controls. One of the wild-type control littermates died on day 7 of unknown cause. The abdominal cavity as well as the anastomotic line had normal appearances, and
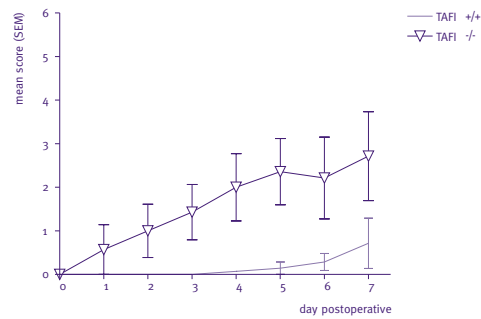
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Figure 6. Clinical appearance of mice following colonic anastomosis.
Course of clinical appearance from the day of surgery (day 0) until the day of sacrifice (day 7). The appearance of each individual mouse was scored by two independent observers, in a semi-quantitative manner. The scoring system used is depicted in table 1; note that the lower the score, the healthier the animal (a normal mouse has a cumulative score of 0). Scores are plotted per genotype and presented as mean ± SEM.

Table 2. Parameters of the anastomotic healing in TAFI-/- mice

	TAFI+/+	TAFI-/-	p
N	14	14	
Mortality	1	4	
Bleeding	0	3	
Mesent thrombosis	0	1	
Dehiscence	0	2	
Weight loss % (mean ± SEM)	4.2 (0.5)	8.2 (1.6)	0.015
Local peritonitis	0	4	
Adhesions	1.6 (0.4)	2.8 (0.8)	0.15
Bursting pressure	193.2 (7.1)	142.6 (19.9)	0.015
On suture	0	4	

no bleeding or coagulation complication could be discerned. In none of the mice mechanical ileus was observed, indicating anastomotic patency in all cases. At sacrifice, 4 knockout mice had clear macroscopic signs of local peritonitis vs. none in the control group. The number of adhesions was not significantly different in the knockout group (2.8 ± 0.8 vs. 1.6 ± 0.4 in the wild-types, p=0.15). In 4/14 knockout mice the rupture of the intestinal segment occurred on the anastomotic line, instead of in adjacent colon tissue. As a consequence, the anastomosis was judged to be weaker than the surrounding tissue in these animals. In the controls, all had an anastomotic line that had become at least as strong as the surrounding tissue one week after surgery, with bursting occurring in the normal intestinal tissue. The bursting pressure in the knockout mice was significantly lower (142.6 ± 19.9 mm Hg vs. 193.2 ± 7.1 in the controls (p=0.015). Histological analysis revealed no gross differences in granulation tissue between genotypes. Increased inflammation was found in cases of intestinal leakage in those animals with bursting pressure measurements corresponding to impaired healing.

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Discussion

There is considerable evidence that TAFI is important for hemostasis and links the coagulation and fibrinolytic systems[7,12]. To study the biological function of TAFI in blood coagulation, fibrinolysis and wound healing, we generated genetically modified mice in which the endogenous TAFI gene is disrupted. In this study, we found that lack of TAFI is compatible with murine life and describe a novel physiological function for TAFI in vivo.

Nagashima et al.[20] studied TAFI deficient mice with respect to coagulation and fibrinolysis, and they did not find any effects on tail bleeding time, venous or arterial thrombosis, thrombin-induced pulmonary embolism, factor X coagulant protein-induced thrombosis, or endotoxin-induced disseminated intravascular coagulation. Swaisgood et al.[15] have recently determined the role of TAFI defiency in modulating the functions of plasminogen. The TAFI knockout mice were tested in a compromised plasminogen background (plasminogen ± mice). This compromised background made the effects of TAFI apparent. It was demonstrated in a pulmonary clot lysis model that fibrinolysis was significantly increased in TAFI knockout mice. Also, in a model of peritoneal inflammation, the leukocyte migration in knockout mice was significantly increased.[14,21-23]

Here we show that mice lacking TAFI have impaired wound healing in two different models of tissue repair. First, skin wound healing was different in TAFI deficient mice. The re-epithelialization by keratinocytes in TAFI-/- mice was delayed and did not follow the normal pattern of migration. In normal secondary wound healing keratinocytes migrate from one wound edge towards the opposing edge. For undisturbed migration the cells need contact with intact ECM over which they migrate. However, in TAFI -/- mice the direction of keratinocyte migration was mostly directed down into the dermal layer, indicating a disruption of functioning ECM. Nevertheless, in both control and TAFI-/- mice cutaneous wound closure had ultimately been completed in all mice within 15 days. Interestingly, fibrinogen deficient mice showed a remarkably similar pattern of cutaneous wound healing[3] as compared with the TAFI deficient mice. We could speculate that, since activated TAFI inhibits fibrin stimulated tPA-mediated plasmin formation, the absence of TAFI can presumably lead to uncontrolled plasmin activation resulting in unbalanced matrix degradation. This may then result in the excessive removal of fibrin and in the observed aberrant effects on keratinocyte migration in the TAFI knockout mice that show similarities to the defects in the fibrinogen deficient mice. In contrast however, PAI-1 deficiency accelerates cutaneous wound healing.[4] PAI-1 does not only regulate plasmin formation, but also inhibits cell migration by blocking the vitronectin receptor $\alpha v \beta 3$ interaction with vitronectin.[24] The interaction of PAI-1 with plasminogen activators results in this setting in a loss of affinity for vitronectin, which restores cell migration. In this respect it is interesting to note that thrombomodulin, a cofactor for thrombin-mediated TAFI activation,[25] is weakly expressed in normal epidermis but markedly upregulated in keratinocytes during cutaneous wound healing. Thrombomodulin is expressed in keratinocytes in areas of stratification within the neoepidermis whereas thrombomodulin is absent in keratinocytes at the margins of the neoepidermis.[26] However, mice deficient in thrombomodulin did not show apparent defects in cutaneous wound healing.[26] This may suggest that other mechanisms, not involving thrombin-thrombomodulin mediated activation of TAFI can occur in vivo. It also indicates a role for other proteases, such as plasmin, in the in vivo activation of TAFI and that TAFI activation might not necessarily be restricted to areas where thrombomodulin is present.

Subsequently, we have shown that end-to-end anastomosis of the ascending colon in those mice resulted in a decreased strength of the anastomosis at sacrifice at day seven postoperatively as compared with the wild-type controls. The healing of colonic anastomoses is a model of wound healing independent of keratinocyte migration but dependent on matrix remodeling and angiogenesis,[27] whereas cutaneous wound healing benefits from diffusion of oxygen through preexisting vasculature[28,29]. Thus, in agreement with Swaisgood et al.[15] it seems that TAFI plays a role in modulating cell migration and/or invasion independent of the cell type.

Proper control of proteolytic degradation of the ECM and ECM remodeling is necessary for secondary wound healing, as well as for anastomotic healing. The number of adhesions surrounding the anastomosis was not significantly different in the knockout group (2.8 ± 0.8 vs. 1.6 ± 0.4 in the wild-types, p=0.15). The presence of adhesions surrounding the suture line is indicative of an inflammatory process, e.g., as caused by intestinal spill and prevents diffuse spreading of inflammation in the abdomen. In addition, based on previous observations we had expected to observe an increase in the number of adhesion as a response to the leakage[6]. Apparently TAFI-/- mice did not form more adhesions in reaction to the observed intra-abdominal leakage.

Another remarkable result of our study, was that in some critically ill mice from impaired colonic healing with dehiscence and subsequent peritoneal leakage, at autopsy a mesenterial thrombosis was suspected in combination with diffuse bleeding from inflicted littermate's bites. In all the dead knockout mice, we found bleeding and ecchymoses at places of mechanical trauma, such as the injection site in the subcutaneous layer of the abdominal wall. This clinical manifestation in the TAFI deficient mice could very well be a result of diffuse intravascular coagulation (DIC). In contrast, mice in whom other components of the fibrinolytic system were disrupted, e.g. antiplasmin[32], PAI-1[33] and PAI-2[34], had normal bleeding phenotypes. Probably, the coagulation system adequately functions under circumstances where the fibrinolytic system is not dramatically challenged. The suspected DIC in the TAFI deficient mice after impaired colonic anastomotic healing might be a result of a critically challenged fibrinolytic system, where other mechanisms insufficiently compensate for the lack of TAFI.

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Based on the impaired healing in two wound healing models in mice lacking TAFI, i.e. the alteration in keratinocyte migration, decreased anastomotic strength of the healed colon, the presence of severe hematological complications in TAFI-/- with peritonitis after impaired colonic healing, and a diminished induction of reactive adhesion formation we could speculate that TAFI might be a player in this delicately regulated process.

In conclusion, our results provide direct evidence for a role for the hemostatic factor TAFI in cellular processes in vivo. Although unchallenged TAFI-/- mice showed no phenotype, we showed that TAFI is involved in tissue repair. Studies to investigate the mechanisms underlying the etiology of the wound healing defect in TAFI deficiency are now warranted.

Acknowledgements

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Chapter 6

Vascular clamping during hepatic surgery for colorectal metastases worsens prognosis

Elisabeth A. te Velde[1,2]**, Paul J. van Diest**[3]**, Bram Fioole**[1]**,
Werner A. Draaisma**[1,2]**, Petra van der Groep**[3,4]**, Astrid E. Greijer**[3,4]**,
Elsken van der Wall**[4]**, Emile E. Voest**[2]**, Inne H.M. Borel Rinkes**[1]**.

Department of Surgery[1], Department of Medical Oncology[2],
University Medical Center Utrecht, Utrecht,
Department of Pathology[3], Department of Medical Oncology[4], VU-UMC, Amsterdam,
The Netherlands

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Abstract

Background Surgical treatment of colorectal liver metastases (CLM) is becoming increasingly important. Routinely, temporary occlusion of the inflow of the liver, i.e. Pringle maneuver (PM), is performed a.o. to reduce peri-operative blood loss. We hypothesized that PM unfavorably affects the prognosis of cancer patients, by inducing temporary hypoxia with upregulation of Hypoxia Inducible Factor-1alpha (HIF1α), increase in Vascular Endothelial Growth Factor (VEGF) levels, and subsequent angiogenesis and growth of residual tumor. We evaluated the effects of PM on 1) tumor recurrence in patients following resection of CLM; 2) intrahepatic HIF1α expression and systemic VEGF-levels in patients undergoing Radiofrequency Ablation, and 3) outgrowth of experimental CLM.

Methods 1) All consecutive patients who underwent hepatectomy for CLM between 1991 and 2000, with or without PM were analyzed for tumor recurrence, disease specific survival (DSS) and disease free survival (DFS); 2) In 10 patients scheduled for Radiofrequency ablation a standard PM of 20 minutes was performed. Intrahepatic HIF1α (immunohistochemistry) and circulating VEGF (ELISA) were measured before and after PM; 3) In 16 mice the growth of CLM was calculated following PM or sham operation.

Findings 1) In the 63 patients analyzed, tumor recurrence was 80 % after PM, vs. 26.3 % in the non-PM group (p=0.000). Logistic regression analysis showed an independent correlation between PM and tumor recurrence (p=0.000). DSS and DFS were significantly worse in the PM group; 2) Directly after PM, intra-hepatic HIF-1α was over-expressed in 8/10 patients and plasma VEGF was increased (p=0.002); 3) PM resulted in a > 2-fold increase in experimental intrahepatic tumor volume (p=0.000).

Interpretation Vascular clamping of the liver's inflow during hepatic surgery for CLM is associated with increased expression of HIF1α and VEGF, increased residual tumor growth and decreased survival, and should therefore be avoided.

Introduction

One of the most common cancers in the Western countries is colorectal cancer.[1] Unfortunately, in almost 75% of patients the cancer will eventually spread to the liver.[2] In the treatment of these metastases, surgery is becoming increasingly important. Despite recent advances in systemic therapies[3-5], partial liver resection for secondary colorectal malignancies still offers the only hope for cure[6]. A novel surgical method of local destruction of liver tumors, Radio Frequency Ablation (RFA), is increasingly performed in those patients with colorectal liver metastases that are irresectable, but confined to the liver.[7-9]

Pringle maneuver, i.e. temporary obstruction of the vascular inflow to the liver by clamping of the portal triad, is routinely applied worldwide.[10] It is predominantly used to reduce intra-operative blood loss, because blood transfusions are known to be associated with poor prognosis.[11] In case of RFA, the additive purpose of Pringle maneuver -or pharmacological reduction of blood flow[12]- is an increase in destructed tumor volume, resulting in larger lesions.[13;14]

Remarkably, although many studies have used Pringle maneuver as an experimental model for ischemia and reperfusion, none have addressed the consequences of Pringle maneuver on tumor growth and recurrence. Authors have reported transiently elevated AST/ALT levels, as well as an increase in the sticking of leucocytes[15], cytokine release[16], and nitric oxide[17]. In addition, ischemia-induced hepatic injury was successfully reduced by endothelin-converting enzyme[15], antioxidants[18], enhancement of glycolytic activity by Insulin[19], as well as L-arginine[20]. Taken together, these studies point towards a direct correlation between Pringle maneuver and intrahepatic hypoxia.

Hypoxia induces the expression of Hypoxia Inducible Factor-1alpha (HIF-1α).[21] HIF-1α is involved in cancer progression in different organs.[22-26] Under physiological circumstances, HIF-1α is rapidly degraded through ubiquitination. However, under hypoxic conditions, the protein is stabilized and transported into the nucleus where it activates a number of genes. One of the most important down stream effects of HIF-1α activation is up-regulation of Vascular Endothelial Growth Factor (VEGF) gene transcription[27], that subsequently gives rise to increased plasma VEGF protein levels. VEGF is a pivotal growth factor that induces angiogenesis[28], which stimulates tumor growth. Indeed, VEGF receptors are expressed in cancers from many organs.[29] A positive relationship between VEGF over-expression in colorectal cancer and the occurrence of liver metastases has been reported.[30;31;32]

All patients undergoing resection or RFA of hepatic metastases have cancer in an advanced stage. As a consequence, they are likely to have micro-metastases in the liver and elsewhere. We therefore hypothesized that Pringle maneuver unfavorably affects the prognosis of cancer patients, by inducing temporary hypoxia with up-regulation of HIF-1α, an increase in circulating levels of VEGF, and subsequent outgrowth of micro-metastases.

The present study was undertaken to evaluate the effect of Pringle maneuver on 1) tumor recurrence in patients following resection of liver metastases, 2) the up-regulation of HIF-1α and VEGF plasma levels in patients undergoing RFA, as well as 3) the outgrowth of experimental intrahepatic metastases.

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Materials and Methods

Effects of Pringle maneuver on tumor recurrence

Retrospectively, records were analyzed of consecutive patients that underwent potentially curative partial hepatectomy for isolated colorectal liver metastases (1991-2000), at the University Medical Center Utrecht, the Netherlands. Patient's characteristics included gender, age, the disease free interval between primary tumor and liver metastases, if the liver metastases was (were) detected at the time of resection of the primary (synchronous) or developed during follow up (metachronous), the number of metastases, and uni- or bilobular distribution. Intra-operative parameters documented were: extent of resection (major defined as three segments or more), duration of surgery, blood loss (units of transfused blood), prolonged periods (> 10 minutes) of hypotension (<95 mmHg systolic) and application of Pringle maneuver or no vascular exclusion. Postoperatively, histological examination of the resection margins was recorded. The duration of the follow-up and the time between hepatectomy and recurrence were registered, as well as the site of recurrence, where recurrence was defined as development of cancer at any site from the same colorectal primary. In addition, survival data were retrieved.

Retrospective anonymous retrieval of patient data and left over tissue material is allowed according to local ethics, and was approved by the Institutional Review Board.

Statistical analysis was used to determine whether the groups with and without Pringle maneuver were comparable for different variables using Chi-square tests, Mann-Whitney test, Fisher Exact-test, as well as independent sample T-tests. Pearson's correlation was determined to identify correlating variables. Logistic regression analysis was used to determine the independence of the different variables on recurrence and survival. For multivariate survival analysis, disease specific survival (DSS, censoring deaths unrelated to the metastases) and disease free survival (DFS) Kaplan-Meijer curves were plotted and differences between the curves were analyzed with the Mantel-Cox test. Data is presented as Mean ± SEM and considered significant when $p < 0.05$.

Effects of Pringle maneuver on HIF-1α and VEGF expression in liver tissue

To prospectively assess the effects of Pringle maneuver on intrahepatic HIF-1α and VEGF expression, a clinical study was performed that allowed sequential liver biopsies during radiofrequency ablation. Patients with liver metastases from colorectal adenocarcinoma -confined to the liver- scheduled for local tumor destruction by Radiofrequency ablation (RFA) were included, after written informed consent. Exclusion criteria were documented extra-hepatic disease, >10 liver metastases, total metastatic involvement of the liver of more than 50%, poor performance status (WHO grade >1), uncontrolled congestive heart failure or angina pectoris, significant neurological or psychiatric disorders, active infection, or major hepatic insufficiency.

RFA consisted of laparotomy, followed by exposure of the entire liver. After intraoperative ultrasound had revealed all detectable lesions, the RFA electrode was positioned under ultrasound guidance. RFA was executed according to the manufacturer's instructions (Surgical Company, Amersfoort, The Netherlands, standard roll on/roll off protocol)

A standard Pringle maneuver was applied in all patients (n=10) by clamping the hepatoduodenal ligament with an atraumatic vascular clamp for 20 minutes. A small surgical liver biopsy (~1cm3) was taken for histological analysis before clamping and 5 min after unclamping. Liver biopsies were divided in two, one part snap frozen in liquid nitrogen, and the other fixed in neutral buffered formaldehyde and paraffin embedded. Blood samples were drawn at the same time points, as well as before incision, one hour post-pringle, and one hour as well as 24 hours postoperatively.

HIF-1α immunohistochemistry We examined liver biopsies before and after Pringle maneuver for nuclear HIF-1α upregulation. Immunohistochemical staining was performed on paraffin embedded samples, as described elsewhere.[24,33] 4-μm-Slides were deparaffinized and rehydrated. Endogenous peroxidase was blocked by Methanol-0.3% H2O2. After antigen retrieval (water bath; 45 min in Target Retrieval Solution (DAKO A/S, Copenhagen, Denmark)) the primary antibody (anti-HIF-1α mouse monoclonal, BD Transduction laboratories, Mississauga, ON, Canada) was applied in a 1:500 dilution for 30 min at room temperature. The signal was amplified with the Catalyzed Signal Amplification System (DAKO) and developed with diaminobenzidine, followed by haematoxylin counterstaining. Between steps, slides were washed in Tris buffered saline, and appropriate positive and negative controls were used throughout.

The slides were scored by two different observers, blinded to the procedure. The percentage of the positive hepatocytes was estimated per specimen. Because the number of unstained endothelial and Kupffer cells is not detectable, the intensity of their staining was semi-quantitatively assessed, as negative (0) to strongly positive (+++).

HIF-1α Western blotting Western blotting was done by standard SDS-polyacrylamide gel electrophoresis. Of each frozen liver specimen 10 5μm slides were sectioned. Tissue was lysed in lysis buffer (1% NP40, 50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail) for 30 min at 4°C followed by 20 min centrifugation. The protein concentration in the supernatant was determined using the BCA protein assay (Pierce, Rockford, IL, USA). A maximum of 300 mg protein in loading buffer was loaded to an 8% SDS-PAGE gel. As a positive control a lysate of MCF7 cells incubated at 1% oxygen was used. Proteins were transferred to nitrocellulose membranes (Hybond, Amersham, Arlington Heights, Illinois, USA). Membranes were blocked in 3% nonfat milk powder dissolved in Phosphate buffered saline with Tween-20 (PBS-T) and incubated for 1 hour with a mouse monoclonal antibody against HIF-1α (BD Transduction laboratories). After washing, the membranes were incubated with

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a secondary antibody conjugated with Horseradish-peroxidase. Chemoluminescent detection of the antibody binding was performed using the super-ECL system (Amersham Biosciences, Piscataway, NJ, USA).

VEGF plasma levels To determine whether the upregulation of HIF-1α in the liver translated to a systemic increase in one of the most important down stream effects of HIF-1α activation, i.e. Vascular Endothelial Growth Factor (VEGF), we measured plasma levels at different time-points after Pringle maneuver. VEGF was measured using a commercially available ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) with duplicate ELISA readings, according to the manufacturer's protocol. The patients' individual VEGF level before Pringle maneuver was set at 100%. Relative VEGF levels for the whole group at the aforementioned different time-points were calculated (mean ± SEM).

Effects of Pringle maneuver on experimental hepatic tumor growth

The murine colon carcinoma cell line C26 was routinely cultured in DMEM supplemented with 10 % heat-inactivated FCS, 100 units/ml penicillin and 100 micrograms/ml streptomycin in a 10% CO_2 environment. Cell viability was determined by trypan blue staining. Confluent cultures were harvested by brief trypsinisation (0.05 trypsin in 0.02% EDTA), to a final concentration of $1x10^5$ cells/100 microliters PBS. Sixteen Balb/C male mice, aged 12 weeks, purchased from Harlan (Leicestershire, England), were housed under standard conditions and allowed food and water ad libitum. Colorectal liver metastases were induced in all mice as follows.[34] Mice were anaesthetized intraperitoneally with fentanyl citrate/fluanisone (0.3 mg/mouse; Janssen-Cilag, Brussels, Belgium) and midazolamchloride (12.5 mg/mouse; Roche, Brussels, Belgium). Through a left lateral flank incision $1x10^5$ C26 colorectal carcinoma cells / 100µl were injected into the spleen parenchyma. After 10 minutes, the spleen was removed to prevent intrasplenic tumor growth.

On day 5, all mice underwent surgery with intravenous injection of heparin (2U/200µl) and half of them underwent 2 x 5 minutes of Pringle maneuver. In a pilot experiment, the maximal tolerable duration of clamping was assessed. The portal vein and hepatic artery were clamped by microscopical vessels clamp. The clamp was released after 5 minutes for 1 minute, to allow recirculation of the congested portal blood from the intestine. Thereafter, the clamp was reapplied for another 5 minutes, hereby resembling the clinical practice of intermitted clamping.

On day 12, the livers were harvested, formaldehyde fixed, and embedded in paraffin. Intrahepatic tumor load was scored as the hepatic replacement area (HRA), the percentage of hepatic tissue having been taken up by metastatic tumor cells. HRA was assessed on non-sequential haematoxylin and eosin stained sections by semi-

automated stereology (Leica-Q-Prodit system, Leica Microsystems, Rijswijk, The Netherlands) using a four points grid overlaid on 100 fields per slide at a magnification of 40x.

Data is a result of two independent experiments. All experiments were performed in accordance with the guidelines of the Animal Welfare Committee of the UMC Utrecht, The Netherlands.

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Results

Effects of Pringle maneuver on tumor recurrence

Sixty-three patients underwent resection of colorectal liver metastases during the studied period. Pringle maneuver was used in 25 of the 63 procedures (36,2 %), whereas the remaining patients had no vascular occlusion. Both patient groups were comparable, except for an age difference of 5 years (p=0.017), as shown in table 1.

Table 1. Pringle maneuver and tumor recurrence

Characteristics of patients undergoing partial hepatectomy for colorectal liver metastases combined with Pringle maneuver (Pringle) and without Pringle maneuver (No Pringle). All variables were comparable (NS), except for age (5 years, p=0.017)

	Pringle (n=25)	No Pringle (n=38)	p
Female	9 (36%)	13 (34%)	
Male	16 (64%)	25 (66%)	1.000
Age	58.0 ± 1.9	63.8 ± 1.4	0.017
Disease free interval primary-liver metastases (months)	14.5 ± 5	19.7 ± 3	0.085
Metachronous	14	29	
Synchronous	11	9	0.105
Number of metastases	1.8 ± 0.2	1.8 ± 0.2	0.485
Lobes involved			
One	15 (79%)	30 (60%)	
Two	10 (21%)	8 (40%)	0.154
Number of segments	3.2 ± 0.2	3.0 ± 0.2	0.676
Resection			
Minor	6 (24%)	14 (37%)	
Major	19 (76%)	24 (63%)	0.408
Duration of surgery (minutes)	266 ± 17.5	241 ± 13.3	0.27
Units of blood	3.8 ± 0.9	2.9 ± 0.5	0.903

Peroperatively, in both groups an equal amount of patients (n=3) experienced a period of hypotension. All resected specimens had negative resection margins on histology. The median follow-up was 36 months.

Overall, metastatic recurrence developed in 30 patients. The recurrence was solely intrahepatic in 10 patients (in additional 8 in combination with other sites), solely intrapulmonary in 2 patients (in 12 in combination with other sites) and elsewhere in 11 patients (in five patients without liver or lung involvement). The percentage of patients that developed recurrence in the group that underwent Pringle maneuver, was 80.0 %, vs. 26.3 % in the group that did not undergo Pringle maneuver (p=0.000, Chi square test). Using Pearson's correlation analysis, there was no significant correlation between any of the other variables listed in table 1 and recurrence, independent of whether patients had undergone Pringle maneuver. Logistic regression analysis revealed a significant association between Pringle maneuver and recurrence, (p=0.000). All other variables per group from table 1 showed no association with recurrence.

Univariate survival analysis for Disease Specific Survival and Disease Free Survival, revealed Pringle maneuver as the only significant variable (p=0.0421, p= 0.0013 resp. log rank test). Multivariate survival analysis (correction for age, 61.0 yr.) showed a significant decrease in DSS (p=0.029) (figure 1a), as well as DFS (p=0.002) (figure 1b) after hepatectomy including Pringle maneuver compared to no Pringle maneuver.
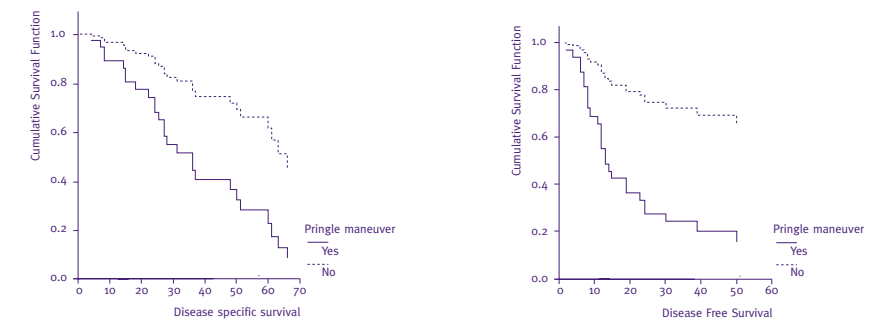

Figure 1. Survival analysis

(a) Disease specific survival (DSS) and (b) Disease free survival (DFS) curves for all 63 patients after partial liver resection for colorectal liver metastases, as a function of Pringle maneuver. Yes represents patients who underwent hepatectomy including Pringle maneuver, and No represents patients who did not. Both DSS and DFS were significantly lower after Pringle maneuver (p=0.029 and p=0.002, resp, multivariate survival analysis using Cox regression).

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Effects of Pringle maneuver on HIF-1α expression

Immunohistochemically, HIF-1α was not detectable before Pringle maneuver in the nuclei of the liver cells of any of the 10 patients (figure 2a). Following 20 minutes of Pringle maneuver and 5 minutes of reperfusion, HIF-1α was clearly over-expressed in 8/10 patients (figure 2b), as was evident from diffuse nuclear staining in hepatocytes (on average 2% of the cells), endothelial cells (++ to +++) and Kupffer cells (++ to +++), and occasionally in the bile-duct epithelium (figure 2c). Of all cells displaying up-regulation of HIF-1α, approximately 10% were hepatocytes, the rest (90%) consisted of endothelial and Kupffer cells. By Western analysis HIF-1α expression was compared between cell lysates of MCF7 cells incubated at 1% oxygen (positive control) and liver biopsies before and after Pringle maneuver. No HIF-1a could be detected in any of the samples either before or after Pringle maneuver. The positive control clearly showed the expected band at 120 kDa.
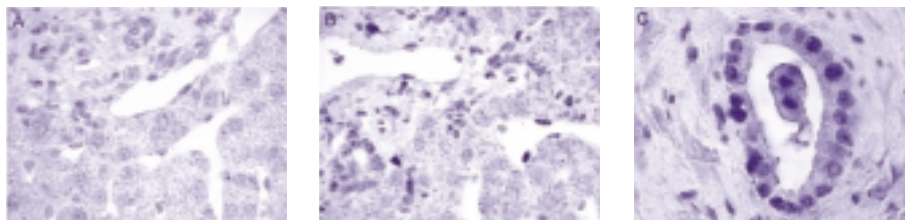

Figure 2. Effects of Pringle maneuver on intrahepatic HIF1α expression. (see color appendix)
Immunohistochemical staining for anti-HIF-1α showed up-regulation of the protein in the nucleus (in brown). (a) In none of the 10 patients, nuclear HIF-1a was detectable before Pringle maneuver. (b) Following 20 minutes of Pringle maneuver and 5 minutes of reperfusion, HIF-1α was clearly over-expressed in 8/10 patients in the nuclei of hepatocytes (on average 2% of the cells), endothelial cells (++ to +++) and Kupffer cells (++ to +++). (c) Occasional nuclear HIF-1α upregulation was found in the bileduct epithelium.

Effects of Pringle maneuver on VEGF plasma levels

The mean relative VEGF levels before incision (125.4 ± 32 %) did not differ from the levels before Pringle maneuver (p=0.5)(figure 3). VEGF levels were significantly elevated directly after Pringle maneuver -allowing 5 minutes of reperfusion- (174.1 ± 24 %, p=0.002). One hour after Pringle maneuver, the mean levels were 388.1 ± 98 % (p=0.002). One hour post-operatively, the levels were increased to a mean of 605.8 ± 310 %. After 24 hours, the mean circulating VEGF levels were 177.9 ± 34 percent of the patient's individual levels before Pringle maneuver (p=0.147).
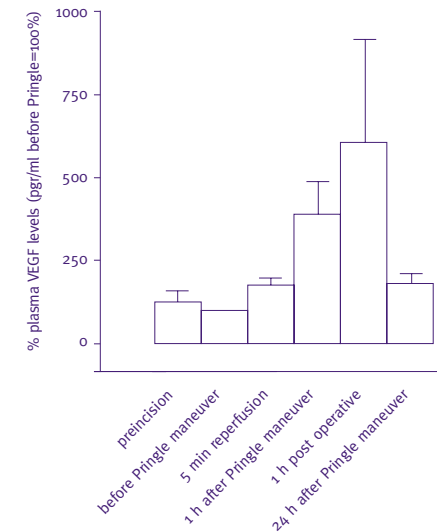

Figure 3. Effects of Pringle maneuver on VEGF expression, as measured by plasma VEGF levels using ELISA.
Each patient's individual VEGF level before Pringle maneuver was set at 100%. Relative VEGF levels for the whole group at different time-points are given (mean ± SEM) The mean relative VEGF levels before surgical trauma, i.e. prior to incision (125.4 ± 32.2 %) did not significantly differ from those before Pringle maneuver (100%) (p=0.5). The VEGF levels were significantly elevated following Pringle maneuver. Directly after Pringle maneuver – and subsequent 5 minutes of reperfusion-, the mean relative plasma VEGF levels (174,1 ± 23.6 %) were elevated in all patients (p=0.002). One hour after Pringle maneuver, the mean levels were 388.1 ± 97.5 % (p=0.002). One hour post-operatively, the levels had increased to a mean of 605.8 ± 309.6 %. After 24 hours, the mean circulating VEGF levels had nearly normalized (177.9 ± 33.7 % of the patient's individual levels before Pringle maneuver, p=0.147).

Effects of Pringle maneuver on experimental hepatic tumor growth

One mouse had died from the procedure, due to cardiopulmonary consequences of Pringle maneuver in combination with spontaneous breathing anesthesia. In the remaining mice (n=15) that underwent Pringle maneuver on day 5 after tumor injection, a significant increase in macroscopic intrahepatic tumor load was seen (figure 4). Correspondingly, the HRA was also significantly increased upon sacrifice one week after Pringle maneuver (67.9 ± 6.4%) compared to control animals that did not undergo Pringle maneuver (31.1 ± 3.5%)(p=0.000)(figure 5).

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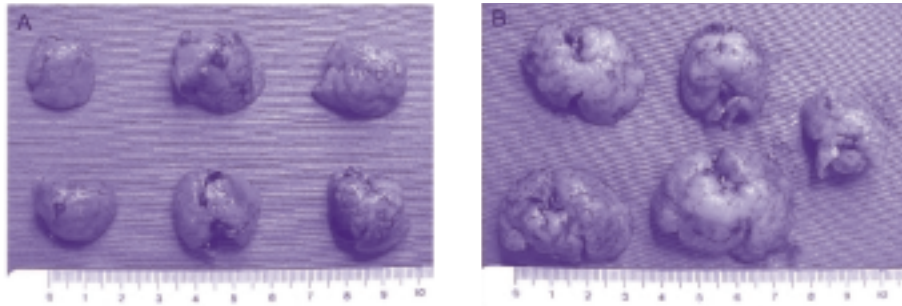
Figure 4. Effects of Pringle maneuver on macroscopic intrahepatic tumorload. (see color appendix)
Effects of Pringle maneuver on experimental hepatic tumor growth as shown by macroscopical appearance of mouse livers of one experiment. In the mice that underwent Pringle maneuver on day 5 after tumor injection, a significant increase in tumor load was observed (B) as compared to the mice that did not (A).
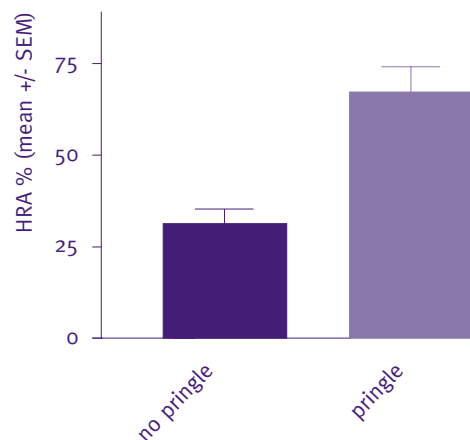

Figure 5. Effects of Pringle maneuver on experimental hepatic tumor growth.
Hepatic replacement area (HRA) of the mouse livers represents the percentage of hepatic tissue having been taken up by metastatic tumor cells. HRA was assessed on H&E stained sections by semi-automated stereology. In the mice that underwent Pringle maneuver on day 5 after tumor injection, a significant increase was observed in microscopical tumor load (67.9 ± 6.4%) as compared to control animals that did not undergo Pringle maneuver (31.1 ± 3.5%) (p=0.000). Data is a result of two independent experiments.

Discussion

Temporary occlusion of the liver's inflow, i.e. Pringle maneuver, is applied worldwide as a routine procedure during liver surgery and RFA.[10;13;14] Here we show that Pringle maneuver is associated with increased metastatic recurrence and decreased survival. Further, we provide evidence that this is likely due to "Pringle-induced" hepatic hypoxia that results in upregulation of HIF-1α in different types of liver cells. This is the only plausible explanation for the observed increase in plasma VEGF levels and outgrowth of micrometastases through induction of angiogenesis. Although we investigated only a relatively small number of patients in a retrospective study comparing two groups of patients with and without Pringle maneuver, the groups were comparable (table 1) and multivariate analysis revealed Pringle maneuver as the only significant prognostic variable, even when patients were matched (data not shown). Based on these considerations and results, we suggest that Pringle maneuver should be omitted in patients where it is not obligatory. Also, we believe that it should be included as an independent prognostic factor in the design of future clinical trials. In addition, the outcome of the retrospective study warrants a prospective study on this matter.

Although we provide the first firm evidence for the disadvantageous effects of Pringle-induced hypoxia, other studies have reported indirect clues to support this. In a retrospective study comparing various forms of vascular clamping, Buell et al found the lowest rate of recurrence in patients undergoing hepatic resection for metastatic cancer when no form of vascular clamping was applied.[35] In ventricular biopsies from patients with coronary ischemia, angiogenic factors such as HIF-1α and VEGF were shown to be increased, as a first adaptation mechanism of human myocardium to deprivation of blood.[36] However, no correlation between (surgery-induced) hypoxia, angiogenic growth factors and tumor growth has been described so far.

The mechanism behind the described sequence of events is probably HIF-1α driven. We have shown an increase in HIF-1α in the major cell types in liver biopsies taken after Pringle maneuver, as proven by immunohistochemistry. Previous studies on the expression of HIF-1α in hepatocytes provided contradictory results, as measured by either mRNA levels or immunohistochemistry.[37;38] However, since hypoxia does not increase HIF-1α mRNA levels, studying hypoxia-induced upregulation of HIF-1α on nuclear protein levels by immunohistochemistry, as opposed to mRNA levels, is more appropriate. In concordance with our findings, the sole study on immunohisto-chemical staining of nuclear HIF-1 in mouse tissue showed an increase in nuclear staining of hepatocytes after a severe hypoxic stimulus.[39] The high HIF-1α expression we found in Kupffer cells -and bile epithelium- has not been demonstrated before, although macrophages were recently shown to express HIF-1α.[40] Since macrophages are known to produce VEGF[41], our findings suggest that the Kupffer cells contribute to the described VEGF production. However, hepatic HIF-1α levels were too low to be detected by Western blotting, although technically well performed. This can be

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explained by the fact that HIF-1α nuclear upregulation was restricted to a small percentage of hepatic cells, and could be detected by enhanced immunohistochemical staining only. Nonetheless, it has been convincingly shown that even limited upregulation of HIF-1α can result in significant downstream effects[42], and this is underlined by the results of our experimental study with significant increase of hepatic tumor volume after Pringle maneuver (figures 4,5).

The elevated plasma levels of VEGF are indicative of a direct effect of HIF-1α upregulation on VEGF production[43]. Although liver endothelial and Kupffer cells showed the most outspoken HIF-1α upregulation, circulating platelets, activated as a result of the stasis induced by Pringle maneuver, may also contribute to the VEGF release[44]. Laparotomy per se was eliminated as a confounding stimulator of VEGF levels, since we found no significant difference between VEGF levels before surgery and those before Pringle maneuver (figure 3).

Studies on spatiotemporal aspects of HIF-1α and/or VEGF upregulation have not revealed for how long these factors must be upregulated before a pro-angiogenic effect is achieved. We found systemic outgrowth of experimental micro-metastases (figures 4,5), even though the plasma VEGF levels were only elevated for several hours (figure 3). Others indeed, gave support for the findings that VEGF has a very early metastatic potential.[45] Furthermore, HIF-1α was shown to prolong the half-life of VEGF in vitro, up to 3 hours, through the stabilization of VEGF mRNA.[46] Therefore, the timing of expression encountered in our studies seems to be of patho-physiological relevance.

It is tempting to speculate that other surgical interventions that hamper oxygen distribution can provide a similar physiologically relevant HIF-1α upregulation and subsequent pro-angiogenic response. For instance, partial bladder outlet obstruction increased angiogenic response in bladder tissue in rats.[47] In addition, a decrease in microcirculation is observed during pneumoperitoneum[48-50], and is evident during vascular surgery. The adverse effects of "surgery-induced" hypoxia on tumor growth, recurrence and survival, as observed in this study, might theoretically be overcome if HIF-1α is blocked completely. Studies using VEGF-antibodies or receptor-blockers or antibodies directed against HIF-1α peri-operatively are warranted.

In conclusion, vascular clamping of the liver's inflow during hepatic surgery for colorectal liver metastases is associated with increased tumor growth, metastatic recurrence and decreased survival. The mechanism behind this is probably HIF-1α driven due to its hypoxia induced up-regulation, leading to increased plasma VEGF levels, which in turn stimulate outgrowth of micro-metastases through induction of angiogenesis.

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Chapter 7

Enhanced antitumor efficacy by combining conventional chemotherapy with angiostatin or endostatin in a liver metastasis model
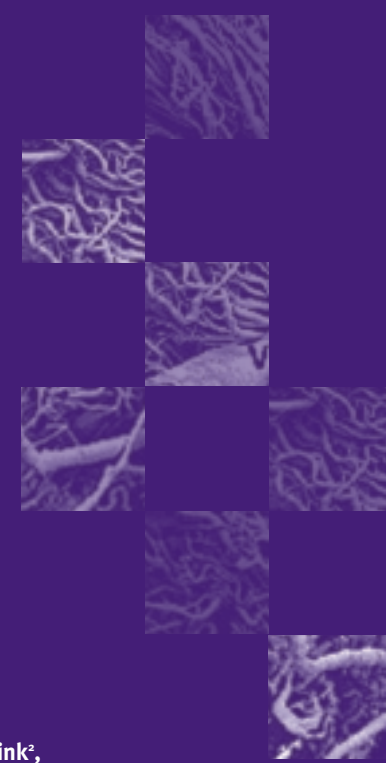
Elisabeth A. te Velde[1,2], J. Mathijs Vogten[1,2], Martijn F. G. B. Gebbink[2], Joost M. van Gorp[3], Emile E. Voest[2] and Inne H. M. Borel Rinkes[1]

Department of Surgery[1], Laboratory of Medical Oncology[2], University Medical Center Utrecht, Department of Pathology[3], Diakonessen Hospital, Utrecht, The Netherlands

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Abstract

Background: Tumor-induced microvascular networks have become attractive targets in cancer therapy. Strategies that target both tumor cells and vasculature have not been investigated in models of early metastatic colorectal disease. The efficacy of a combination of conventional chemotherapy with a potent angiogenesis inhibitor (endostatin or angiostatin) in a murine model of early colorectal liver metastasis was studied.

Methods: Sixty-six mice were subjected to intrasplenic injection of C26 tumor cells to induce colorectal liver metastases. Control animals received phosphate-buffered saline (n = 8) or citrate buffer (n = 8). Treatment included conventional chemotherapy (n = 9), endostatin (n = 8), high-dose (n = 5)or low-dose (one-tenth of optimal dose; n = 10) angiostatin, as well as the combination of either of these drugs with chemotherapy (n > 5). Clinical appearance was scored daily using a semiquantitative scale. Liver weight, macroscopic and histological tumor involvement (hepatic replacement area; HRA) were measured upon death at day 12.

Results: Treated mice displayed significantly better clinical scores than controls, except for those animals treated with low-dose angiostatin with or without chemotherapy. Treatment with conventional chemotherapy resulted in a decrease in HRA from 42 ± 3 to 29 ± 1 per cent (P < 0.001). The addition of angiostatin or endostatin to conventional chemotherapy improved antitumoral efficacy, in a multiplicative manner, resulting in a HRA of approximately 3.5 per cent (P < 0.001).

Conclusion: The addition of angiostatin or endostatin to conventional chemotherapy enhanced antitumoral efficacy in a murine model of early colorectal liver metastasis.

Introduction

Colorectal cancer is one the most prevalent malignancies in developed countries.[1] Secondary malignant hepatic disease will develop in as many as 50-80 percent of these patients[2], and is the sole site of initial recurrence in approximately 30 percent[3]. Once colorectal liver metastases have developed, the natural course of the disease is associated with a poor survival rate.[4] Partial liver resection offers the only hope of cure for these patients[5], but is, unfortunately, restricted to selected patients, many of whom will still have recurrence within the liver or develop extrahepatic metastases.

As a result of the use of adjuvant chemotherapy, survival rates of patients with colorectal carcinoma have improved. As a consequence, adjuvant chemotherapy has become standard practice in high-risk patients after operation. Studies of chemotherapy before or after liver resection for secondary malignant disease, however, have been contradictory,[6-8] although responses to schemes including oxaliplatin have recently been shown to increase the resectability rate of liver metastases[7]. These results indicate that there is room for improvement, particularly regarding perioperative systemic treatment. A search for alternative treatment modalities for colorectal liver metastases is therefore warranted.

One promising antitumor strategy involves antiangiogenic treatment. This approach aims to suppress metastatic outgrowth by inhibiting the formation of a tumor-induced vascular network. Many antiangiogenic agents are being tested in preclinical studies and clinical trials.[9] Experimental models have shown that when tumors are deprived of new blood vessels they remain in a microscopic state of dormancy.[10] As antiangiogenic drugs are directed against components of developing vasculature, they may be expected to lead to stable disease, rather than complete remission, and may have little or no effect on gross or bulky (metastatic) disease. Moreover, discontinuation of antiangiogenic therapy may allow the tumor or its metastases to resume growth.[10,12] Hence, patients most suitable for long-term antiangiogenic treatment may be those with `early metastatic' or `minimal residual' disease.

Combining conventional chemotherapy with antiangiogenic compounds might lead to improved antitumor efficacy by targeting both tumor cell and endothelial cell compartments.[13-18] Strategies combining these two targets have not, however, been investigated thoroughly in models of early widespread metastatic colorectal liver disease[19-24].

In this study, the efficacy of combinations of conventional chemotherapy and strong angiogenesis inhibitors was assessed in a murine model of early metastatic colorectal cancer. The effects of the combination therapies were compared with those of conventional and antiangiogenic agents alone.

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Materials and methods

Drugs

Angiostatin Human angiostatin was purified as described by O'Reilly et al.[25], with minor modifications[12]. Outdated human plasma was applied to a lysine-Sepharose column (Pharmacia, Uppsala, Sweden). Plasminogen was eluted with e-aminocaproic acid 0.2 mol/l and dialysed against distilled water (molecular weight cut-off: 6-8000 Spectra/Por (Spectrum Laboratories, Rancho Dominguez, California, USA); dilution greater than $4x10^7$; 4°C), buffered with Tris 7.6 20 mmol/l, followed by proteolytic digestion with plasminogen porcine pancreatic elastase (Calbiochem, San Diego, California, USA) 0.8 units/mg (shaken overnight at 37°C; 120 r.p.m.). The solution was applied to the same column and eluted with ε-aminocaproicacid 0.2 mol/l, while the flow-through was collected and treated as recently cleaved plasminogen, in order to collect angiostatin fragments with low lysine affinity. The angiostatin was dialysed and freeze-dried. The angiostatin and its flow-through were subsequently combined. The bioactivity of the angiostatin was confirmed by means of the mouse cornea neovascularization assay, as described elsewhere[12].

In all experiments the administration of angiostatin was as follows. Mice were given a dorsal osmotic pump (Alzet™ pump, type 2001; Alza, Palo Alto, California, USA)subcutaneously for continuous administration of angiostatin in a dose of 100 mg/kg daily. In previous work this dose was shown to elicit maximal antitumor effects[12]. This concentration is subsequently referred to as 'high dose'. In addition, one-tenth of this dose (10 mg/kg daily) was used to study any additional effect over conventional chemotherapy of low amounts of angiostatin.

Endostatin Human recombinant endostatin™ produced in Pichia pastoris (Entremed, Rockville, Maryland, USA) was administered by daily subcutaneous injection of 100 ml in a dose of 500 μg/day. Control animals received the solvent citrate buffer (17 mmol/l citric acid, 59 mmol/l sodium chloride, 66 mmol/l sodium phosphate, pH 6.2) 100 μl/day subcutaneously.

Chemotherapy Doxorubicin® (Pharmacia and Upjohn, Woerden, TheNetherlands), in a dose of 10 mg/kg body-weight, was administered intravenously by tail vein injection once on postoperative day 3[26]. Controls received an equal volume of PBS intravenously.

Tumor cell line

The murine colonic carcinoma cell line C26 was cultured in Dulbecco's modified Eagle's medium supplemented with 10 percent heat-inactivated fetal calf serum, penicillin 100 units/ml and streptomycin 100 μg/ml in a 10 per cent carbon dioxide environment. Cell viability was determinedby trypan blue staining. Confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02 per cent ethylenediamine tetra-aceticacid), to a final concentration of 1×10^5 cells per 100 μl PBS.

Animals

The animals studied were male Balb/c mice, aged 10 weeks, purchased from the General Animal Laboratory of the University Medical Center Utrecht. They were housed under standard conditions and allowed food and water adlibitum. All experiments were carried out according to the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht. Mice were anaesthetized intraperitoneally with fentanylcitrate fluanisone (0.3 mg per mouse; Janssen-Cilag,Brussels, Belgium) and midazolam chloride (12.5 mg per mouse; Roche, Brussels, Belgium). The spleen was exteriorized through a left lateral flank incision. Some 1×10^5 C26 colorectal carcinoma cells per 100 ml were injected into the spleen parenchyma using a 27-G needle. After 10 min, the spleen was removed to prevent intrasplenic tumor growth. The peritoneum and skin were closed in two layers with 5-0 polyglactin.

Study design

Mice were subsequently divided into the following groups: controls received PBS (n = 8) or citrate buffer (n = 8). Conventional chemotherapy alone consisted of doxorubicin (n = 9). Single-agent antiangiogenic therapy included high-dose angiostatin (n = 5), low-dose angiostatin (n = 10) or endostatin (n = 8). Combination therapies included treatment with high- or low-dose angiostatin combined with doxorubicin (n = 4 and n = 9, respectively) or endostatin combined with doxorubicin (n = 5). The treatment was initiated 6 h after tumor cell injection based on calculations for tumor cell arrest and extravasation[27]. The clinical appearance of the mice was scored daily by two independent observers blinded to the treatment, according to the scoring system provided by the animal facility (Table 1).

All mice were killed on day 12 after tumor implantation. Livers were collected for the analysis of wet liver weight and hepatic replacement areas (HRAs).

Histological analysis

Formalin-fixed livers were sectioned into 4-μm slices and embedded completely in paraffin. For each liver, four non- sequential 4-μm slides were stained with haematoxylin and eosin, and analysed. The HRA was defined as the percentage of normal hepatic tissue that had been taken up by metastatic tumor cells, and assessed morphometrically in a semi-quantitative manner (Leica-Q-Prodit, Leica Microsystems, Ryswyk, The Netherlands). A four- grid analysis was used to determine the area ratio in 100 fields per slide. According to this protocol, a 5 per cent confidence interval can be achieved.[28]

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Table 1 Clinical appearance scoring system

	Score
Appearance	
Normal	0
Fur untreated	1
As above + red spots around eyes	2
As above + abnormal posture	3
Behaviour	
Normal	0
Minor changes	1
Decreased activity	2
Immobility	3
Reactivity	
Normal	0
Increased or reduced	1
As above but more so	2
As above but even more so	3
Body-weight	
Normal	0
Approx. 10 per cent weight loss	1
Approx. 20 per cent weight loss	2
More than 20 per cent weight loss	3

Statistical analysis

For differences in clinical scores, the post hoc test of repeated measurements analysis of variance (ANOVA) was performed. The independent t test was used to compare the various treatment groups. The interaction effects between the different antiangiogenic drugs in combination with conventional chemotherapy were tested with ANOVA. A multiplicative effect was defined as an enhanced effect of antiangiogenic therapy in the presence of chemotherapy, or vice versa. Intrahepatic tumor load (HRA) was expressed as a percentage of the total liver area. Relative HRA was defined as the HRA resulting from a particular treatment modality in relation to the HRA following conventional chemotherapy alone (100 per cent). Results are presented as mean ± SEM. Differences were considered significant when $P < 0.003$ for t test calculations (Bonferroni correction for high number of tests) and when $P < 0.05$ for ANOVA.

Results

Clinical appearance

Mice treated with either drug displayed a significantly better clinical score than the (citrate) control animals, except for those treated with low-dose angiostatin or its combination with chemotherapy. The clinical appearance scores showed an increase on days 3-5 for all mice treated with chemotherapy, indicating a transient adverse effect on clinical condition on the days immediately after intravenous injection (Figure 1).
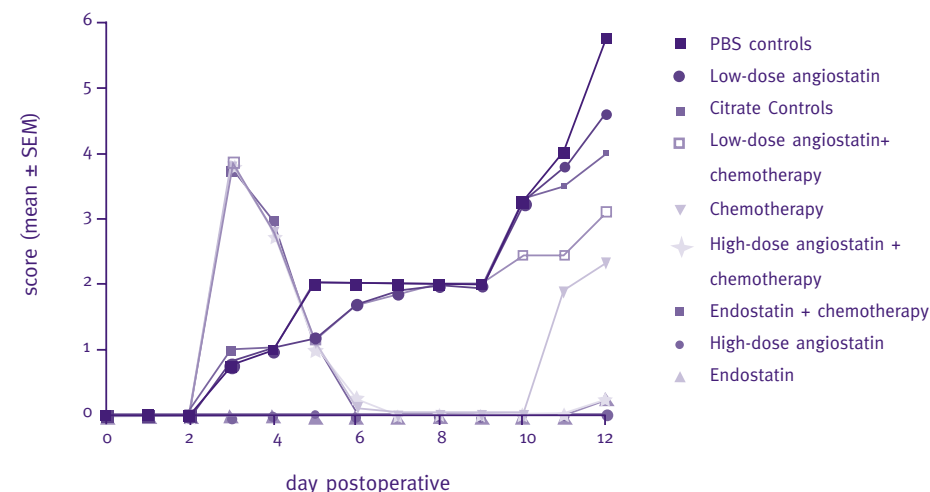

Figure 1 Course of clinical appearance
From the day of operation (day 0) until the day of death (day 12). The appearance of each mouse was scored by two independent observers in a semi-quantitative manner. The scoring system used is depicted in Table 1; note that the lower the score, the healthier the animal (a normal mouse has a cumulative score of 0). Scores are plotted per treatment group; mean values are given. PBS, phosphate-buffered saline

Macroscopic appearance and liver weight

The macroscopic appearance of tumor-bearing control livers was distinctive from that of treated livers (Figure 2). Livers of untreated animals were enlarged, showing an irregular liver surface caused by extensive tumor growth, whereas those of treated animals were smaller in size, having a macroscopically normal appearance with smoother, elastic, normal texture. The mean(±SEM) liver weight of mice treated with endostatin (1.4(0.1) g) or with endostatin in combination with chemotherapy (1.2(0.1) g) was significantly decreased compared with that in untreated tumor-bearing livers (2.5(0.2) g) (P = 0.002 and P < 0.001 respectively). Non-tumor-bearing control age-matched mice had a mean liver weight of 1.2(0.1) g (data not shown).
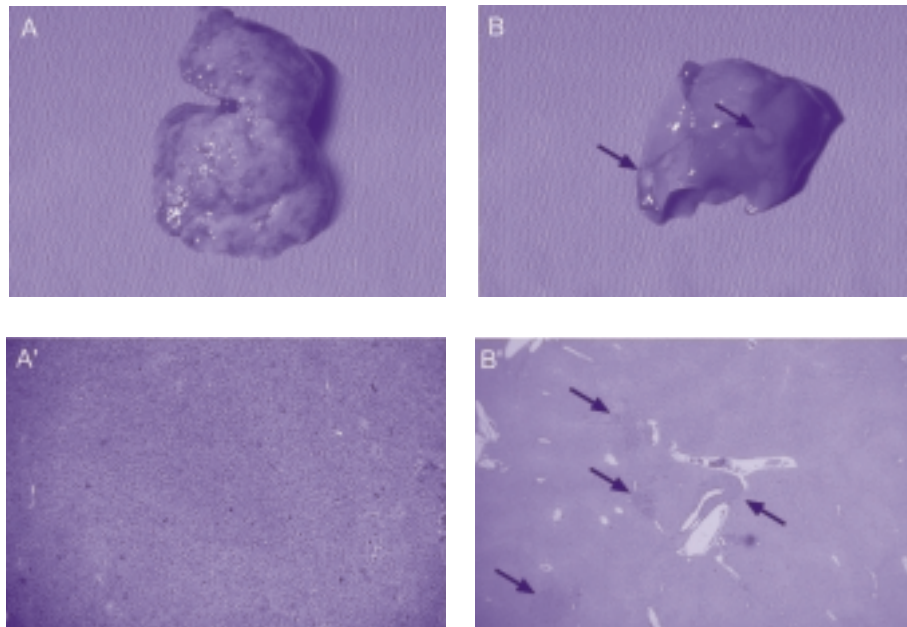
Figure 2. The macroscopic and histological appearance of resected livers. (see color appendix)
*A. The irregular white surface of the liver is due to excessive tumor growth B. This is signifi-
cantly reduced by endostatin and chemotherapy. A'. Normal liver architecture is highly disturbed
by tumor (hepatic replacement area (HRA) 53 per cent)(stained with haematoxylin and eosin).
B'. After combination treatment infiltration by tumor is significantly less (HRA 5 per cent).
Arrows indicate metastatic lesions.*

Antitumor effect

Table 2 and Figure 3 summarize the results of the histological analysis of the
HRAs in the various groups. Respective P values for comparisons between individual
treatment groups are outlined in Table 3. All single-agent drugs gave superior results
with regard to antitumor efficacy compared with no treatment (P < 0.001).

Chemotherapy monotreatment In a pilot experiment, the optimal antitumor dose
(HRA 25-30 per cent) resulted in a high clinical score (greater than 6), indicating a
toxic response to chemotherapy (data not shown). A suboptimal antitumor dose,
resulting in a significant antitumor effect but with diminished, moderate toxicity, was
determined, as judged by a clinical score below 4 (Figure 1). All experiments were
carried out with this suboptimal reduced-toxicity dose of chemotherapy. The treatment
of liver metastases with this dose of doxorubicin monotherapy (n = 5) resulted in a

Table 2. Tumor hepatic replacement area
*Per treatment group on day 12 after C26 intrasplenic tumor injection to induce liver metastases.
Values are mean ± SEM*

	Hepatic replacement area (%)		
	No chemotherapy		**Chemotherapy**
Controls	42.3(2.0)		29.1(0.9)
High-dose angiostatin	8.6 (2.6)		3.3(1.4)
Low-dose angiostatin	27.2(1.0)		8.7(0.7)
Endostatin	12.4(1.4)		3.8(1.1)
Citrate	42.1(5.9)		

decrease in mean ± SEM HRA, from 42.3(2.0) (95 per cent confidence interval (c.i.)
37.5 to 47.0) per cent in non-treated controls to 29.1(0.9) (95 per cent c.i. 27.2 to
31.1) per cent (P < 0.001) (Tables 2 and 3; Figure 3).

Angiostatin   As shown in Tables 2 and 3, and in Figure 3, high-dose treatment (n = 5)
resulted in a mean(s.e.m.) HRA of 8.6(2.6) (95 per cent c.i. 1.4 to 15.8) per cent (P <
0.001). Low-dose angiostatin (n = 10) resulted in a HRA of 27.2(1.0) (95 per cent c.i.
25.0 to 29.4) per cent (P < 0.001). The addition of high- and low-dose angiostatin to
the conventional chemotherapy improved antitumor efficacy, resulting in HRAs of
3.3(1.4) (95 per cent c.i. 1.1 to 7.6) and 8.7(0.7) (95 per cent c.i. 7.1 to 10.2) per cent
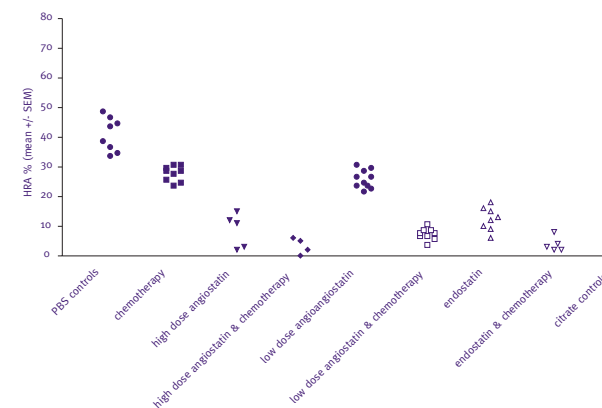respectively. High-dose angiostatin added to conventional chemotherapy was not

Figure 3. Results of tumor hepatic replacement area (HRA).
*Each treatment group on day 12 after C26 intrasplenic tumor injection to induce liver
metastases. PBS, phosphate-buffered saline*

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Table 3. Statistical analysis of hepatic replacement area per treatment group on day 12 after C26 intrasplenic tumor injection to induce liver metastases

Agents	P*
Controls versus all agents	‹ 0.001
Citrate versus endostatin	0.001
Citrate versus endostatin + chemotherapy	‹ 0.001
Chemotherapy versus high-dose angiostatin + chemotherapy	‹ 0.001
Chemotherapy versus low-dose angiostatin + chemotherapy	‹ 0.001
Chemotherapy versus endostatin + chemotherapy	‹ 0.001
High-dose angiostatin versus high-dose angiostatin + chemotherapy	0.135
Low-dose angiostatin versus low-dose angiostatin + chemotherapy	‹ 0.001
Endostatin versus endostatin + chemotherapy	0.001

Independent t test with Bonferroni correction

Figure 4. Results of the univariate analysis of variance.
Calculation of the interaction effect of low-dose angiostatin (10 mg per kg per day, continuously) in combination with conventional chemotherapy (doxorubicin). The solid line represents the effect of chemotherapy alone on the hepatic tumor load (hepatic replacement area; HRA) and the dashed line shows the effect of low-dose angiostatin in combination with chemotherapy on the HRA. The multiplicative effect of the combination was significant (P = 0.029, univariate analysis of variance).

significantly more effective than high-dose angiostatin monotherapy (P = 0.135). However, low-dose angiostatin combined with conventional chemotherapy significantly enhanced antitumor efficacy when compared with low- dose angiostatin alone (P ‹ 0.001). When compared with chemotherapy alone, the effect of addition of angiostatin to chemotherapy was multiplicative for both high-dose (P = 0.047) and low-dose (P = 0.029) angiostatin, as shown in Figure 4. The effect of the addition of low-dose angio-statin to chemotherapy on the relative HRA was also multiplicative (P ‹ 0.001).

Endostatin   Endostatin monotherapy resulted in a mean(s.e.m.) HRA of 12.4(1.4) (95 per cent c.i. 9.1 to 15.7) per cent (P ‹ 0´001) (Tables 2 and 3; Figure 3). Addition to the conventional chemotherapy regimen further reduced the hepatic tumor load to a HRA of 3.8(1.1) (95 per cent c.i. 0.7 to 6.9) per cent (P ‹ 0.001). Again, co-administration of the antiangiogenic agent and conventional chemotherapy was multiplicative with regard to the effect on the relative HRA (P = 0.017).
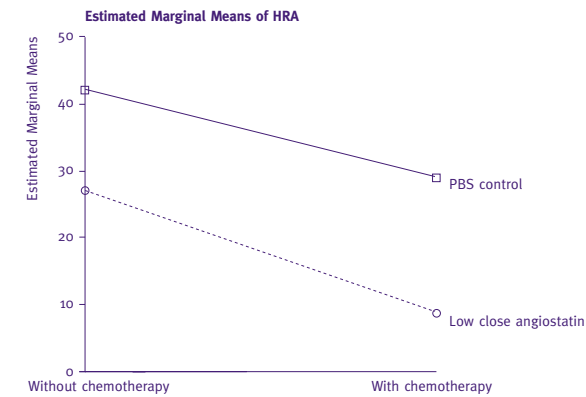
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Discussion

In this murine model of early colorectal liver metastases, the addition of angiostatin or endostatin to a conventional chemotherapeutic agent improved antitumor efficacy compared to chemotherapy alone. These results support the concept that combined cytotoxic and antiangiogenic therapy may improve results in the treatment of microscopic colorectal liver metastases. Compared with monotherapy chemotherapeutic regimens, the antitumor effect of combined chemotherapeutic and antiangiogenic agents was multiplicative (Fig. 4); the combination was more effective than the sum of effects of either monotherapies, suggesting synergy between the agents. Thus, the rationale of the combination strategy, i.e. targeting two separate cell populations (tumor cells and endothelial cells), is supported by this study. In apparent contrast to these findings, some authors have speculated that treatment targeting the vasculature (vascular endothelial growth factor) could hamper other blood-borne therapeutic agents from reaching tumor cells.[29,30] A combination of tumor necrosis factor alpha and melphalan, however, was shown to generate a higher intratumoral concentration of the cytotoxic agent than chemotherapy alone.[31] Recently, Jain[32] suggested that antiangiogenic therapy may lead to `normalization' of tumor vasculature before its destruction. The effect of chemotherapy during the normalization phase might be enhanced, rather than being inhibited at a later stage once vasculature has been destroyed. Another advantage of combination strategies might be a dose reduction of the individual drugs involved, without impairing antitumor efficacy. Based on experimental data, antiangiogenic agents are believed generally to be safe, although some caution should be exercised, because the authors' group recently showed that angiostatin, administered in a high dose immediately after primary colonic anastomosis, results in impaired healing.[33] In the present experimental study, treatment with low-dose angiostatin combined with relatively low doses of conventional chemotherapy was effective, and the mice displayed only minor toxicity throughout the treatment period. Antiangiogenic scheduling of treatment by lowering the dose with increased frequency of administration, has also proved effective.[34]

This study employed an experimental model that resembles the most suitable patient category (`early' metastatic disease). This is important because most angiogenesis inhibitors that are currently in clinical trials are being administered to patients with bulky end-stage tumors. This is also true for the combination strategies currently under study.[35] Only a few trials are evaluating the efficacy of the combination of antiangiogenic and chemotherapeutic agents in an adjuvant setting.[15,35] Based on the action of ant angiogenic therapy, i.e. preventing outgrowth of microscopic tumor deposits, few or no antitumor effects may be anticipated in bulky disease, and the selection of suitable patient categories is required before efficacy can be established. Although doxorubicin is not used conventionally in the treatment of human colorectal cancer, there is a large body of literature confirming the biological relevance of this murine model for colorectal metastatic disease[26,36]. The results of the present study on experimental early colorectal liver metastases indicate that antiangiogenic therapy may be highly attractive for perioperative treatment schedules. Future clinical studies evaluating the effectiveness of combination strategies should be undertaken in a perioperative setting so that assessement can be made of antiangiogenic agents in multidrug therapy of patients with `early' metastatic or `minimal residual' disease.

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Chapter 8

Early Endostatin Treatment Inhibits Metastatic Seeding of Murine Colorectal Cancer cells in the liver and their Adhesion to Endothelial cells
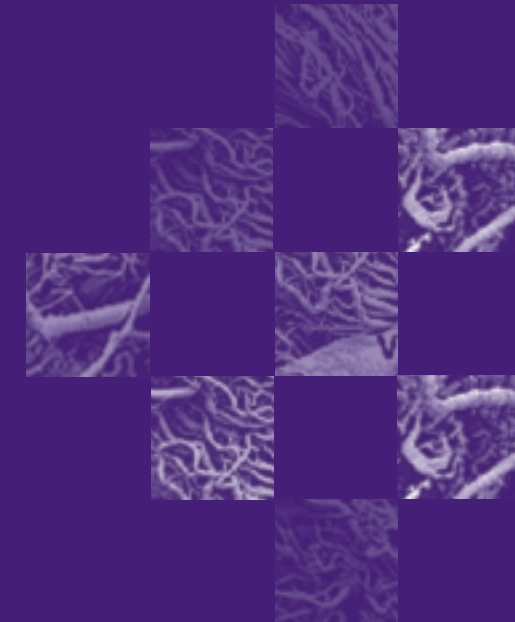
Elisabeth A. te Velde [1,2]**, Arie Reijerkerk** [2] **, Dieta Brandsma** [2,3]**, J. Mathijs Vogten** [1,2]**, Yaping Wu** [4]**, Onno Kranenburg** [2]**, Emile E. Voest** [2]**, Martijn Gebbink** [2]**, Inne H.M. Borel Rinkes** [1]

Department of Surgery [1], Department of Medical Oncology [2], Department of Neurology [3], Department of Hematology [4], University Medical Center Utrecht, Utrecht, The Netherlands

Submitted

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Abstract

Endostatin (rh-E), a carboxyterminal fragment of collagen XVIII potently inhibits angiogenesis and tumor growth, presumably through induction of apoptosis in endothelial cells and/or inhibition of their migration. Here we show that the anti-tumor activity of endostatin in a liver metastasis model of colorectal cancer cells depends on the time of its administration.

First, 500 µg/day rh-E was administered on different time-points to mice before and/or after intrasplenic injection of C26 coloncarcinoma cells metastasizing to the liver. Hepatic tumor volume replacement (HRA) was assessed on day 12 (n=24). Quantitative analysis (t=15 and t=60 min) of intrahepatic fluorescent C26 cells by use of in vivo microscopy (IVM) (n=20), as well as [125I]iododeoxyuridine-labeled C26 cells (n=40) was performed, following rh-E treatment of the mice 2 hours before tumor cell injection. In vitro, the effects of 100/200/400 µg/ml rh-E on C26 cell death and migration, and effects of 2-hour pre-incubation with rh-E on the adhesion of C26 tumor cells to TNF-alpha stimulated HUVEC's under flow conditions (n=15) were assessed.

rh-E was most effective when administered early, even if rh-E was only continued until day 4 (HRA on day 12 was 15 ± 3.5 %, vs. 60 ± 2.9. in the controls, p=0.001). Rh-E did not inhibit tumor growth when administered later than 4 days after tumor injection. IVM and radioactive tumor cell labeling experiments revealed that the effect of endostatin-pretreatment was mainly due to inhibition of tumor cell arrest in the liver (IVM: 3.2 ± 0.5 cells / high power field vs. 7.3 ± 0.6 / high power field in the citrate-controls, p<0.001; [125I]iododeoxyuridine-labeled C26 cells 34.4 ± 5.6 % vs. 57.44 ± 2.5 % in controls, p=0.001). These results are supported by in vitro perfusion experiments showing that endostatin induced a two fold decrease of tumor cell adhesion to endothelial cells under flow conditions (103 ± 18 vs. 180 ± 15 adhered cells/mm², p=0.007).

Our results show that endostatin, in addition to its effects on the developing tumor vasculature, also affects tumor cell adhesion in the very early phases of metastasis formation. Possible mechanisms include inhibition of integrin dependent adhesion of tumor cells to endothelial cells, in addition to inhibition of angiogenesis. These data can be helpful in the design of trials to augment surgical treatment of colorectal carcinoma and liver metastases.

Introduction

Anti-angiogenic drugs are directed against components of the developing vasculature. Based on the working mechanism of these drugs, i.e. preventing outgrowth of microscopic tumor deposits, little or no anti-tumor effects might be anticipated in gross or bulky (metastatic) disease. In the case of established (unresectable) tumors, treatment with anti-angiogenic agents will -at best- lead to disease stabilization, rather than to complete tumor remission. Furthermore, discontinuation of therapy will allow the tumor or its metastases to resume their outgrowth.[1] Taken together, initiation of the treatment already during the initial phase of tumor growth can be essential in optimizing anti-angiogenic therapy.

Endostatin, a naturally occuring fragment of collagen XVIII, is one of the most effective inhibitors of angiogenesis and dramatically reduced tumor growth in several mouse models with no serious side effects observed.[1-3] However, endostatin was ineffective in other studies, and these data have prompted the discussion about the efficacy of endostatin[4]. In a previous report by our group, endostatin resulted in significant anti-tumor effects in a model of murine colorectal liver.[5] These effects, as measured 12 days following tumor cell injection by histology, were achieved with administration of endostatin from day 0 until day 12. Others found that the efficacy of endostatin improved when administered before tumor cell injection, as measured by tumor load 21 days following tumor cell injection.[6] In a pilot study, we have observed different biological activities of endostatin that depended on the timing of its administration. We were not able to show regression of already established colorectal liver metastases when endostatin treatment was initiated 7 days after tumor cell injection.

The ability to optimize the timing of endostatin administration will largely depend on its working mechanism. So far, endostatin is known to have anti-angiogenic properties, i.e. its anti-tumor effects depend on inhibition of developing tumor vasculature. This action has predominantly been ascribed to inhibition of apoptosis and –migration of endothelial cells.[7,8] Many studies point towards an effect of endostatin on the adhesion of endothelial cells to other endothelial cells[9]: These inhibitory effects may be mediated by direct blocking of specific integrins[10,11], by disassembly of focal adhesions and actin stress fibers[12], and/or by antagonizing the Wnt pathway[13]. Based on these studies we hypothesized that (anti-adhesive) anti-tumor properties of endostatin could play a role even shortly after administration, before new vessel formation is needed for progression of malignancy.

In this study, we have investigated the influence of endostatin on the early spatio-temporal fate of murine colon carcinoma cells metastasizing to the liver. We show that 2-hr pretreatment in vivo with rh-E resulted in reduced intrahepatic tumor growth, mainly due to early (<15 minutes) inhibition of tumor cell seeding in the liver; and induced a two fold decrease of tumor cell adhesion to endothelial cells under flow conditions.

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Materials and Methods

Endostatin. Human recombinant endostatin was produced in Pichia pastoris (Courtesy of Entremed, Rockville, USA). In mice, 500 μg was administered by daily subcutaneous (SC) injection of 100 μl. Control animals received the equal amount of solvent citrate buffer (17 mM citric acid, 59 mM NaCl, 66 mM Na2PO4, pH 6.2). For the *in vitro* experiments, three equivalent dosages of endostatin and its control were used, i.e. 400 μg/ml, 200 μg/ml and 100 μg/ml.

Tumor Cell Culture. The murine colon carcinoma cell line C26 was routinely cultured in dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin and 100 microgram/ml streptomycin in a 10% CO_2 environment. Cell viability was determined by trypan blue staining. Confluent cultures were harvested by brief trypsinization (0.05 trypsin in 0.02% EDTA), and resuspended in Phosphate Buffered Saline (PBS) to a final concentration of 10^6 x 1.0 cells/ml (unless stated otherwise).

Endothelial cell Culture. Human vascular endothelial cells (HUVEC) were isolated from human umbilical veins. Cells were cultured in endothelial basal medium (EBM) containing EGM-2 medium (Clonetics, Bio Whitaker) supplemented with 5% FBS, gentamicin, amphothericin B, hydrocortisone, ascorbic acid, and the following growth factors: VEGF, bFGF, hEGF, and IGF-1. Cells of passage two were seeded on fibronectin coated glass coverslips and grown until confluency.

In vivo Liver Metastases Model. Balb/C male mice, aged 10 weeks, purchased from Harlan (Leicestershire, Great Britain), were housed under standard conditions and allowed food and water ad libitum. Colorectal liver metastases were induced in all mice as follows[5]. Mice were anaesthetized intraperitoneally with fentanyl citrate/fluaisone (0.3 mg/mouse; Janssen-Cilag, Brussels, Belgium) and midazolamchloride (12.5 mg/mouse; Roche, Brussels, Belgium). Through a left lateral flank incision C26 colorectal carcinoma cells were injected into the spleen parenchyma. In case mice were allowed to survive 24 hours, the spleen was removed after 10 minutes to avoid intrasplenic tumor growth. All experiments were performed in accordance with the guidelines of the Animal Welfare Committee of the UMC Utrecht, The Netherlands.

Histological Analysis. To determine the effects on tumor load of different schemes of endostatin administration, mice (n=24) were randomly assigned to the following experimental groups: endostatin treatment starting 2 hours before tumor cell injection, 8 hours after tumor cell injection, 4 and 7 days after tumor cell injection. The treatment was discontinued on day 4, day 12. Lower concentrations of tumor cells were used for treatment groups until day 19 (10^5 x 1.0 /ml). For histological analysis of tumor burden, the mice were sacrificed on day 7, day 12 or day 19 (n=3 per

group). The livers were harvested, formaldehyde fixed, and embedded in paraffin. Intrahepatic tumor load was scored as the hepatic replacement area (HRA), the percentage of hepatic tissue having been taken up by metastatic tumor cells. HRA was assessed on non-sequential haematoxylin and Eosin stained sections by semi-automated stereology (Leica-Q-Prodit system, Leica Microsystems, Rijswijk, The Netherlands) using a four points grid overlaid on 100 fields per slide at a magnification of 40x.

Intrahepatic Detection of Radiolabeled Tumor Cells. To determine the spatiotemporal fate of tumor cells metastasizing to the liver we used radiolabeled tumor cells. To prepare labeled cells for intrasplenic injection, C26 tumor cells were co-cultured with 1 mCi/ml [125I] iododeoxyuridine (Amersham Biosciences, Weert, the Netherlands) in DMEM containing 5% FCS for 72 hours at 37°C in 10% CO2 environment. The cells were washed in PBS, detached with trypsin-EDTA, washed with serum-containing media and twice with PBS and resuspended to a final concentration of 10^6 x 5.3 cells/ml in PBS.

Intrasplenic injection of [125I] iododeoxyuridine-labeled tumor cells was followed by hepatectomy at t=15 minutes and t=1 hour, respectively (n=5 per group per time-point per experiment). The resected livers were rinsed in 70% ethanol. Radioisotope levels were measured by a gamma counter and expressed as percentage of the input dose (Cpma). The input dose was determined by measuring two 100-ml aliquots of PBS containing [125I] iododeoxyuridine-labeled tumor cells in parallel with the liver samples (100 %).

In Vivo Microscopy. To examine the fate of tumor cells in the liver after intrasplenic injection by in vivo microscopy, C26 cells were labeled with a fluorescent probe, carboxyfluorescein succinyl ester (CFSE, Molecular Probes, Leiden, the Netherlands). Tumor cells were incubated for 15 minutes at 37ºC with 20 mL of 4% CFSE in phosphate buffered saline containing 0.1% bovine serum albumin (BSA). Cells were centrifuged, resuspended in DMEM and incubated for 30 minutes at 37ºC. Cells were then centrifuged and resuspended for intrasplenic injection. A fraction of tumor cells was cultured in parallel to confirm fluorescence in 99% of the cells.

At 15 minutes and 1 hour after intrasplenic injection of C26 tumor cells intravital fluorescence microscopy was performed using a Nikon TE-300 inverted microscope (Uvikon, The Netherlands) equipped with a filter set for fluorescein (excitation 450-490 nm, emission ›515 nm.). Images were registered with a charge coupled device camera (Exwave HAD, Sony, the Netherlands) and recorded with a s-VHS VCR (Panasonic). Using a high magnification 40x lens, 10-15 randomly selected fields were chosen in each animal (n=20 mice); images were recorded for one minute and were analyzed off-line. At indicated time points, the extent of metastasis was measured as number of tumor cells per high power field (hpf).

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Tumor Cell Migration Assay. The C26 tumor cells were grown to 75-90% confluency. After washing, the cells were trypsinized, pelleted and resuspended in DMEM / 0.1% BSA. The cells were labeled in situ with 10 μM Calcein-AM in DMEM for 15 minutes at 37°C and subsequently added to 8 μm FALCON® HTS FluoroBlok™ Cell Culture Insert (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), at a density of 100.000 cells/insert. DMEM supplemented with 10% FCS was used as a chemoattractant in the lower wells, while DMEM/0.1% BSA was added to the control wells. Different concentrations of endostatin (100mg/ml and 400mg/ml) or equal concentrations of control buffer were added. The inserts were incubated for 15 minutes, 1 hour, 4 hours and 24 hours at 37°C. The number of migrated cells was measured using a PE Biosystems CytoFluor 4000 plate reader at a gain setting of 54.

Tumor Cell Death Assay. Tumor cells were exposed to different concentrations of endostatin (400 μg/ml, 200 μg/ml and 100 μg/ml) in culture medium for 24 hours. The total pool of adherent and detached cells was obtained by collecting the detached cells. The remaining adherent cells were trypsinized and added to the detached cells in the medium. One half of the cells was stained with 0.02% Trypan blue and the percentage dead (Trypan blue-positive) cells was assessed using a Bürker glass counter chamber. Duplicate samples were analyzed. In addition, the other half of the cells was fixed in the culture medium using 3.7% formaldehyde and analyzed.

Tumor Cell Adhesion Assay under Flow Conditions. The effects of endostatin on the in vitro adhesion of C26 tumor cells to stimulated HUVEC's were assessed under flow conditions. The HUVEC's were stimulated with TNF-alpha (PeproTech, London, England) at a concentration of 10 Ng/ml for three hours before tumor cell perfusion. Two hours before perfusion, the stimulated HUVEC's were incubated with endostatin in a concentration of 400 μg/ml, an equal concentration of BSA or citrate buffer only.

First, the adhesion of C26 colon carcinoma cells (10^6 x 4.0 cells/ml, 1% FCS, 25mM Hepes buffer) to endostatin (n=7) versus citrate buffer (n=8) treated TNF-alpha stimulated HUVEC's was investigated using a modified form of a transparent perfusion chamber (14). Data from two independent experiments was pooled. The microchamber has a slit height of 0.2 mm and width of 2 mm and contains a plug on which a coverslip (18 mm x 18 mm) with confluent HUVEC was mounted. C26 colon carcinoma cells were aspirated from a reservoir through the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, MA). In this way, the flow rate through the chamber could be controlled precisely. The wall shear stress (t) was 50mPa. During the perfusion, the flow chamber was mounted on a microscope stage (DM RXE, Leica, Wetzlar, Germany), equipped with a B/W CCD-video camera (Sanyo, Osaka, Japan) connected to a VHS video recorder. Perfusion experiments were recorded real-time on videotape. Video images were analyzed off-line for the number of adherent

C26 colon carcinoma cells with a Quantimet 570C image-analysis system (Leica Cambridge, Cambridge, UK). The number of surface-adherent C26 cells per mm^2 was measured after 5 minutes of perfusion at a minimum of 25 fields (total surface 1.0 mm^2) using custom made software developed in Optimas 6.1 (Media Cybernetics Systems, Silverspring, MD, USA).

Second, adhesion of C26 colon carcinoma cells to HUVEC was determined in whole blood.[15] The HUVEC's were seeded on glass coverslips sized 60x24 mm and grown to confluency. They were perfused in a small parallel-plate perfusion chamber with a slit height of 0.1 mm and a slit width of 2 mm corresponding with flow rates of 15 μL/min (shear rate 10s). Fresh blood from healthy donors was anticoagulated with 1/10 volume of 150 U/mL Orgaran (a low molecular weight heparinoid [LMWH]; Organon, Oss, the Netherlands). Blood was prewarmed at 37°C for 10 minutes and was then drawn through the perfusion chamber by a Harvard infusion pump (pump 22, model 2400-004;Natick, MA). Tumor cells (10^6 x 6.0 /ml) were incubated with 200 μg/ml dihydroethidium (3,8-Diamino-5,6-dihydro-5-ethyl-6-phenylphenanthridine 2,7-Diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine Hydroethidine, Sigma-Aldrich, Inc). Aliquots of 15 ml tumor cells in 150 ml blood were used per perfusion. Real-time images were recorded using a VCR system and were analyzed off-line and the number of adhered tumor cells per mm^2 confluent HUVEC layer was calculated. After perfusion, full blood perfused coverslips were fixed in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Staining of cytoskeletal actin was done by Phalloidin-TRITC. Hoechst 33342 was used for blue nuclear staining, and anti-fibrinogen was stained green (primary antibody Rabbit anti-human anti-Fibrinogen). The coverslips were mounted by fluorescence medium, FluorSaveTM (Calbiochem 345789, La Jolla, CA, USA). The stained cover slips were analyzed using Multi-photon laser scanning microscopy.

Statistical analysis. For comparison of the endostatin treatment and the controls, the independent sample *t* test was used. Data was given as mean ± SEM and was considered significant when p <0.05.

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Results

Histological Analysis of Liver Metastases. Intra-hepatic arrest and outgrowth of circulating tumor cells is a multi-step process. Mice were injected with C26 tumor cells in the spleen to induce liver metastases and treated with endostatin at different time points. After 12 days, livers were removed and analyzed for the extent of replacement of liver tissue by tumor cells (HRA). On day 12, the HRA of the controls (buffer treated) was 60 ± 2.9 (figure 1A). The HRA in mice that were treated with endostatin from 2 hours prior to tumor cell injection until sacrifice was the lowest: 19 ± 2.1 % (p<0.001). When treatment was started 2 hours before tumor cell injection and replaced by citrate buffer from day 4 until day 12, the HRA was still significantly decreased (15 ± 3.5 %, p=0.001). When treatment was started 8 hours after tumor cell injection and continued until day 12, there was a significant anti-tumor effect as well (HRA 32 ± 3.4 %, p=0.002). However, initiation of the treatment on day 4 or later after tumor cell injection did not result in anti-tumor efficacy as reflected by HRA (36.7 ± 10.1 %, p=0.139). Treatment from day 7 until day 19 did not reduce tumor load and resulted in a HRA of 38.3 ± 7.3 % vs. 41 ± 10 % in the citrate buffer treated controls (p=0.8) (figure 1A and 1B.).
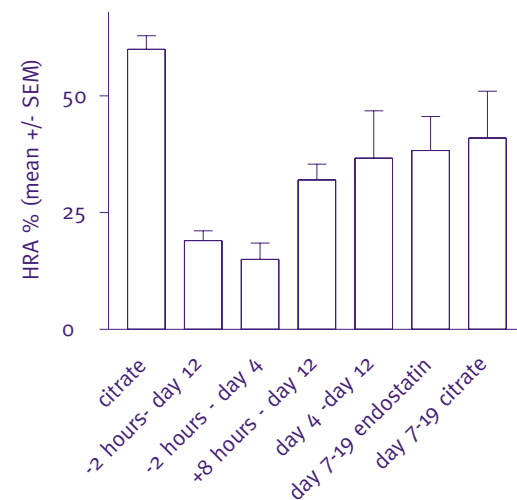
Figure 1A. *Hepatic replacement area (HRA) for all different treatment groups. To determine the anti-tumor effects of different schemes of endostatin administration we examined the intra-hepatic tumor load (i.e. HRA) after tumor cell injection in the spleen. Endostatin was most effective when administered early, even if only continued until day 4 (as measured by hepatic tumor volume replacement on day 12, 15 ± 3.5 %, vs. 60 ± 2.9. in the citrate buffer treated controls, p=0.001). Endostatin did not inhibit tumor growth when administered later than 4 days after tumor injection. Data is plotted as mean ± SEM.*
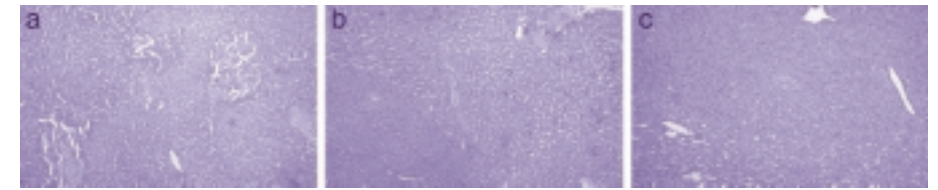

Figure 1B. (see color appendix)

Effects of endostatin on already established tumors in the liver, measured by histological hepatic replacement area (HRA) (H&E staining). Fig. 1B-a shows three small intrahepatic tumor lesions (dark nodules) 7 days after intrasplenic injection of tumor cells. Fig. 1B-b. Tumor-bearing livers 19 days after tumor cell injection. Endostatin was administered daily from day 7-day 19. The tumors continued to grow under endostatin treatment. Fig 1B-c. No significant anti-tumor effect was achieved as compared with the citrate buffer treated control (HRA 38.3 ± 7.3 % vs. 41 ± 10 %, p=0.8).

Intrahepatic Detection of Radiolabeled Tumor Cells. The above results suggest that endostatin acts at a very early stage of metastasis formation. To investigate the initial arrest of circulating C26 tumor cells in the liver of endostatin-pretreated mice, we measured radiolabeled tumor cells after injection into the spleen. Livers were collected at 15 and 60 minutes after tumor cell injection and the radioactivity was subsequently determined. Data from two independent experiments was pooled. Fifty percent of the intrasplenic injected tumor cells was arrested in the liver during the first 15 minutes, as measured by radioactivity. Endostatin pre-treatment reduced the percentage of injected dose in the liver to 34.4 ± 5.6 % at 15 minutes after tumor cell injection vs. 57.4 ± 2.5 % in the controls (figure 2, p=0.001).). When radiolabeled cells in the liver were measured after 1 hour, these percentages had not significantly changed (54.8 ± 3.2 % vs. 37.6 ± 5.2 %).

In Vivo Microscopy. Initial tumor cell arrest in the liver was studied in more detail by in vivo microscopy. C26 tumor cells were fluorescently labeled with calcein and injected into the spleens of recipient mice. Tumor cells reaching the liver were easily discernable from the hepatic tissue (figure 3). Two independent experiments were pooled (n=20). Fifteen minutes after injection of tumor cells into the spleen, the control mice showed a mean of 7.3 ± 0.6 fluorescent tumor cells per high power field (figure 3A) whereas in the mice that were treated with endostatin 2 hours prior to injection less arrested intrahepatic cells (3.2 ± 0.5 cells / hpf) were counted (p<0.001)(figure 3B.). When images were made one hour after tumor cell injection, this reduction was unchanged (endostatin treated livers 2.7 ± 0.3 arrested cells / hpf vs. controls 7.4 ± 0.7 cells /hpf, p<0.001). No differences were observed within either
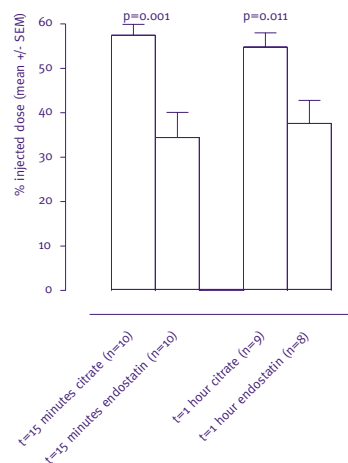
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Figure 2.

Radioactive labeled tumor cells in the liver, *15 minutes and 1 hour after intrasplenic injection, as measured by gamma counter and represented as percentage of injected (^{125}I) iododeoxyuridine-labeled tumor cells. Fifteen minutes after tumor cell injection in the spleen, the percentage of injected dose in the liver was 34.4 ± 5.6 % in the mice that were treated with endostatin 2 hours prior to tumor cell injection, vs. 57.4 ± 2.5 % in the controls (p=0.001). After 1 hour, these percentages had not significantly changed.*
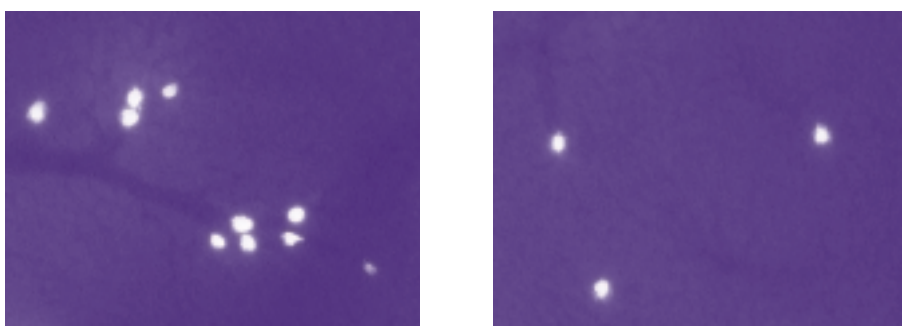
Figure 3A. (see color appendix)

Intravital microscopy images recorded 15 minutes after injection of fluorescent tumor cells into the spleen of (a) Control liver and (b) liver after 2 hour endostatin pre-treatment. The control mice showed a mean of 7.3 ± 0.6 fluorescent tumor cells per high power field whereas mice treated with endostatin 2 hours prior to injection showed a lower number of arrested fluorescent cells in the liver (3.2 ± 0.5 cells / per high power field, p<0.001).
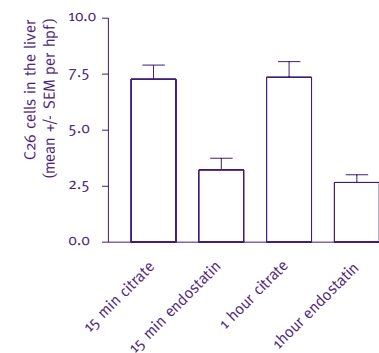
Figure 3B.

Number of fluorescent tumor cells that are present in the liver per high power field as measured by intravital microscopy 15 minutes and 1 hour after intrasplenic injection. No differences were observed within either treatment group between the two time-points (controls 15 minutes vs. 1 hour p=0.93; endostatin 15 minutes vs. 1 hour p=0.39).

treatment group between the two time-points 15 minutes and 1 hour after tumor cell injection (controls 15 minutes vs. 1 hour p=0.93; endostatin 15 minutes vs. 1 hour p=0.39).

Tumor Cell Assay. Significant Calcein-AM labeled C26 chemotaxis was not detected until 2 hours of incubation. The migration of endostatin-treated as compared to the control tumor cells was similar at all time-points measured. At the time of maximal migration -at 4 hours- the lowest concentration endostatin resulted in a fluorescent signal of the migrated cells of 6165 ± 926 vs. 5620 ± 765 in the controls (p=0.6). The highest concentration of endostatin did not influence the migration of tumor cells either (5562 ± 1018 vs. 5268 ± 623 in the controls, p=0.8). There was no direct effect of endostatin on cell death of C26 murine colon carcinoma cells (data not shown).

Tumor Cell Adhesion Assay under Flow Conditions. Since endostatin treatment decreased early intrahepatic tumor cell arrest and did not directly influence tumor cell death or migration, we investigated tumor cell- endothelial cell interactions. Fluorescent-labeled tumor cells were perfused over a confluent layer of endothelial cells. Two hours pre-treatment with endostatin of stimulated HUVEC led to a more

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than two-fold decrease of tumor cell adhesion under flow conditions (103 ± 18 adhered cells/mm²) as compared to pre-treatment with control buffer (180 ± 15 adhered cells/mm², p=0.007). Shear forces did not induce rolling of the C26 cells, but rather a direct tethering, followed by firm tumor cell adhesion under both control and endostatin conditions. BSA did not inhibit adhesion of tumor cells to HUVEC (data not shown). In concordance, after perfusion with full blood, the adhesion of C26 cells to HUVEC was inhibited with endostatin pre-treatment (143 ± 20 adhered cells/mm²) vs. controls (239 ± 41 adhered cells/mm²)(p=0.0231). Multi Photon Laser Scanning Microscopy visualized the adhesion of tumor cells to endothelial cells (figure 4).
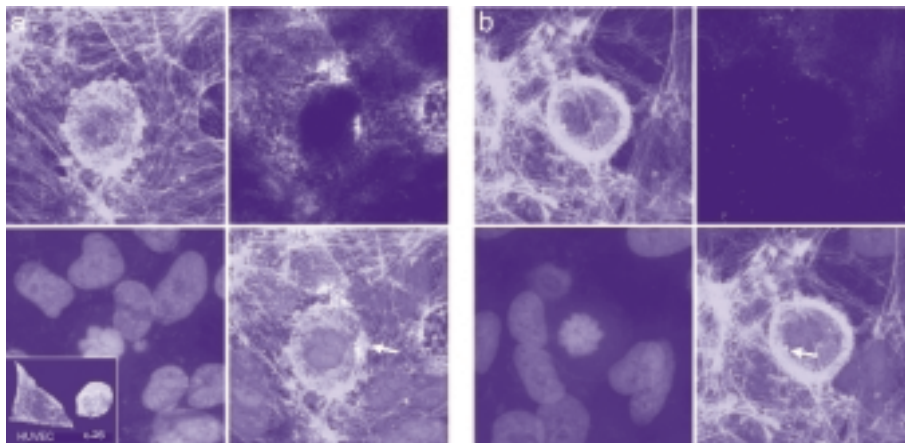
Figure 4. (see color appendix)
Immunohistochemistry and multi-photon laser scanning microscopy visualized tumor cell adhesion to HUVEC. *After perfusion with tumor cells in full blood, the coverslips were fixed and stained. The number of adhered tumor cells to cover slips of stimulated HUVEC after two hours pre-treatment with endostatin (b) was less than to those pre-treated with citrate buffer control (a). Platelets are indicated by arrows, the green colors represent anti-fibrinogen.*

Discussion

Several mechanisms have been proposed that might explain the anti-angiogenic activity of endostatin.[7-13] However, it is unclear whether and to what extend these activities contribute to early anti-angiogenic- and anti-metastatic effects. Undisturbed early tumor cell metastasis is a multi-step process that requires both intravascular arrest of tumor cells, as well as their adhesion to endothelial cells, followed by trans-endothelial migration. All of these aforementioned steps -in theory- can be potential targets by which early metastasis is inhibited.

In this study we show that early treatment with endostatin enhanced anti-tumor efficacy, in conjunction with an inhibition of the number of arrested tumor cells in the liver, as soon as within 15 minutes after intra-splenic tumor cell injection. In vitro, endostatin did neither affect the migration nor cause cell death of the tumor cells. There was, however, a strong inhibitory effect of endostatin pre-treatment on the adhesion of tumor cells to endothelial cells under flow conditions. This data cannot be explained by classical "anti-angiogenesis". Therefore, our studies provide evidence for a novel working mechanism of endostatin, in addition to direct effects on angiogenesis and endothelial cells.

In vitro experiments mainly focused on endostatin and endothelial cell-cell adhesion revealed an integrin dependent interaction.[10,11] Possibly our findings demonstrating that endostatin inhibits adhesion of tumor cells to endothelial cells and diminishes intra-hepatic tumor cell arrest are integrin mediated. This explanation is supported by the findings of Rehn et al[10], who demonstrated that endostatin interacts with $a_5\beta_1$, $a_v\beta_3$, and $a_v\beta_5$ integrins on the surface of HUVEC. Others found that endostatin activity is mediated by an integrin dependent inhibition of adhesion of endothelial cells to collagen I.[11] Interestingly, in a study on the mapping of endostatin binding sites in intact human tissues, endostatin was predominantly found to bind to blood vessels.[16] Inhibitory effects of tumor cell adhesion to HUVEC by endostatin are not likely to be explained by a direct effect of endostatin on TNF-alpha up-regulation, since this pathway was observed not to be influenced by endostatin[17].

Furthermore, the ability of endostatin to bind to the proteoglycan heparin[2] points to a possible involvement of heparin sulfate proteoglycans (HSPG's) in endostatin activity. HSPG's are expressed on the cell surface or present in the extracellular matrix (ECM) and mediate the adhesion of cells to other cells or ECM.[18,19] Indeed, heparin and heparan sulphate can inhibit experimental tumor metastasis (reviewed in[20]).

On the other hand, one can not rule out that alternative steps to adhesion could be of importance in early metastasis. Intra-vascular tumor cell arrest, through direct interactions of tumor cells with components of the blood, can mediate dissemination. Tumor cell arrest can be affected by fibrin deposition[21], as well as platelets-tumor cell interaction[22]. We could speculate that the binding of platelets to the tumor cells is inhibited, possibly mediated by integrins as well. The relative contribution of fibrinogen-, platelet- and endothelial cell-mediated interactions with tumor cells will have

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to be elucidated, in in vivo experiments using mice that lack components of the fybri-nolytic system.

As in a previous study, we found that endostatin is effective against colorectal liver metastases.[5] Solaun et al. showed that the antiangiogenic mechanism (i.e. endothelial cell apoptosis) was selective for sinusoidal-type metastases, in which the neovasculature originating from sinusoidal endothelium cells was targeted by endostatin.[6] Differences in the tumor's microenvironment are known to determine anti-tumor efficacy of the treatment.[23,24] The precursor of endostatin, the long form of collagen XVIII, is almost exclusively found in the liver[25] and mainly expressed by hepatocytes[26,27]. Continuous capillaries contain both XV and XVIII collagen, but fenestrated capillaries (e.g. liver sinusoids, glomeruli, lung alveoli, and splenic sinusoids) express only type XVIII.[28] In our hands, endostatin had no effect on subcu-taneous tumors (data not shown). We could speculate that this is mediated by dominant negative competition with its precursor. Such insights on organ-specificity might be useful in the design of future clinical trials. Furthermore, since we found optimal anti-tumor effects even as early as within 15 minutes after intrasplenic tumor cell injection, endostatin prophylaxis might prove very promising to augment surgical treatment of colorectal carcinoma and liver metastases. Even in no-touch surgery, preventing metastatic seeding and combined with mesenterical vessel ligation, 12.5% of patients have detectable tumor spill in the circulation.[29] Overall, as many as 75% of colorectal cancer patients develop liver metastases.[30] Others also found that the efficacy of endostatin improved when administered before tumor cell injection, as measured by tumor load 21 days following tumor cell injection.[6] Bergers et al. corres-pondingly showed that endostatin, amongst others, was capable of treating early stages of cancer.[31] In vitro studies provide evidence to support an early effect of endostatin: disassembly of focal adhesions and actin stress fibers in endothelial cells in vitro was described to be already visible 1 hour after endostatin administration.[12] In addition, early response genes responsible for cell-matrix interactions were down-regulated rapidly after endostatin treatment.[32]

In conclusion, 2-hr pretreatment in vivo with endostatin inhibits intra-hepatic tumor growth, mainly due to early (<15 minutes) inhibition of tumor cell seeding in the liver. It also induces a two fold decrease of tumor cell adhesion to endothelial cells under flow conditions. This appears a novel working mechanism of endostatin, non-angiogenesis related, possibly mediated by integrin dependent adhesion of tumor cells to endothelial cells. These data can be helpful in the design of trials to augment surgical treatment of colorectal carcinoma and liver metastases.
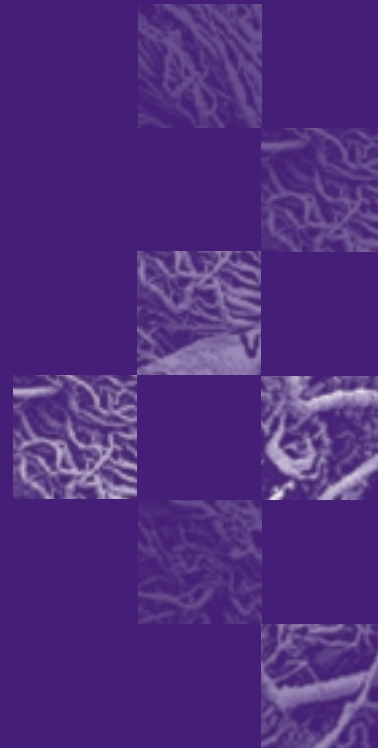
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Chapter 9

Summary

Early anti-metastatic potential of endostatin E. A. te Velde, A. Reijerkerk, D. Brandsma, et al.

Anti-angiogenic agents are promising new drugs in the fight against cancer. They might improve anti-tumor efficacy of current treatment in patients with (disseminated) colorectal cancer. Keeping in mind it is a highly idealistic prospective, optimization of therapy requires rationale and knowledge of 1) selection of suitable patients with 2) sensitive tumor types, 3) the mechanistic background of the drug(s) used, 4) the appropriate timing of administration, and 5) a safe combination with surgery. To understand the interactions of anti-angiogenic agents and surgery, we have studied the effects of surgery on angiogenesis in tumor growth and during wound healing.

Antiangiogenic cancer therapy is likely to be administered long term for sustained suppression of tumor outgrowth. Surgeons will encounter more patients undergoing such therapy. Therefore, it is essential to know the effects of antiangiogenic agents on physiological angiogenesis, as occurs during the healing of colonic anastomoses. As shown in *chapter 3*, angiostatin impairs anastomotic healing in mice. Histological examination revealed a decreased number of newly formed vessels in the wound and abundant granulation tissue as a result of the impaired healing (*chapter 4*). However, on discontinuation of antiangiogenic therapy, normal anastomotic healing is promptly restored.

Plasmin and other components of the plasminogen activation system play an important role in tissue repair by regulating extracellular matrix remodeling, including fibrin degradation. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a procarboxy-peptidase that, after activation, can attenuate plasmin-mediated fibrin degradation by removing C-terminal lysine residues from fibrin. These C-terminal lysines play a role in the binding and activation of plasminogen. To test the hypothesis that TAFI is an important determinant in the control of tissue repair we investigated the effect of TAFI-deficiency on the healing of cutaneous wounds and colonic anastomoses in *chapter 5*. Histological examination revealed inappropriate organization of skin wound closure in the TAFI knockout mice, including an altered pattern of epithelial migration. The time required to overall heal the cutaneous wounds was slightly delayed in TAFI deficient mice. Healing of colonic anastomoses was also impaired, as was reflected by a decreased strength of the tissue at the site of the suture as well as by bleeding complications in 3 out of 14 animals. Together, these abnormalities resulted in increased mortality in TAFI deficient mice after colonic anastomoses. Although our study shows that tissue repair, including re-epithelialization and scar formation, can ultimately proceed in some TAFI deficient mice, TAFI appears to be important for appropriate organization of the healing process.

Surgical treatment of colorectal liver metastases (CLM) is becoming increasingly important. Routinely, temporary occlusion of the inflow of the liver by clamping of the portal triad, i.e. Pringle maneuver (PM), is predominantly used to reduce intra-operative blood loss. In case of local destruction of liver metastases by Radiofrequency Ablation (RFA) the additive purpose of PM is an increase in destructed tumor volume, resulting in larger lesions. In *chapter 6*, we hypothesized that PM unfavorably affects the prognosis of cancer patients, by inducing temporary hypoxia with upregulation of Hypoxia Inducible Factor-1alpha (HIF1a), increase in Vascular Endothelial Growth Factor (VEGF) levels, and subsequent angiogenesis and growth of residual tumor. We evaluated the effects of PM on 1) tumor recurrence in patients following resection of CLM; 2) intrahepatic HIF1a expression and systemic VEGF-levels in patients undergoing RFA, and 3) outgrowth of experimental CLM.

Vascular clamping of the liver's inflow during hepatic surgery for CLM is associated with increased expression of HIF1a and VEGF, increased residual tumor growth and decreased survival. Therefore, Pringle maneuver should be avoided whenever possible. Experimental studies on therapeutical application of VEGF-antibodies or receptor-blockers perioperatively whenever Pringle maneuver is inevitable are indicated.

In our study we show that new drugs directed against tumor vasculature (anti-angiogenic therapy) provide us with promising combination strategies in the fight against cancer. The currently available treatment for intestinal cancer that has spread to the liver consists of surgery and is restricted to selected patients only. Chemotherapy is used a.o. to increase the number of patients eligible for resection. Strategies that target both tumor cells and vasculature have not been investigated in models of early metastatic colorectal disease. The efficacy of a combination of conventional chemotherapy with a potent angiogenesis inhibitor (endostatin or angio-statin) in a murine model of early colorectal liver metastasis was studied in *chapter 7*. We descibed that after twelve days of treatment, the liver metastases were signifi-cantly decreased in the mice that had received combination strategies. The addition of angiostatin or endostatin to conventional chemotherapy enhanced anti-tumoral efficacy in a murine model of early colorectal liver metastasis. Since this is an experi-mental study, the outcome of the combination of anti-angiogenic therapy with surgery and conventional chemotherapy in upcoming clinical trials in patients must be awaited.

Endostatin (rh-E), a carboxyterminal fragment of collagen XVIII, potently inhibits angiogenesis and tumor growth, presumably through induction of apoptosis in endothelial cells and/or inhibition of their migration. Inhibitory effects on endothelial cell adhesion play a role in this context. Reports on the in vivo effects of rh-E, however, have been contradictory. As we describe in *chapter 8*, we have observed different biological activities of rh-E depending on the timing of administration. Consequently, we hypothesized that part of the in vivo tumor inhibition of rh-E takes place during the initial phase of metastasizing, by inhibiting tumor cell arrest in the

liver and tumor-endothelial cell interaction. Two-hr pretreatment of mice or HUVEC with rh-E enhanced anti-tumor efficacy; resulted in early (<15 minutes) effects on tumor cell arrest in the liver; and induced a two fold decrease of tumor cell adhesion to stimulated HUVEC's under flow conditions. Possible mechanisms include inhibition of integrin dependent adhesion of tumor cells to endothelial cells or hampered intra-vascular arrest, in addition to anti-angiogenesis. Potential clinical application might be endostatin prophylaxis in preventing microscopic metastasis in patients subjected to surgery for colorectal carcinoma.

Chapter 10

General Discussion and Conclusions

General discussion

Administration of anti-angiogenic agents in a safe combination with surgery might improve anti-tumor efficacy of (disseminated) colorectal cancer. We have investigated whether the antiangiogenic agent angiostatin affects physiological angiogenesis, in addition to the already known effects on tumor endothelium. Since surgeons will encounter more patients undergoing such therapy, knowledge about the effects of anti-angiogenic therapy on physiological angiogenesis in wound healing is essential. No data existed on angiostatin in wound-healing assays. It was assumed that it would specifically affect activated, proliferating, and migrating endothelial cells and thus would lack any major side effects during treatment. Indeed, endostatin, another strong inhibitor of angiogenesis, was reported to have no adverse effects on physiological angiogenesis in cutaneous wound healing[1,2]. In mice, Bloch et.al. observed reduced connective tissue density in early and late wounds. They concluded that endostatin treatment even improved the quality of the healed wound. According to the authors an indirect effect based on the reduced number of functional blood vessels, supposedly caused these effects. Furthermore, the authors suggested that the large number of newly formed functional vessels in the wound is not required for healing under normal circumstances and that endostatin administration might be safe to combine with surgery. However, these wounds were created in cutaneous tissue. In contrast to the healing of cutaneous wounds, we considered the healing of colonic anastomoses far more dependent on angiogenesis and less dependent on diffusion of oxygen through pre-existing vasculature. Thus, our model could be more sensitive to angiogenesis-directed interventions compared with cutaneous wound healing. Indeed, short-term administration of two mildly angiosuppressive agents seem to suggest possible adverse effects during the early phase of wound healing[3,4] This healing of colonic anastomoses therefore served as a model for physiologically critical angiogenesis in *chapter 3*[5]. Strong evidence is provided here that angiostatin impairs anastomotic healing in mice when administered continuously during the phase of postoperative repair. Although the exact working mechanism of angiostatin has not been elucidated, it was recently shown to have a direct effect on endothelial cells.[6] Our immunohistochemical evaluation in *chapter 4* showed a reduction in the number of newly formed vessels in the granulation tissue surrounding the anastomotic line. On the basis of this data, we speculate that the formation of new vessels at the site of the anastomosis is impaired by angiostatin. This in turn may lead to a decrease in delivery of oxygen and nutrients to the healing colon. The adverse effects of angiostatin on physiological angiogenesis suggest mutual underlying mechanisms for tumor-induced and physiological angiogenesis. This assumption is supported by the knowledge that many other factors involved in angiogenesis, such as vascular endothelial growth factor, do indeed affect both physiological and pathologic angiogenesis.[7] We could speculate that the anti-tumor effect and the effects on physiological angiogenesis of angiostatin are both mediated through a direct effect on endothelial cells.

Angiostatin did not impose toxicity on any organ besides the anastomosis. However, mice with peritoneal leakage were severely ill, and consequently their skin wounds (laparotomy scar) showed impaired healing as well. Although we did not study cutaneous wound healing under angiostatin treatment, we do feel that administration of anti-angiogenic agents to patients following major surgery (colonic anastomosis) should be done with caution.

In addition, in *chapter 5* we showed that mice lacking TAFI have impaired wound healing in two different models of tissue repair. End-to-end anastomosis of the ascending colon in those mice resulted in a decreased strength of the anastomosis upon sacrifice at day seven postoperatively as compared with the wild-type controls. Cutaneous wound healing was different in TAFI deficient mice as well. It appeared that re-epithelialization by keratinocytes in TAFI-/- mice is delayed and furthermore did not follow the normal pattern of migration. In normal secondary wound healing keratinocytes migrate from one wound edge towards the opposing edge. For undisturbed migration the cells need contact with intact ECM over which they migrate.[8] However, in TAFI -/- mice the direction of keratinocyte migration was mostly directed down into the dermal layer, indicating a disruption of functioning ECM. Nevertheless, in both control and TAFI-/- mice cutaneous wound closure had ultimately been completed in all mice within 15 days. Interestingly, fibrinogen deficient mice showed a remarkably similar pattern of cutaneous wound healing[9] as compared with the TAFI deficient mice described in *chapter 5*. Another remarkable result of our study, was that at autopsy a mesenterial thrombosis was suspected in combination with diffuse bleeding from inflicted littermate's bites. These findings were in critically ill mice with impaired colonic healing, colonic dehiscence and subsequent peritoneal leakage This clinical manifestation in the TAFI deficient mice could very well be a result of diffuse intravascular coagulation (DIC). In concordance with the phenotype described by others[10], mice in whom components of the fibrinolytic system are disrupted, e.g. antiplasmin [11], PAI-1[12], and PAI-2[13], had normal bleeding phenotypes. Probably, the coagulation system adequately functions under circumstances where the fibrinolytic system is not dramatically challenged. The suspected DIC in the TAFI deficient mice after impaired colonic anastomotic healing might be a result of a critically challenged fibrinolytic system, where other mechanisms insufficiently compensate for the lack of TAFI.

Proper control of proteolytic degradation of the ECM and ECM remodeling is necessary for secondary wound healing as well as anastomotic healing. Based on the impaired healing in mice lacking TAFI and the observed alteration in keratinocyte migration we could speculate that TAFI might be a player in this delicately regulated process. Further studies should address the molecular and cellular mechanisms that lead to TAFI activation. A possibility is that TAFI may control angiogenesis, a key process in many (patho) physiological processes, in which the fibrinolytic system plays an important role.

When is it presumed safe to administer anti-angiogenic agents to patients perioperatively when they are subjected to major surgery? In an attempt to answer this

question, we have examined the kinetics of the adverse effects of angiostatin on the healing of colonic anastomoses (*chapter 3*). This was undertaken by administering the agent before surgery and discontinuing the treatment on the same day as the surgical procedure. Administration of angiostatin immediately before surgery had no negative effect on the healing of the anastomoses. These results in mice suggest that surgical patients who are treated with angiostatin can safely be operated on as soon as the treatment is discontinued. Assuming that antiangiogenic treatment is used perioperatively in patients undergoing colonic resection, it should preferentially be discontinued as briefly as possible, in an attempt to keep any metastatic tumor cell deposits in a state of dormancy. Because levels of circulating endogenous antiangiogenic agents produced by the primary tumor diminish after resection of the primary, this leads to accelerated metastatic outgrowth[6,14]. Considering the short half-life of angiostatin (4 to 6 hours), one might speculate that brief discontinuation of angiostatin treatment and resuming its administration shortly after anastomotic healing could circumvent the risk of anastomotic leakage while still effectively suppressing metastatic outgrowth.

Resuming administration "as soon as possible" implies that the surgeon should wait until anastomotic integrity is restored. When will that be? In wound healing the inflammation phase starts directly after wounding and continues until approximately the seventh day. The angiogenesis takes place during the proliferation phase, which begins with the appearance of fibroblasts. After 3 weeks the fibroblast and macrophages disappear, while remodeling and maturation of the ECM are the most predominant features of the healing process. The mucosa of the colon is healed within several days following anastomosis. The strength of the healed colon, however, is not dependent on mucosal healing but dependent on submucosal healing with fibroblast infiltration, ECM remodeling and smooth muscle cells[8]. In animal models, the strength of the colon is restored approximately one week following anastomosis[5,15]. In individual patients sub-clinical leakage can occur as late as two to three weeks after surgery. Moreover, it depends on the sort of agent used which process in the wound healing is potentially influenced. In our study, angiostatin did not cause increased endothelial cell apoptosis in mice at sacrifice one week after intestinal wounding (*chapter 4*). MMP-inhibitors might affect the healing process even unto a much later stage, since they are potentially influencing ECM remodeling in the maturation phase of wound healing. In daily practice, for every individual patient, the surgeon will decide on the postoperative clinical course when the anastomosis may be considered clinically healed and administration of the anti-angiogenic agents can be safely resumed.

What about the administration of anti-angiogenic therapy during pregnancy and embryogenesis? Similar to effects on wound healing, possible adverse effects on embryogenesis should be excluded before pregnancy could even be considered in disease-free patients undergoing prolonged exposure to anti-angiogenic agents. Endothelial cells and the formation of vessel structures are important features in the formation of chorionic villi in the development of a human placenta. As we showed by immunohistochemistry, in the majority of early abortions, especially the vessel formation was hampered, indicating a prerequisite for normal villous development.[16] The relative safety of pregnancy in cancer patients receiving anti-angiogenic agents will have to be investigated for every compound used and/or pathway intervened.

In the second part of this thesis, the interactions between surgery and angiogenesis is addressed. Surgery may cause selective tissue hypoxia, but it is not known whether this hypoxia leads to subsequent tumor angiogenesis.

Temporary occlusion of the liver's inflow, i.e. Pringle maneuver, first described by H. Pringle in the Annals of Surgery in 1909, is applied worldwide as a routine procedure during liver surgery. It's purpose is to reduce intraoperative blood loss during hepatectomy and to increase lesion size during local ablation techniques by preventing heat transport by the flowing of blood[17-19]. In *chapter 6* we show that Pringle maneuver is associated with increased metastatic recurrence and decreased survival. Further, we provide evidence that this may well be due to "Pringle-induced" hepatic hypoxia that results in upregulation of HIF-1α in different types of liver cells. This could lead to the observed increase in plasma VEGF levels and outgrowth of micrometastases through induction of angiogenesis. Based on these considerations and results, we suggest that Pringle maneuver should be omitted in patients where it is not obligatory. Also, we believe that it should be included as an independent prognostic factor in the design of future clinical trials. In addition, this outcome warrants a prospective study on this matter.

On the other hand, prospective studies on the effects of Pringle maneuver to increase lesion size during local ablation of liver tumors have recently opened for accrual. Local ablative techniques are a relatively new surgical interventions that are used to locally destroy (secondary colorectal) hepatic malignancies.[20-25] These techniques are restricted to those patients not eligible for standard surgical resection, with metastases confined to the liver. On interim analysis these studies show an increase in lesion size and a short-term decrease in local recurrence of the ablated lesion (unpublished data). Data on long-term effects on distant recurrence or intrahepatic new lesions as well as survival, however, is not available yet. In studies on the effects of vascular occlusion on enhanced lesion size during local ablation, the contribution of vascular occlusion of the hepatic artery was significantly less than the effects of venous clamping.[26,27] Some authors even claimed that the occlusion of the artery alone did not significantly increase lesion size in rabbits.[28] It might very well be that the effects of vascular clamping on intrahepatic hypoxia are predominantly caused by clamping of the hepatic artery instead of the portal vein. We would like to emphasize that studies, experimental and/or clinical, on clamping of the hepatic artery versus portal vein or both and intrahepatic hypoxia are indicated before Pringle maneuver is implicated as a standard measure during local ablation of liver tumors to

increase local control. We would like to postulate that Pringle maneuver used to increase lesions during the local ablative techniques should exclusively be applied to the portal vein. In this way, larger lesions are obtained, local recurrence controlled, without long-term adverse effects of clamping on recurrence and survival.

Although we provide the first firm evidence for the disadvantageous effects of Pringle-induced hypoxia, other studies have reported indirect clues to support this. In a retrospective study comparing various forms of vascular clamping, Buell et al found the lowest rate of recurrence in patients undergoing hepatic resection for metastatic cancer when no form of vascular clamping was applied.[29] In ventricular biopsies from patients with coronary ischemia, angiogenic factors such as HIF-1α and VEGF were shown to be increased, as a first adaptation mechanism of human myocardium to deprivation of blood.[30] However, no correlation between (surgery-induced) hypoxia, angiogenic growth factors and tumor growth has been described so far. It is tempting to speculate that other surgical interventions that hamper oxygen distribution can provide a similar physiologically relevant HIF-1α upregulation and subsequent pro-angiogenic response. For instance, partial bladder outlet obstruction increased angiogenic response in bladder tissue in rats.[31] In addition, during pneumoperitoneum in laparoscopic surgery a decrease in microcirculation in intra-abdominal tissues was observed[32-34], and the consequent hypoxia was a cofactor in adhesion formation[32]. Experimental studies on surgery-induced hypoxia and subsequent HIF-1α upregulation might be worthwhile to pursue.

We have shown an increase in HIF-1α in the major cell types in liver biopsies taken after Pringle maneuver, as proven by immunohistochemistry. The elevated plasma levels of VEGF are indicative of a direct effect of HIF-1α upregulation on VEGF production. Although liver endothelial and Kupffer cells showed the most outspoken HIF-1α upregulation, circulating platelets, activated as a result of the stasis induced by Pringle maneuver, may also contribute to the VEGF release[35]. In mice, we found systemic outgrowth of experimental micro-metastases, even though the plasma VEGF levels in patients were only elevated for several hours. HIF-1α was shown to prolong the half-life of VEGF in vitro, up to 3 hours, through the stabilization of VEGF mRNA.[36] The survival curves, however, showed a negative effect of the Pringle maneuver for as long as years after surgery. It is not known whether these effects are the result of several hours of VEGF upregulation, or that short-term intrahepatic hypoxia induced other events leading to enhanced carcinogenesis.

The adverse effects of "surgery-induced" hypoxia on tumor growth, recurrence and survival, as described in *chapter 6*, might theoretically be overcome if HIF-1α is blocked completely. Further experimental studies on therapeutical application of VEGF-antibodies or receptor-blockers perioperatively are indicated to test whether VEGF inhibition can overcome the enhanced tumor growth after Pringle maneuver.

In the first part of this thesis we have studied the interaction between surgery and (anti) angiogenesis. Knowing that physiological angiogenesis can be affected as well, and that certain types of surgical interventions can stimulate angiogenesis, ultimately, the ultimate goal is optimal use of antiangiogenic agents in the treatment of cancer. Surgical treatment is the only curative option for patients with colorectal liver metastases. Studies on the treatment of patients prior or post liver resection for secondary malignant disease with conventional chemotherapy, have produced contradictory results[37-39]. More and more studies are being published on increased resectability rates of previously unresectable colorectal liver metastases, be it all by the same group[38,40-42]. Overall, it seems that nearly one third of the patients with previously unresectable colorectal liver metastases can be converted to resectable disease with preoperative chemotherapy. Nevertheless, alternative treatment modalities for colorectal liver metastases are still warranted. This was investigated in the latter part of this thesis.

Combining conventional chemotherapy with anti-angiogenic compounds might lead to improved anti-tumor efficacy by targeting both tumor cell- and endothelial cell-compartments. In concordance with this hypothesis, co-administration of angiogenesis inhibitors with other drugs in cancer treatment is briefly mentioned and advised in many reviews.[43-47] The rationale of such combination strategies is supported by our study described in *chapter 7*[48]. We showed that, compared with monotherapy chemotherapeutic regimens, the anti-tumor effect of combined chemotherapeutic and anti-angiogenic agents was even multiplicative. In apparent contrast to these findings, some authors have speculated that treatment that targets vasculature could hamper other blood-borne therapeutics in reaching tumor cells.[49,30] Many in fact questioned: "how chemotherapeutics may reach the tumor when anti-angiogenic therapy has destroyed the transporting vascular structures." Recent unpublished data show that the EGFR-receptor blocker "Iressa" in combination with conventional chemotherapy does not prolong survival in patients compared with chemotherapy alone. On the other hand, a combination of tumor necrosis factor a and melphalan was shown to generate a higher intra-tumoral concentration of the cytotoxic agent than chemotherapy alone.[50] An explanation for the beneficial effects of combination strategies was given by Jain, who recently suggested that antiangiogenic therapy might lead to `normalization' of tumor vasculature before its destruction.[51a] The effect of chemotherapy during the normalization phase might be enhanced, rather than being inhibited at a later stage once vasculature has been destroyed. In large tumors inhibition of angiogenesis could kill the center of the tumor, while the rim of the tumor remains viable through co-option of the preexistent vessels. Under those circumstances, chemotherapy could act as a cytotoxic agent for the peripheral part of the tumor.

An additional advantage of combination strategies might be a dose reduction of

the individual drugs involved, without impairing anti-tumor efficacy. Treatment with low-dose angiostatin combined with relatively low doses of conventional chemo-therapy was effective, and the mice displayed only minor toxicity throughout the treatment period. Antiangiogenic scheduling of treatment by lowering the dose with increased frequency of administration has also proved effective.[51]

So, whilst several animal studies show beneficial effects of combination strategies, clinical evaluation remains to be awaited. Our study employed an experimental model that resembles the most suitable patient category (early metastatic disease). This is important because most angiogenesis inhibitors that are currently in clinical trials are being administered to patients with bulky end-stage tumors as is true for the combi-nation strategies currently under study. Few or no anti-tumor effects may be antici-pated in bulky disease, and the selection of suitable patient categories is required before efficacy can be established. Furthermore, as we showed in *chapter 7* for high dose angiostatin a multiplicative effect of a particular drug is difficult to prove when the effect of that particular single drug is already very significantly high. In the deter-mination of efficacy of combination regimens, this implies a statistical problem.

The timing of administration is another important issue to be addressed in this respect. What comes first, chemotherapy of anti-angiogenic therapy? If indeed anti-angiogenic therapy normalizes the tortuous and leaky tumor vasculature, than it should perhaps proceed chemotherapy. Other arguments in favor off that order might be that anti-angiogenic therapy, as we stated above, is more effective in early disease.

What if anti-angiogenic agents are to be administered as adjuvant of neo-adjuvant therapy, i.e. in combination with surgery? In *chapter 8*, we showed that pre-treatment with endostatin inhibits hematogenic tumor cell seeding and adhesion to the liver. Endostatin prophylaxis could prove very promising in the treatment of patients under-going colorectal surgery of the primary tumor. Even in no-touch colorectal surgery, with mesenterical vessel ligation, 12.5% of patients have tumor spill in the circulation.[52] Indeed, overall, as many as 75% of the patients develop liver metastases. Given the half-life of endostatin (8 hours) adverse effects on colonic healing are not to be expected when endostatin is administered perioperatively. As we showed in *chapter 8*, even within 15 minutes tumor cell adhesion in the liver is dimin-ished. Furthermore, anti-VEGF might prevent the outgrowth of micrometastases after partial liver resection with inevitable clamping of the portal triad (*chapter 6*). The results of *chapter 7* on experimental early colorectal liver metastases indicate that anti-angiogenic therapy may also be highly attractive for peri-operative treatment schedules (minor residual disease (R1 resections)).

Differences in anti-tumor efficacy of anti-angiogenic compounds also depend on the tumor's microenvironment.[53] Paget developed the "seed and soil" theory in 1889. He stated that some tumor cells find certain organs more fertile an environment for metastatic growth. In addition, the organ environment is capable of modulating the

tumor cell response to chemotherapy.[54] Orthotopic tumors behave different from ectopic tumors, with respect to production and receptors of angiogenic growth factors.[55,56] Hence, anti-angiogenic therapy may produce a different response at different sites.[57] Even more specific, anti-angiogenic therapy may produce a different response at the same site for different tumor cells.[58] In that study it was suggested that the tumor cells altered the endothelial cell phenotype, mediating sensitivity to anti-angiogenic therapy.

In *chapter 8* we showed that endostatin works mainly on the initial phase of metastasis, i.e. seeding of tumor cells and adhesion to the vessel wall, and does not regress already established tumors in our models. Maybe this is also true for other agents called anti-angiogenic agents. It has been speculated that the in vivo target of angiostatin is not the mature endothelial cell, but the circulating endothelial progenitor cells (EPC's).[59] The growth of EPC's isolated from human subjects were, in contrast to mature endothelial cells, exquisitely sensitive to angiostatin. These results suggested that angiostatin may exert its biological effects by inhibiting the contri-bution of EPC's to angiogenesis and not by altering the growth of mature endothelial cells. Again, this favors for treatment with anti-angiogenic compounds in a very early phase of cancer, or even maybe prophylactic.

Conclusions

The combination of angiogenesis with surgery emerges when surgery induces physiological angiogenesis (wound healing), induces hypoxia with subsequent angiogenesis, or when anti-angiogenic therapy is added to the treatment of surgical patients. More and more frequently this will be the case. We must attempt to understand the molecular biological mechanisms behind the efficacy of anti-angiogenic agents, their intervention in early metastatic disease, as well as their safety in combination with surgery. However, the more insight we have gained regarding anti-angiogenic treatment, the more complicated it seems to get. "Anti-angiogenic" therapy comprises a very heterogeneous group of agents. It can also affect physiological angiogenesis. Currently, properties that have been attributed to anti-angiogenic therapy now include amongst others: regression of already established tumors by attacking putative targets as endothelial cells, or ECM; prevention of outgrowth of microscopic tumor deposits by inhibition of tumor cells adhesion to endothelial cells; and normalizing tumor vessels in order to make the tumor more susceptible for chemotherapy.

Anti-angiogenic therapy is, or should we perhaps say, "was" considered one of the most promising new developments in cancer treatment. At first, the media claimed that Judah Folkman was going to cure cancer within ten years. The results of the current treatments with respect to anti-tumor efficacy did, however, not live up to our expectations. "Angiogenesis" as such is often oversimplified. Translation of the experimental models to the clinic proved to be difficult. Clinical trials will eventually answer important questions as to selection of suitable patients, drugs, and timing of administration.

The following conclusions may be drawn from the work presented here:
- Anti-angiogenic agents can affect physiological angiogenesis. The administration of anti-angiogenic agents to patients following major surgery (colonic anastomosis) should be done with caution.
- Extra cellular matrix remodeling in secondary wound healing and skin wound healing is disturbed in mice lacking Thrombin-activatable fibrinolysis inhibitor, resulting in impaired wound healing.
- Surgically induced hypoxia by clamping of blood flow can be associated with increased expression of HIF1α and VEGF, increased residual tumor growth and decreased survival. Therefore, Pringle maneuver should be avoided whenever possible. VEGF might be a potentially interesting therapeutical target in this respect.
- Antiangiogenic therapy is most likely to be maximally effective in patients with early metastatic or minor residual disease. The combination of conventional chemotherapy and anti-angiogenic therapy is more effective against colorectal liver metastases than the sum of effects of either monotherapies.
- Endostatin, in addition to its effects on the developing tumor vasculature, also affects tumor cell adhesion in the very early phases of metastasis formation. Anti-tumor efficacy could be improved by preventing microscopic liver metastasis during colorectal surgery.

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Chapter 11

Nederlandse samenvatting

Acknowledgements

Curriculum vitae

List of publications

Nederlandse samenvatting

Anti-angiogene therapie is een veelbelovende nieuwe ontwikkeling in de behandeling van kanker. De middelen uit deze categorie medicijnen zijn gericht tegen de bloedvat-nieuwvorming (angiogenese) van kanker gezwellen, ofwel tumoren. De klassieke theorie van de anti-angiogene therapie, gesteld door Dr. J. Folkman, is als volgt. Door langdurige behandeling met anti-angiogene medicijnen wordt een tumor verhinderd om groter te groeien en via de bloedbaan uit te zaaien (metastaseren) naar andere organen. In de westerse wereld komt kanker van de dikke darm, colon kanker, veel voor. Curatieve behandeling van colon kanker of uitzaaiingen daarvan in de lever, lever metastasen, is primair chirurgisch. Het uiteindelijke streven, zij het idealistisch, is het nog beter behandelen van de individuele patiënt met kanker. Dit kan bijvoorbeeld door gebruik te maken van een combinatie van (nieuwe) behandelingsstrategieën. We hebben hiervoor meer kennis nodig over onder andere het volgende: Welke patiënten komen in aanmerking voor anti-angiogene therapie; welke tumor typen; hoe werken die middelen het beste; op welk tijdstip in de behandeling kunnen ze het beste toegediend worden; en kunnen ze veilig gecombineerd worden met chirurgische behandeling?

In het eerste gedeelte van het proefschrift wordt aandacht besteed aan de veiligheid van de combinatie chirurgie en anti-angiogene therapie. In *hoofdstuk 3* werden muizen geopereerd als hadden ze colon kanker. Ze werden bovendien gelijktijdig behandeld met angiostatine, een krachtig anti-angiogeen middel. Het bleek dat de genezing van de darmnaden slechter verliep in die muizen die angiostatine kregen gedurende het genezingsproces. In *hoofdstuk 4* werden de weesfels van die muizen onderzocht of we een aanwijzing konden vinden waarom angiostatine de genezing negatief beïnvloedde. Het bleek dat het aantal nieuwgevormde bloedvaten, dat in de darm speciaal van belang is voor het genezingsproces, minder was. Andere werkingsmechanismen van angiostatine die in de literatuur beschreven zijn konden we niet in onze weefsels terug vinden.

Wondgenezing is een complex proces, waarbij ook componenten van de stolling (fibrinolyse) en het steunweefsel (ECM) tussen de cellen een belangrijke rol spelen. Ook hierbij is angiogenese van belang. Een eiwit dat onderdeel uitmaakt van zowel de fibrinolyse als de ECM is Thrombin-activatable fibrinolysis inhibitor (TAFI). We weten nog niet waarvoor TAFI in het menselijk lichaam belangrijk is. Om dit te onderzoeken werden in *hoofdstuk 5* muizen gemaakt zonder TAFI (TAFI knock-outs). Bij die muizen werden zowel huidwonden als darmnaden gemaakt. Het bleek dat TAFI een rol speelt bij het wondgenezingsproces, aangezien de muizen zonder TAFI slechter genazen. Gedetailleerd onderzoek onder de microscoop (histologie) liet een vertraging van de genezing in de huid zien met een afwijkend patroon van migratie van de oppervlakte bedekkende cellen (keratinocyten).

In het tweede gedeelte van het proefschrift wordt aandacht besteed aan de inter-actie tussen chirurgie en angiogenese. Indien een levermetastase is ontstaan is de enige kans op genezing voor de patiënt om de laesie chirurgisch te laten verwijderen. Wanneer die laesie technisch niet weggesneden kan worden, is er een nieuwe behan-deling mogelijk middels locale ablatie met radiofrequente golven (RFA). Tijdens operaties van de lever wordt veelal gebruik gemaakt van het afklemmen van de bloed doorstroming van de lever. Dit wordt gedaan ten einde bloedverlies te beperken. Bovendien vergroot het de laesie die met RFA wordt gemaakt om de tumor te vernie-tigen. In *hoofdstuk 6* hebben we de hypothese opgesteld dat het afklemmen van de lever doorstroming (Pringle manoeuvre) een vermindering van de zuurstof in de lever veroorzaakt (hypoxie). Hypoxie geeft aanleiding tot angiogenese. Door angiogenese vindt namelijk weer meer zuurstof transport plaats, zijnde een compensatie mecha-nisme. Echter, angiogenese en verhoogde aanmaak van groeifactoren kan weer aanleiding geven tot meer tumor groei. We hebben derhalve gekeken naar de effecten van de Pringle manoeuvre op 1) tumor recidief bij patiënten na resectie van de lever metastase(n) van colon kanker; 2) groeifactor waarden bij patiënten tijdens RFA; en 3) de groei van lever metastasen in muizen. De Pringle manoeuvre bleek verhoogde expressie te geven van de angiogene factoren Hypoxia Inducible Factor-1alpha (HIF1α) en Vascular Endothelial Growth Factor (VEGF), en gepaard te gaan met meer tumor recidief en tumorgroei, alsmede met een slechtere overleving. Derhalve zijn wij van mening dat de Pringle manoeuvre -indien mogelijk- dient te worden vermeden. Verdere studies naar of anti-VEGF de negatieve gevolgen van de Pringle manoeuvre kan tegen gaan dienen ons inziens te worden verricht. Bovendien lijkt het raadzaam de effecten van selectieve Pringle manoeuvre (alleen afklemmen van de ader, en niet de slagader) nader te onderzoeken, aangezien de Pringle manoeuvre bij locale tumor destructie veelvuldig wordt toegepast.

Optimaal gebruik van de anti-angiogene middelen is het onderwerp van het laatste gedeelte van het proefschrift. In theorie is het zeer aantrekkelijk om twee soorten medicijnen te combineren die op verschillende punten aangrijpen. Naast chirurgische resectie van lever metastasen bij colon kanker wordt er steeds meer gebruik gemaakt van chemotherapie. Die heeft onder andere geresulteerd in een vergroting van het aantal patiënten dat na chemotherapie alsnog in aanmerking komt voor resectie. De combinatie van chemotherapie -hoofdzakelijk gericht tegen de tumor cellen- en anti-angiogene therapie -gericht tegen de bloedvaten van de tumor- werd onderzocht in *hoofdstuk 7*. In de ideale situatie zal anti-angiogene therapie toege-diend moeten worden wanneer de tumor niet groot (meer) is, inmiddels is wegge-haald, en/of om metastasen te voorkomen. In een muizenmodel van de vroege lever metastasen hebben wij chemotherapie gecombineerd met angiostatine of endostatine. Na twaalf dagen behandelen met beider combinatie bleek dat er significant minder van de lever ingenomen werd door tumor weefsel. Bovendien was de som van de

effectiviteit van de chemotherapie en de anti-angiogene therapie samen groter dan van beiden apart. Mogelijk is dit gebaseerd op het normaliserende effect van de anti-angiogene therapie op de tumorvaten, waardoor de chemotherapie beter wordt opgenomen. Bij het combineren van anti-angiogene therapie met conventionele middelen dient derhalve -afhankelijk van het werkingsmechanisme- de volgorde en tijdschema van toedienen zorgvuldig te worden gekozen.

Endostatine is een van de potente anti-angiogene middelen. Het werkingsmecha-nisme wordt voornamelijk toegeschreven aan de direct remmende werking op bloedvat cellen (endotheel cellen). Endostatine remt met name de hechting van deze endotheelcellen in kweek. In een levend organisme zoals de mens of de muis (in vivo) is dat echter niet helemaal opgehelderd. Zoals we beschrijven in *hoofdstuk 8* werkt endostatine bij de behandeling van kanker in de muis soms wel en soms niet, hetgeen onder andere afhankelijk bleek van het moment van toedienen. Na verder onderzoek vonden we dat endostatine in een hele vroege fase van de tumor groei en metastasering het grootste deel van het remmende effect sorteerde. Dit in tegen-stelling tot het klassieke concept van de anti-angiogene therapie waarbij de tumor eerst groter moet worden om afhankelijk te zijn van bloedvaten. Bovendien remde endostatine de vroege hechting van tumor cellen aan endotheel cellen, een proces dat noodzakelijk is voor de metastasering. Het toedienen van endostatine voordat tumor cellen losraken en in de bloedbaan komen, bijvoorbeeld tijdens chirurgische manipulatie van dikke darmkanker, zou een belangrijke therapeutische aanvulling kunnen zijn op de huidige behandeling van patiënten met colon kanker.

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Curriculum vitae

De auteur van dit proefschrift werd geboren in Leiden, op 28 februari 1970. In 1988 voltooide zij de middelbare school (gymnasium β, eerst gymnasium Sorghvliet, Den Haag en later het Stedelijk gymnasium te Haarlem). Nadat zij twee keer werd uitgeloot voor geneeskunde, studeerde zij gedurende een jaar biologie aan de Vrije Universiteit in Amsterdam. Via de hardheidsclausule en de commissie van beroep van het ministerie van onderwijs, volgde het jaar daarop alsnog toelating tot de studie geneeskunde, Vrije Universiteit in Amsterdam.

Onderzoek naar Groei-hormoon-respons op hypoglycaemie, afd. Endocrinologie, Vrije Universiteit in Amsterdam (*Prof. dr. E.A. van der Veen*), alsmede de vascularisatie van de placenta in eerste trimester (Afd. Klinische Pathologie Spaarne Ziekenhuis Heemstede, mijn vader, *dr. J. te Velde en dr. N. Exalto*) werd gevolgd door voorzetting van het voorgenoemde onderzoek aan de Vrije Universiteit in Amsterdam, afdeling pathologie (*Prof dr. P.J. van Diest*).

In 1997 haalde zij haar artsenbul en nog diezelfde dag begon zij als arts assistent chirurgie in het Diaconessen Ziekenhuis Heemstede (huidige Spaarne Ziekenhuis, *dr. H. Siebbeles en dr. A. Labrie*). In 1998 werd zij aangenomen als arts assistent chirurgie in het Academisch Ziekenhuis Utrecht (huidige UMC Utrecht) door Prof dr. Th J M V van Vroonhoven, waar ze in 1999 werd aangenomen voor de opleiding tot chirurg.

In aansluiting op een aantal kortlopende traumatologische studies (*Prof dr Chr. Van der Werken*) werden gedurende drie opeenvolgende jaren (2000-2003) de hier voor genoemde studies verricht, resulterend in het alhier beschreven proefschrift, bij de afdeling chirurgie UMC Utrecht (*Prof dr. I.H.M. Borel Rinkes*), in samenwerking met het laboratorium voor medische oncologie (*Prof dr. E.E. Voest*). Dit werd gesubsidieerd door het Nederlands Wetenschappelijk Onderzoeks Instituut (NWO), in de constructie van AGIKO (assistent geneeskunde in opleiding tot klinisch onderzoeker).

Van 2003 tot 2007 zal zij haar klinische opleiding voltooien in het Diakonessen Ziekenhuis in Utrecht (*dr. G.J. Clevers*), gevolgd door het UMC Utrecht (*Prof dr. I.H.M. Borel Rinkes*).

In 1999 werd voor de opzet naar dit proefschrift de Klopper Prijs voor het beste onderzoeksvoorstel verkregen, beschikbaar gesteld door de Stichting Wetenschappelijk onderzoek Heelkundige Specialismen (SWOAHS), waarvoor onze hartelijke dank.

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- **E.A. te Velde,** N. Exalto, P. Hesseling, J.C. vd Linden. First trimester development of human chorionic villous vascularization studied with CD34 immunohistochemistry. *Human Reproduction* 1997; 12(7):1577-1581.
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- **E.A. te Velde**, , E.E. Voest, J. v Gorp, A.Verheem, J. Hagendoorn, M. Gebbink I.H.M. Borel Rinkes. Adverse effects of the antiangiogenic agent angiostatin on the healing of experimental colonic anastomoses. *Annals of Surgical Oncology* 2002 Apr-May;9(3):303-9.
- **E.A. te Velde**, J.M. Vogten, M. Gebbink, J. v Gorp, E.E. Voest, I.H.M. Borel Rinkes. Enhanced anti-tumor efficacy by combining conventional chemotherapy with angio-statin or endostatin in a liver metastases model. *British Journal of Surgery* 2002 Oct;89(10):1302-9.
- **E.A. te Velde***, G.T.M. Wagenaar*, A. Reijerkerk, M. Girma, I.H.M. Borel Rinkes, E.E. Voest, B.N. Bouma , M.F.B.G. Gebbink*, J.C.M. Meijers*. Mice lacking TAFI: impaired healing of cutaneous wounds and colonic anastomoses. *Equal contribution. *Journal of Thrombosis and Haemostasis 2003, accepted.*
- **E.A. te Velde**, P. J. van Diest, B. Fioole, W.A. Draaisma, P. van der Groep, A.E. Greijer, E. van der Wall, E.E. Voest, I.H.M. Borel Rinkes. Vascular Clamping during hepatic surgery for colorectal metastases worsens prognosis. *Submitted to the Lancet.*
- **E.A. te Velde**, A. Reijerkerk, D. Brandsma, J.M. Vogten, Y. Wu, O. Kranenburg, E.E. Voest, M. Gebbink, I.H.M. Borel Rinkes. Endostatin Prophylaxis Inhibits Metastatic Seeding of Murine Colorectal Cancer cells and their Adhesion to Stimulated Endothelial cells under flow conditions. *Submitted to Cancer Research in addapted form.*
- Vogten JM, Drixler TA, **E.A. te Velde,** Schipper, ME, van Vroonhoven ThJMV, Voest EE, Borel Rinkes IHM. Angiostatin inhibits experimental liver fibrosis in mice. *Submitted.*
- **E.A. te Velde,** B. Kusters, C. Maas, R. de Waal, I.H.M. Borel Rinkes. Histological analysis of defective colonic healing as a result of angiostatin treatment. *Journal of Experimental and Molecular Pathology 2003, in press.*
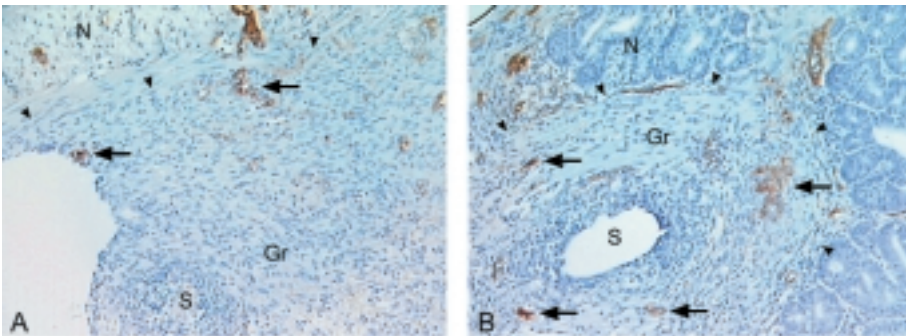
Chapter 12

Color appendix

Chapter 1
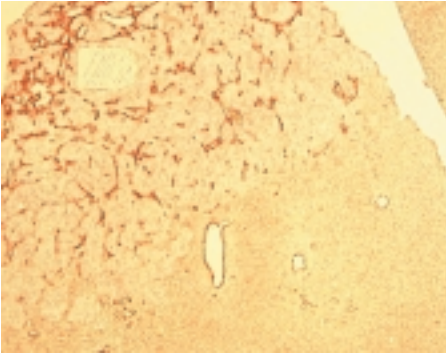
Chapter 1
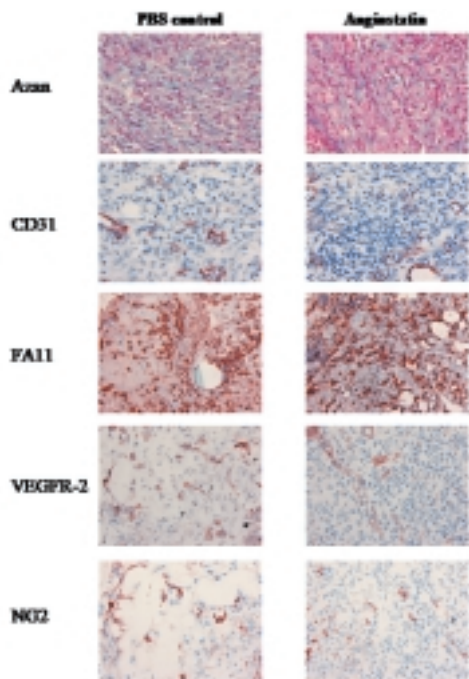*Figure 1. **Example of the "angiogenic switch"** page 8*

Chapter 3

Chapter 3
*Figure 3. **Anastomotic healing on day 7 after surgery** page 33*
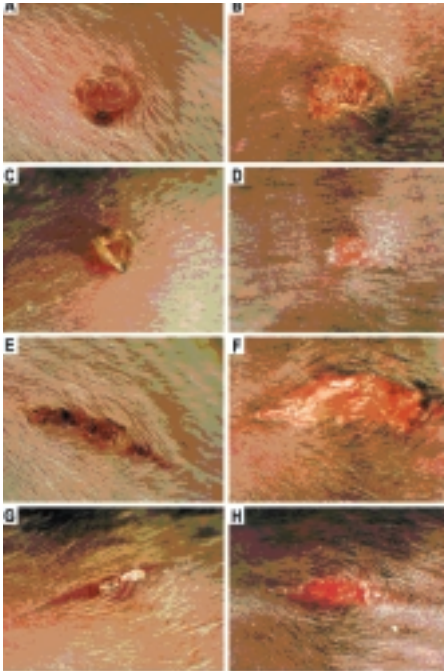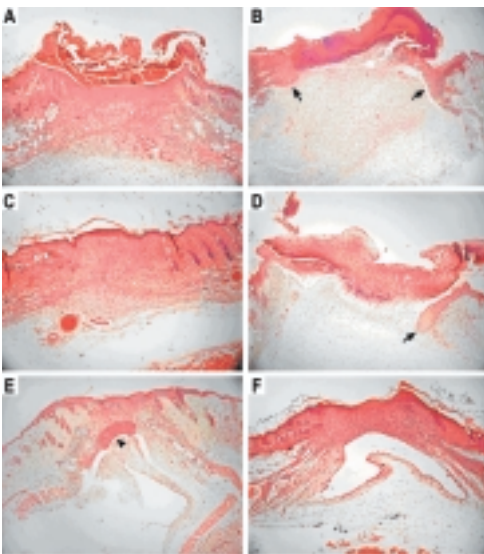
Chapter 4

Chapter 4

Figure 1. ***Comparison of granulation tissue in colonic wound healing in control mice and angiostatin treated mice*** *page 43*
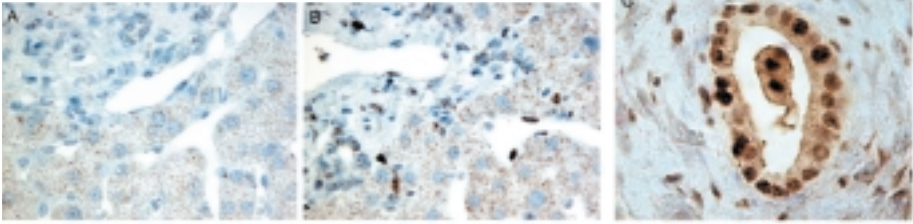
Chapter 5

Chapter 6

Chapter 6

*Figure 2. **Effects of Pringle maneuver on intrahepatic HIF1α expression** page 80*
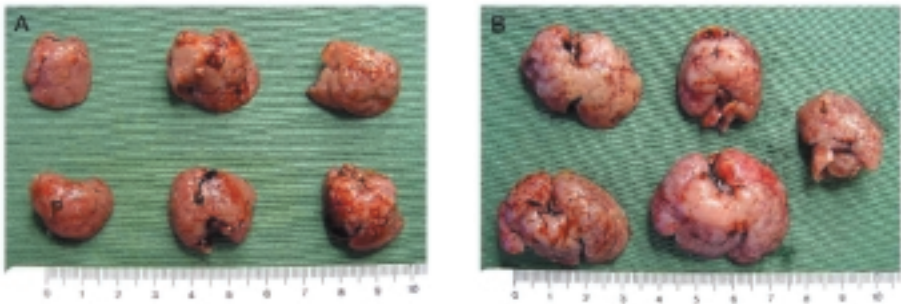
Chapter 6

*Figure 4. **Effects of Pringle maneuver on macroscopic intrahepatic tumorload** page 82*
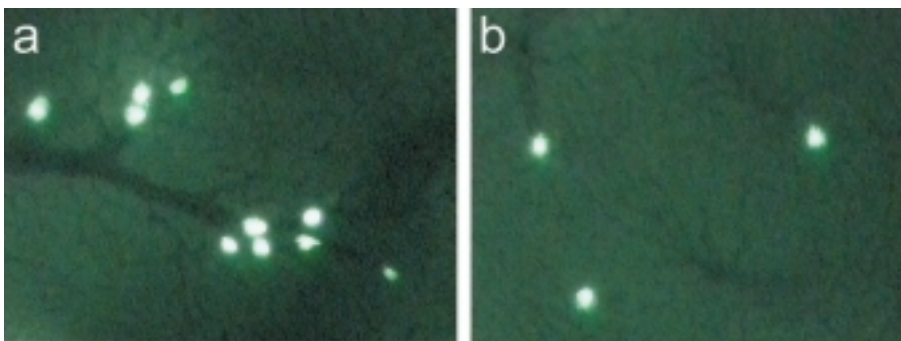
Chapter 7

Chapter 7

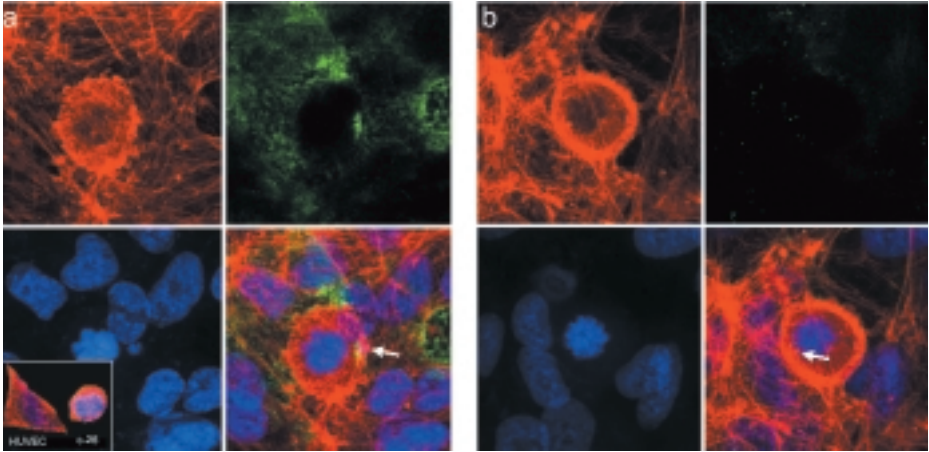*Figure 2.* **The macroscopic and histological appearance of resected livers** *page 96*

Chapter 8

Chapter 8

Figure 4. **Immunohistochemistry and multi-photon laser scanning microscopy visualized tumor cell adhesion to HUVEC.** *page 116*