Angiogenesis and hepatic colorectal metastases

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Angiogenesis, or the generation of new blood vessels from an existing vascular bed, is a tightly controlled dynamic process required for tumor growth and development. Normal adult vasculature is typically quiescent except when physiologic proliferation of blood vessels is triggered during certain conditions, such as a woman's menstrual cycle and wound repair. Angiogenesis is also implicated in several pathologic conditions, including retinopathies, rheumatoid arthritis and malignancy [1]. Many studies have also shown that tumor metastasis is an angiogenesis-dependent process [2,3]. The understanding that tumor growth and metastasis depends on angiogenesis has led to the development of novel treatment strategies directed at tumor vasculature. This article focuses on evidence implicating angiogenesis in colorectal cancer metastasis and reviews the initial results of antiangiogenic therapy in the treatment of this disease.

Biology of tumor-associated angiogenesis

A population of transformed cells, ultimately destined to be a clinically relevant cancer, is initially limited in size based on oxygen and nutrient delivery from neighboring normal host blood vessels. Dormancy of either a primary cancer or a micrometastasis can ensue in a scenario where cell proliferation is balanced by apoptosis until the minimal cell mass is either eradicated by the immune system or supported for further growth through the recruitment of new blood vessels. A shift in the local equilibrium between negative and positive regulators of angiogenesis must result for normally quiescent endothelium (with turnover time measured in years) to begin the process of neovascularization (where turnover time is only a few

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days). Tumor cells may develop this “angiogenic phenotype” in several manners, including overexpression of proangiogenic factors, recruitment of host cells capable of altering the angiogenic factor milieu, mobilization of angiogenic proteins from the extracellular matrix, or a combination of these processes. The creation of new blood vessels occurs by a series of steps. Eventually, cords of new endothelial cells develop lumens and create new networks of blood vessels in which blood flow supplies the tumor cell mass. Tumors that have undergone neovascularization may not only enter a phase of rapid growth but may also have increased metastatic potential.

**Assessment of angiogenesis**

The evaluation of the angiogenic response has proven difficult. Serum markers, such as measurement of angiogenesis-related proteins, are elevated in several diseases, including colon cancer, but have not proven particularly effective as biomarkers of disease [4]. Several radiologic methods to assess tumor vasculature have been evaluated, including dynamic CT, MRI, and ultrasound, but these are still under investigation. To date, there exists no standard for following the angiogenic response during therapy with angiogenesis inhibitors, although this continues to be an active field of investigation.

One method of evaluating tumor-associated angiogenesis is determination of microvessel density (MVD) in histologic tissue sections immunostained with antibodies against endothelial antigens. This technique, originally evaluated in breast cancer, has been shown to correlate with invasion, metastasis, and prognosis for most solid malignancies, including colorectal cancer [5,6]. A significant difference in both survival and incidence of recurrence is also seen in colorectal cancer patients with elevated tumor vascularity compared with those with lower microvessel counts [7,8]. Furthermore, tumor-associated MVD correlates with both tumor and endothelial cell proliferation [9]. Because the fraction of proliferating endothelial cells is higher in the tumor than in adjacent normal mucosa, therapeutic targeting of this population becomes a viable possibility.

**Proangiogenic factors**

Several proangiogenic regulators have been implicated in the development of an angiogenic response. Among these are several small-molecule growth factors, including basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) [10]. VEGF remains the most characterized of these proangiogenic factors. This diffusible endothelial cell–specific mitogen is the most potent direct-acting angiogenic protein known [11]. VEGF also increases vascular permeability, thus serving to enhance diffusion of nutrients and proteins...
needed for growth and metastasis [12]. Several VEGF receptors, expressed primarily on endothelial cells, have been identified. This group of tyrosine kinases includes VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4).

Several studies have evaluated the relationship between VEGF and colon cancer. It has been established that expression of VEGF is increased in histologic tissue sections from patients with recurrent node-negative colon cancer compared with those patients who did not experience recurrence [8]. The colon adenoma-carcinoma sequence has been further evaluated in patients with colonic adenomas, nonmetastatic colon cancer, or metastatic colon cancer [13]. A significant difference in VEGF and VEGFR-2 immunostaining in metastatic tumors was seen when compared with nonmetastatic lesions and adenomas. These results correlated strongly with both tumor microvessel counts and proliferative index, with metastatic lesions having significantly higher numbers than nonmetastatic tumors or adenomas. VEGF expression has also been evaluated in colorectal cancer liver metastases [14]. Intense staining for VEGF occurred in the carcinoma cells of liver metastases but not in tumor stromal cells or in normal liver tissues. Most tumor-associated endothelia also expressed VEGF receptors, whereas no significant staining occurred in tumor cells or in normal liver tissue.

Because VEGF and its receptors appear to be important in colorectal cancer-associated angiogenesis, several animal studies have evaluated agents targeting this growth factor. Strategies of VEGF inhibition can be divided into the following categories: (1) neutralization of the VEGF ligand, (2) blockade of the VEGF receptor, and (3) inhibition of VEGF receptor signal transduction. Agents aimed at both VEGF and its receptor as potential targets have been evaluated in experimental models of metastatic colorectal cancer. Treatment with a monoclonal antibody to VEGF has been evaluated in athymic mice inoculated with a colorectal cancer cell line by splenic-portal injection [14]. Treatment resulted in a decrease in both the size and number of liver metastases. In addition, strong labeling of the mouse VEGF receptor flk-1 was seen in the tumor-associated vessels of the control mice, but not in the treated animals. Monoclonal antibody to the VEGF receptor has also been evaluated in a similar model [15]. Tumor burden, MVD, and tumor cell proliferation were all decreased by treatment, correlating with an increase in tumor apoptosis. Another study evaluated two inhibitors of VEGFR-2 [16]. SU5416 is a selective inhibitor of the VEGFR-2 receptor whereas the orally bioavailable SU6668 inhibits multiple tyrosine kinase receptors, including VEGF, bFGF, and PDGF [16,17]. Treatment with either drug resulted in a significant decrease in tumor burden, MVD, and tumor cell proliferation, with a corresponding increase in both tumor and endothelial cell apoptosis. There are yet to be reports of VEGFR signal transduction inhibitors as therapeutic antiangiogenic agents, but it is likely that the next class of drugs will selectively exploit these pathways.
Among the most characterized angiogenesis inhibitors in clinical trials is Bevacizumab (Genentech, Inc, San Francisco, CA), a recombinant humanized monoclonal anti-VEGF antibody [18]. This drug appears to be well tolerated, with the most common toxicity being hypertension (20%) and a few reports of thromboembolic events and serious bleeding [19]. Currently, bevacizumab is being evaluated in several phase 2 and 3 studies of colon cancer in combination with cytotoxic chemotherapy. The first of these trials, although too small to compare efficacy, suggested an improved response rate and lengthened time to progression in patients with untreated metastatic colorectal cancer receiving 5-fluorouracil (5-FU) and leucovorin (LV) with bevacizumab versus those receiving chemotherapy alone [20,21]. There are two ongoing phase 3 trials in patients with metastatic colorectal cancer evaluating bevacizumab in combination with either irinotecan or oxaliplatin. Results of these trials will provide further information as to the efficacy of bevacizumab in this patient population.

Several inhibitors of the VEGF receptor are currently under investigation in clinical trials. Most of these are still in phase 1 and 2 trials with advanced solid tumors, of which patients with colorectal cancers make up a large proportion. These inhibitors include the oral VEGFR-2 inhibitors ZD6474 (Astra-Zeneca, Inc, Wilmington, DE), PTK787/ZK222584 (Novartis, Inc, Basel, Switzerland), and SU6668 (Sugen, Inc, South San Francisco, CA). The selective VEGFR-2 inhibitor SU5416 (Sugen, Inc, South San Francisco, CA) is the best studied of these agents. An ongoing phase 2 trial with SU5416 in advanced colorectal cancer has reported initial findings in 15 patients [22]. Seven patients achieved disease stabilization, with two patients developing toxicity (fatigue and hepatotoxicity), which precluded further treatment. Initial results of a phase 1 and 2 study of SU5416 in combination with 5-FU, LV, and irinotecan in advanced metastatic colorectal cancer revealed the drug to be well tolerated with neutropenia as the dose-limiting toxicity [23]. Of five evaluable patients, there were two partial responses and three with stable disease. There are several ongoing trials evaluating SU5416 in colorectal cancer, including two phase 3 trials examining efficacy of this drug in combination with adjuvant chemotherapy in patients with metastatic colorectal cancer. One of these, evaluating 5-FU/LV with or without SU5416, has completed enrollment with more than 700 patients worldwide. Another trial comparing 5-FU/LV/irinotecan with or without SU5416 continues with an anticipated accrual of 1200 patients.

Endogenous inhibitors of angiogenesis

Tumor dormancy provides the conceptual framework to explain a prolonged quiescent state in which liver micrometastases are present but tumor progression is not clinically apparent. Although a dormant population of tumor cells within the liver might remain clinically undetectable during initial treatment of the primary cancer, these cells can be activated and thus pose a constant risk for tumor recurrence.
Interferons are one group of endogenous regulators of cell growth and differentiation. These antiangiogenic agents inhibit in vitro angiogenesis and decrease the expression of several proangiogenic regulators. The antiangiogenic effects of interferon alpha-2a have been investigated in an animal model of hepatic metastasis from colorectal cancer [24]. Treated mice had a significant decrease in both number and volume of hepatic metastases. This reduction in tumor burden appeared independent of the antiproliferative effects of interferon and correlated with a decrease in MVD, bFGF, and matrix metalloproteinase 9 (MMP-9), directly implicating the antiangiogenic properties of interferon in this process. To date, clinical trials with interferons have been disappointing, largely because of drug toxicity seen in currently used regimens. Future trials with alternative dosing schedules may prove more efficacious with fewer limiting side effects.

Several protein fragments that act as endogenous inhibitors of angiogenesis have also been identified [25]. These agents were initially found in scenarios where primary tumors inhibited the growth of associated metastases [26]. Administration of these agents caused tumor regression to dormant microscopic lesions that exhibit a high proliferation rate balanced by apoptosis in tumor cells. Angiostatin is a 38-kDa internal fragment of plasminogen, which was first isolated from the serum and urine of tumor-bearing mice. It specifically inhibited endothelial cell proliferation and angiogenesis in multiple in vitro assays. Angiostatin therapy also inhibits tumor growth in multiple murine models, without development of drug resistance or toxicity [27]. A recombinant form of this agent is currently under investigation in human clinical trials.

Endostatin is one of the most potent endogenous inhibitors of angiogenesis identified to date. It is a 20-kDa C-terminal fragment of collagen XVIII, originally isolated from conditioned growth media of a murine hemangiendothelioma cell line, which also causes endothelial cell apoptosis [28]. Systemic administration of recombinant endostatin to mice bearing syngeneic tumors resulted in potent inhibition of tumor growth without the development of drug resistance [29]. In addition, human colon cancer cells transfected with the endostatin gene exhibited significant reduction in xenograft growth following inoculation into the flanks of mice [30]. Furthermore, injection of the endostatin gene into mice produced measurable protein in the circulation capable of inhibiting systemic angiogenesis, primary tumor growth, and the development of metastases [31]. Clinical development of this drug continues and two phase 1 trials in patients with advanced solid cancer have recently been completed.

Cell adhesion molecules

Critical to the successful development of an angiogenic response is cell adhesion. Integrin family receptors mediate cell adhesion and are expressed on the surface of endothelial cells [32,33]. Several integrins have emerged as
important in the angiogenesis cascade. Among these, the role of integrin $\alpha_v\beta_3$ has been best characterized. $\alpha_v\beta_3$ acts as an endothelial receptor for several angiogenesis-associated ligands, and its concentrations are significantly increased during angiogenesis, as evidenced by increased expression in granulation tissue [32,34,35]. The antibody to $\alpha_v\beta_3$ has also been shown to inhibit angiogenesis in a chick chorioallantoic membrane (CAM) model and an $\alpha_v$ knockout mutation proved lethal in approximately 80% of mice, suggesting a critical role for $\alpha_v\beta_3$ in embryologic angiogenesis [34,36]. In cancers, inhibition of angiogenesis has been shown to reduce both tumor growth and local invasion in breast carcinoma when treated with $\alpha_v\beta_3$ antibodies [37]. In colon cancer, immunostaining for $\alpha_v\beta_3$ yielded greater staining in tumor-associated vessels than in normal mucosa [4]. Anti-integrin therapy in vivo has been evaluated in a murine model of metastatic colon cancer. Treatment with a combination of an $\alpha_v\beta_3$ antagonist and a tumor-specific antibody—interleukin 2 (IL-2) fusion protein—revealed significant regression of both tumor and associated vessels, a finding not seen with IL-2 alone [38].

More recently, another integrin, $\alpha_5\beta_1$, has been implicated in the angiogenic cascade. This integrin also binds several angiogenesis-associated proteins, but its role has been less well characterized. Up-regulation of $\alpha_5\beta_1$ has been demonstrated on neovessels of the chick CAM and $\alpha_5\beta_1$ antagonists inhibit bFGF-induced angiogenesis in the CAM [18]. The integrin $\alpha_5\beta_1$ is also expressed on tumor-associated vasculature but not normal colonic mucosa [18]. Therapy targeted at $\alpha_5\beta_1$ in colon cancer has also been evaluated. A murine model of hepatic colorectal metastasis was used to evaluate ATN-161, a specific inhibitor of $\alpha_5\beta_1$, alone or in combination with 5-FU [39]. Results showed that combination therapy yielded several long-term survivors but monotherapy with either agent had none.

The in vitro and in vivo results have led to clinical evaluation of integrins as cytotoxic chemotherapy. Vitaxin (Applied Molecular Evolution, San Diego, CA), the first of these agents to be evaluated in clinical trials, is a humanized version of the $\alpha_v\beta_3$ antibody LM609. A phase 1 study has been completed in patients with stage IV malignancy and progressive disease, several of whom had metastatic colon cancer [40]. Although there was only one partial response, several patients achieved stable disease while on treatment, and treatment-related toxicity was minimal. These results have led to an ongoing phase 1/2 trial with Vitaxin in patients with irinotecan-refractory metastatic colorectal cancer. Patients will be treated with Vitaxin weekly for 52 weeks, with response rate, response duration, and time to progression the primary clinical objectives of this study.

**Matrix proteins**

Invasion is a required aspect of tumor metastasis and associated angiogenesis. Matrix metalloproteinases (MMPs) are a family of proteases
characterized by their ability to degrade the extracellular matrix [41]. To date, more than 25 MMPs have been identified. These can be subdivided into four groups based on enzyme specificity: collagenases, gelatinases, stromelysins, and membrane-associated metalloproteinases. There also exists a family of endogenous inhibitors of MMPs termed tissue inhibitors of metalloproteinase (TIMPs). Matrix stability depends on a finely coordinated balance between these two groups of enzymes.

MMP expression has been evaluated in colorectal cancer. Immunostaining reveals that expression of gelatinase A (MMP-2) is significantly increased in colon cancer when compared with normal mucosa, and there is a trend toward increased expression of gelatinase B (MMP-9) [42]. Expression of gelatinases A and B are also increased in linear fashion in the adenoma-carcinoma sequence when evaluated by immunohistochemistry [43]. In addition, expression of MMP-1 has correlated with both the incidence of hematogenous metastasis and prognosis in colorectal cancer [44,45].

MMP inhibitors have been studied in several animal models of colorectal cancer. BB-94 (Batimistat), an inhibitor of several MMPs, decreases the number and size of liver metastases and inhibits lung metastases in murine models [46]. In an orthotopic model of colon cancer, there was a significant decrease in the growth of primary tumor and incidence of metastasis with the use of Batimistat, which corresponded to an improved median survival time [47]. Batimistat has also been evaluated in a rat model of colon cancer peritoneal carcinomatosis. Similar to that seen in mice, the treated group had a significant decrease in disease, which translated into an increase in survival [48]. Another MMP inhibitor, KB-R7785, also has been evaluated in colon cancer. Treatment decreased both tumor growth and functional vascular area in an in vivo transparent chamber model of colon cancer. It also significantly decreased the number of lung metastases in a model of metastatic disease [49].

Several MMP inhibitors have been evaluated in phase 1 and 2 trials of advanced solid cancer, with patients with metastatic colorectal cancer making up a significant number of patients being evaluated. The first generation of compounds includes Batimistat, and the closely related, orally available agent Marimistat (BB-2516). Marimistat has been shown to have a dose-dependent biologic effect in patients with recurrent colorectal cancer as measured by rise of carcinoembryonic antigen when compared with pre-enrollment rate of increase, suggesting a potential therapeutic effect [50]. These drugs have been limited by significant musculoskeletal toxicity, however, particularly arthritis. Newer generations of MMP inhibitors are now being investigated that have specific MMP activity but minimally affect other metalloproteinases, such as sheddases, responsible for musculoskeletal toxicity of older agents.

Another therapeutic strategy is to combine MMP inhibitors with traditional cytotoxic chemotherapy. This has been done in a phase 1 study
with MMI270, an orally available drug with activity against several MMPs, combined with 5-FU and folinic acid in metastatic colon cancer. With this regimen in 18 patients, there were 2 patients with partial responses and 10 patients with stable disease [51].

Other antiangiogenic strategies

Cyclooxygenase

Cyclooxygenase (COX) is an important enzyme in arachidonic acid metabolism, and inhibition of this enzyme reduces production of the inflammatory mediator prostaglandin. Two subtypes of COX exist, termed COX-1 and COX-2. Constitutive expression of COX-1 is present in most human tissues whereas COX-2 expression appears only after up-regulation by inflammatory mediators and tumor-related factors [52]. In colon cancer, COX-2 is detectable in 80% to 90% of colorectal adenocarcinomas, and expression is increased in colorectal adenomas when compared with normal mucosa [53,54]. COX-2 expression has also correlated with invasiveness of colorectal cancer cell lines in vitro and overexpression is associated with an increase in production of MMP-2, both of which are modulated by administration of nonsteroidal anti-inflammatory drugs [55]. COX-2-expressing colon cancer cells have also been shown to induce endothelial tube formation in vitro, which is inhibited by a selective COX-2 inhibitor [56]. Further evidence to suggest inhibition of angiogenesis may be an important activity of COX-2 inhibitors is that selective COX-2 antagonists decrease growth of colon cancers that do not express COX [57]. In vivo, COX-2 inhibition decreases polyp formation in the Min mouse model of intestinal adenoma, decreases lung metastasis in a murine model of hematogenous metastasis, and decreases tumor proliferation while increasing apoptosis in human colon cancer cell xenografts implanted in mice [58–60]. This evidence has led to several ongoing clinical trials evaluating COX-2 inhibitors in the prevention of colonic polyps in patients with hereditary nonpolyposis colon cancer and those with sporadic adenomatous polyps. As evidence continues to mount regarding not only the chemopreventive effects of these drugs but also the antineoplastic activity associated with them, it is likely they will be used in the adjuvant setting for colorectal cancer as well.

TNP-470

One of the first angiogenesis inhibitors to be evaluated, TNP-470, is a synthetic analog of fumagillin, a naturally occurring antibiotic secreted by Aspergillus fumigatus [61]. This drug inhibits proliferation of human umbilical vein endothelial cells in vitro and decreases angiogenesis in several in vivo models [62,63]. In an orthotopic model of colorectal metastasis, treatment with TNP-470 yielded a significant decrease in the
number of liver metasases [64]. In a similar study, efficacy of TNP-470 was compared with that of mitomycin C using the same model [65]. Again, the group treated with TNP-470 had a lower incidence of liver metastasis than either the control or the mitomycin C groups. It also appears that timing of administration of TNP-470 is important in the inhibition of liver metastasis. This has been evaluated in a rat model of metastatic colon cancer where treatment was begun either immediately after tumor inoculation or after tumor nodules had already appeared in the liver (15 days after inoculation) [66]. There was a significant decrease in the number of liver metastases in the group treated immediately but no decrease in the number of metastases in the group whose treatment was begun after liver disease had already been confirmed, a finding that may have implications in the clinical use of this drug. Clinical trials are currently underway investigating TNP-470 in several diseases.

Summary

Angiogenesis is a critical step in the metastatic cascade of colorectal cancer. Several angiogenesis inhibitors have been evaluated in animal models and have shown efficacy, but challenges remain in using these drugs effectively in the clinical setting. Although several of these angiogenesis inhibitors are currently being evaluated in clinical trials, alone or in combination with cytotoxic chemotherapy, early results suggest that angiogenesis inhibitors alone, when used for advanced disease, have minimal activity. It is likely that this class of drugs will prove more efficacious when used either in the setting of minimal disease as agents that may promote tumor dormancy or in combination with other conventional forms of therapy. In addition, strategies such as metronomic therapy have been proposed whereby lower doses of cytotoxic chemotherapy, given more frequently, may act via an antiangiogenic mechanism [67,68].

Another challenge is identifying methods of assessing response to antiangiogenic therapy. To date, traditional methods of identifying response to treatment have not proven effective. Several investigators are working toward identifying circulating endothelial or tumor-associated factors that may be useful in following treatment. Novel imaging techniques are also being evaluated with enhanced CT and MRI, and newer modalities. Hepatic colorectal metastases provide an opportune setting in which to accomplish these challenges because the high incidence of disease and the ability to measure tumor with a variety of techniques lend themselves to evaluation of antiangiogenic therapy.

References


