ANGIOGENESIS, THROMBOSPONDIN-1 AND CERVICAL CARCINOGENESIS

Ming-Ping Wu[1–3], Cheng-Yang Chou[3,4]*

[1]*Department of Obstetrics and Gynecology, Chi Mei Foundation Hospital,*
[3]*Institute of Clinical Medicine, Medical College, National Cheng Kung University,*
[4]*Department of Obstetrics and Gynecology, National Cheng Kung University Hospital, Tainan, and*
[2]*College of Medicine, Taipei Medical University, Taipei, Taiwan.*

SUMMARY

Angiogenesis, the growth of new vessels from existing vasculature, plays an essential role in tumor development. The process involves interaction among cancer cells, endothelial cells, and components of the extracellular matrix, and is regulated by the balance of angiogenesis activators and angiogenesis inhibitors. This review profiles some fundamental concepts of angiogenesis, the importance of angiogenesis in cervical neoplasm, and the role of thrombospondin-1 as an angiogenesis inhibitor in cervical carcinogenesis. The usefulness and limitations of microvessel density in evaluation of angiogenic status are also discussed. Recent research and evolving concepts have led to a paradigm shift in anticancer therapy, from conventional cancer-centered chemotherapy to angiogenic or "metronomic" chemotherapy and/or combined angiogenesis inhibitors. The epigenetic strategy, which views the tumor system as a whole, transcends the cancer gene-centered approach. [*Taiwanese J Obstet Gynecol* 2005;44(2):128–138]

Key Words: angiogenesis activator, angiogenesis inhibitor, cervical cancer, metronomic chemotherapy

Angiogenesis Overview

Tumor growth is angiogenesis dependent

Angiogenesis is defined as the formation of new blood vessels by proliferation of new capillaries from existing microvessels. This process is distinct from vasculogenesis, which is defined as the formation of blood vessel *de novo* from angioblasts [1,2]. Angiogenesis involves degradation of the basement membrane surrounding an existing capillary or venule, migration of endothelial cells through the basement membrane to create a sprout, proliferation of endothelial cells, formation of a lumen within the new sprout and joining of two sprouts to form a functional capillary loop, and vessel maturation [3,4]. The idea that tumor growth is dependent on angiogenesis was first proposed in 1971, allowing anti-angiogenic therapy to be used to treat cancer [5]. The development of a solid tumor progresses from a prevascular phase to a vascular phase. The prevascular tumor does not induce angiogenesis, is limited in size, and rarely metastasizes. The vascularized tumor induces host microvessels to undergo angiogenesis. One genetic proof that tumor growth is angiogenesis dependent is the induction of tumor angiogenesis by *ras* oncogene [6]. *ras* induces the sequential activation of *myc* via a mechanism that enables it to repress the expression of endogenous angiogenesis inhibitors, e.g. thrombospondin-1 (TSP1) [7], and to activate angiogenesis activators, e.g. vascular endothelial growth factor (VEGF) [8]. Thus, blocking angiogenesis can result in tumor dormancy and tumors that cannot expand beyond a microscopic size [9]. Within dormant tumors, proliferating tumor cells are balanced by apoptotic tumor cells and few, if any, microvessels [10].

**Correspondence to:* Dr. Cheng-Yang Chou, Department of Obstetrics and Gynecology, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan 704, Taiwan.
E-mail: chougyn@mail.ncku.edu.tw
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Angiogenesis activators are produced by tumor cells while inhibitors are endogenous

In the early 1980s, it became clear that tumor cells can produce specific angiogenesis activators that stimulate the proliferation of capillary endothelial cells [11]. The growth of the tumor vasculature is no longer regarded as an inflammatory reaction. Angiogenesis activators are stored in the extracellular matrix (ECM) in "stand-by mode" before they become activated by specific enzymes when angiogenesis is required, physiologically or pathologically [9]. Angiogenesis inhibitors can be expressed by tumor cells or by normal host cells and are endogenous molecular defense barriers in pathologic hotspots of angiogenesis [9]. They are formed from cryptic fragments of matrix protein, e.g. angiostatin from plasminogen [12], endostatin from a fragment of collagen XVIII [13], and tumstatin from collagen IV [14]. The existence of naturally occurring angiogenesis inhibitors, which a tumor must overcome to induce angiogenesis, forms the basis of the concept of the "angiogenic switch" [2,15].

Angiogenic switch depends on the balance of angiogenesis activators and inhibitors

Bouck proposed that the onset of angiogenesis is the result of a shift in the balance of angiogenesis activators and inhibitors, which is controlled by oncogenes and tumor suppressor genes [15]. The balance of activators and inhibitors governs the angiogenic switch [2], which is characterized by downregulation of angiogenesis inhibitors or upregulation of angiogenesis activators, or both. When inhibitors exceed activators, the switch is "off". When activators exceed inhibitors, the switch is "on" [2]. Change in the balance of activators and inhibitors activates the angiogenic switch, before stabilizer molecules activate the maturation of nascent blood vessels [3]. This change takes place within the tumor cell itself and between the tumor cell's angiogenic proteins and the host's anti-angiogenic proteins [2]. For a tumor to switch to the angiogenic phenotype, it must overcome natural angiogenesis inhibitors in the host's circulation, the ECM or tumor cells. Rastinejad et al demonstrated that tumor cells did not become angiogenic until they had significantly reduced their own production of TSP1 [16]. In certain tumors, the angiogenic switch also involves downregulation of endogenous angiogenesis inhibitors, in addition to increased expression of angiogenesis activators. For example, *ras* transfection decreases expression of TSP1 and increases VEGF expression [17].

Angiogenic process relies on complex tumor–host interaction

The mechanisms of angiogenesis involve angiogenic activity arising from at least two sources: tumor cells that mediate the release of angiogenesis activators and host cells (e.g. macrophages) recruited by the tumor from the surrounding host ECM [6]. During angiogenesis, there is a complex interaction among cell components such as tumor, stromal, endothelial, and inflammatory cells, growth factors, and the ECM, which are regulated by angiogenesis activators and inhibitors [18].

Endothelial cells control the size of both normal tissue and tumor

Tissue mass, whether it is neoplastic or normal, may be regulated by microvascular endothelial cells [9]. Both tumors and organs may limit their own growth by increasing the production of angiogenesis inhibitors with size, until a critical size is reached where further growth is actively self-inhibited [19]. Tumor mass, as well as normal organ size, is under the tight control of the microvascular endothelium [20]. If normal cells are similarly dependent on endothelium-derived paracrine factors, the ratio of endothelial cells to normal parenchymal cells is likely to be lower than for tumor cells. Nevertheless, the regulation of tissue mass or organ size by vascular endothelial cells may be based on mechanisms that also operate in tumors [21]. Cancer growth requires the proliferation of endothelial cells in addition to malignant cells [22]. Without the proliferation of endothelial cells, a tumor cannot grow beyond the size of a colony. Folkman proposed the "two compartment model" to explain the interaction of endothelial cells and tumor cells, which constitute two important compartments of a tumor. Within a tumor, these two cell compartments can stimulate each other's growth via a perfusion or paracrine effect [22]. Coordinated tumor/vascular growth exploits an ultimate limitation to tumor size under angiogenic control, where opposing angiogenic stimuli come into dynamic balance [23].

During tumor angiogenesis, there is continued recruitment of endothelial cells and continued expansion of the tumor mass. Ultimately, each newly recruited endothelial cell can support a large population of tumor cells. This leverage may be exploited to cancer therapeutic advantage [19]. Administration of an angiogenesis inhibitor, which is not directly cytotoxic to tumor cells, can increase tumor cell apoptosis and inhibit tumor growth by inhibiting endothelial proliferation and migration, or by inducing apoptosis in endothelial cells [24]. By administering angiogenesis inhibitors to shift the stimulatory climate in the tumor back to inhibition, recruited endothelial cells are removed, followed by loss of the relatively abundant supported tumor cells [19,23].

Angiogenic Switch in Cervical Neoplasm

Angiogenesis plays an important role in cervical carcinogenesis

Cervical cancer is the second most common malignancy among women worldwide, with nearly 80% of cases arising in less developed countries. There are approximately 500,000 diagnoses of cervical cancer per year, leading to 200,000 deaths each year [25]. Cervical cancer develops usually by a sequence of gradual, stepwise events starting with low-grade squamous intraepithelial lesion (LSIL) and progressing through high-grade squamous intraepithelial lesion (HSIL) to invasive cancer [26]. Tumor development and metastasis is a complex process that includes transformation, proliferation, neovascularization, and metastatic spread. Angiogenesis is now regarded as one of the most important events occurring in the neoplastic process, and its role in cervical neoplasm is evident [27,28]. Hockel et al reported an association between tumor hypoxia and progression of cervical cancer, and this would seem to correlate with the angiogenic potential of the tumor [29]. However, trying to connect this finding with actual vessel counts or angiogenic factors has proven difficult. Two commonly used tumor vascularity measurements are microvessel density (MVD), the hotspot method that provides a histologic assessment of tumor angiogenesis, and intercapillary distance (ICD), which is thought to reflect tumor oxygenation [30]. Accumulating evidence has shown that angiogenesis is related to cervical neoplasm in histologic parameters, prognosis, survival [31], and therapeutic efficacy [32,33].

Timing of angiogenic switch in cervical carcinogenesis

The timing of the angiogenic switch during cervical carcinogenesis remains controversial. There is debate about the ability of cervical intraepithelial neoplasm (CIN) to induce angiogenesis [34–36]. Smith-McCune and Weidner found a significant increase in MVD in CIN III lesions compared with underlying low-grade lesions such as condyloma and CIN I [34]. Sotiropoulou et al reported that there was also a significant difference in the number of vessels between carcinoma *in situ* (CIS) and controls, but no significant correlation was found in relation to depth of invasion and histologic grade of the microinvasive carcinoma [28]. On the contrary, Abulafia et al showed that microinvasive squamous cell carcinoma (SCC), but not CIS, is angiogenic [36]. In order to eliminate the inborn heterogeneity in angiogenesis, Wu et al examined surgical specimens with lesions of different severity within the same histologic slide, so that each lesion could be used as an internal control for the others [37]. Their data showed that the angiogenic switch occurred during the transition from low-grade CIN to high-grade CIN, and that neovascularization was largely confined to a narrow zone immediately underneath the dysplastic epithelium [37]. This is in concordance with the results of Smith-McCune and Weidner [34] and Sotiropoulou et al [28]. Altogether, it seems that the onset of angiogenesis in cervical cancer is an early event, usually at a pre-invasive stage, which supports that cervical carcinogenesis is angiogenesis dependent [9,28].

MVD and Other Modalities in Evaluation of Angiogenic Status

Usefulness of MVD

One often-quantified aspect of tumor vasculature is MVD. However, whether MVD measurement reflects every aspect of angiogenesis remains controversial. MVD measurement within isolated regions of high vessel concentration (i.e. hotspots) is a significant and independent prognostic indicator in early-stage breast carcinoma [38]. MVD is a measure of the number of vessels per high-power field and reflects the ICD. ICDs are determined at the local level by the net balance between angiogenesis activators and inhibitors in each microenvironment; they do not reflect the angiogenic activity or angiogenic dependence of a tumor [39]. Thus, MVD may be a useful prognostic indicator but it may not be useful in monitoring anti-angiogenic treatment efficacy [39]. Though both vascularity measurements, ICD and MVD, provide independent prognostic information in multivariate analysis in cervical neoplasm, there is only significant correlation between tumor hypoxia and ICD, not MVD [40].

MVD used as prognostic indicator in cervical neoplasm

MVD serves as a prognostic indicator in cervical SCCs [27,35,41]. Together with depth of invasion, regional lymph node status, and vascular invasion, it is a strong independent prognostic indicator for overall survival in patients with clinical stage IB cervical carcinoma [42]. MVD may play a role in predicting recurrence and survival in patients with stage II SCC [31]. Nevertheless, two reports independently show that high MVD predicts improved survival in cervical cancer patients treated with radiotherapy [32] or intra-arterial chemotherapy [33]. Vascular densities were significantly higher in the effective group than the non-effective group. Rutgers et al also reported that increased angiogenesis in SCC of the cervix is not associated with a worsened prognosis

[43]. Another study showed that MVD was not a significant prognostic factor, although it might be associated with the presence of lymph node metastases and vascular space invasion by tumor cells [44].

It is possible that the discrepancies in results from MVD assays are due to the methodology used. For example, different types of antibody, as well as whether MVD is assessed at the periphery or center of the tumor, can influence its value as a prognostic indicator. Vieira et al compared the performance of three different monoclonal antibodies, anti-CD31, anti-CD34, and BNH9, in the quantification of angiogenesis in cervical cancer [45]. MVD estimated using anti-CD34 and BNH9 was significantly higher than that estimated using anti-CD31. Furthermore, the agreement between anti-CD34 and BNH9 to quantify MVD was higher than that between anti-CD31 and other antibodies.

MVD may not be an indicator of anti-angiogenic treatment efficacy

It is a common misconception that anti-angiogenic treatment can only be used in cancers that have high MVD. However, MVD does not reflect the angiogenic activity or angiogenic dependence of a tumor [39]. Since virtually all tumors are angiogenesis dependent, low MVD within a tumor is not a sufficient criterion to exclude patients from treatment with angiogenesis inhibitors [19]. Also, MVD is not predictive of tumor response under anti-angiogenic treatment and, therefore, is not useful for stratifying patients for clinical trials [39]. There is a considerable degree of heterogeneity in the intensity of angiogenesis within each tumor. In many situations, the MVD in a tumor is lower than that in its corresponding normal tissue [46]. Also, rapid tumor growth does not imply high MVD, because tumor cells can remain viable at lower oxygen concentrations. In other words, they can exist at greater distances from the vasculature than can their normal counterparts.

Individual tumors can make a wide variety of angiogenesis activators, and the relative expressions of these factors can change over time. The net angiogenic influence of the tumor microenvironment should be thought of as the sum of the angiogenesis activators and inhibitors that arise from both tumor cells [23] and host tissues [47]. Tumor MVD may not vary in accordance with the tissue or blood levels of any single angiogenesis activator [39]. Anti-angiogenic therapy has an ultimate limitation to tumor size under angiogenic control, where opposing angiogenic stimuli come into dynamic balance [23]. All tumor vessels are not equal in their ability to provide oxygen and nutrients to the tumor cells they support. Some can be ineffective. Thus, inhibition of ineffective vessels has little effect on the reduction of tumor growth [39]. The lack of a parallel relationship between tumor size and MVD points to the error in interpreting the lack of a decrease in MVD during therapy as a failure to inhibit vascularization. Thus, the efficacy of anti-angiogenic agents cannot be simply visualized using alterations in MVD during treatment [39].

Other models for studying angiogenesis

Though MVD offers a convenient way to evaluate angiogenesis, it has limitations. Hypoxia may not be morphologically detectable and is more likely to be detected by functional imaging than by histologic tissue assessment [48]. Cheng et al developed a novel method to evaluate *in vivo* angiogenesis in patients with cervical carcinoma [49]. Using color Doppler ultrasound and quantitative image processing, they found that the intratumoral vascularity index (VI), but not resistance index, showed good correlation with tumor stage, tumor size, depth of stromal invasion, lymphovascular emboli, and pelvic lymph node metastases. VI is well correlated with the conventional indicator of tumor angiogenic activity, MVD. The combined use of three-dimensional (3D) imaging and power Doppler provides the possibility of assessing cervical cancer volume and quantifying the power Doppler signal in the whole target organ, in contrast to two-dimensional (2D) ultrasound, where information on vascularization and blood flow is restricted to one subjectively chosen 2D plane. We have demonstrated that 3D power Doppler ultrasound accurately measures cervical cancer volume and provides a global assessment of vascular heterogeneity [50]. Therefore, it can be used to monitor response to, and vascular changes in tumors after, anti-angiogenesis therapy. Tumor vascularity and oxygenation have long been implicated as important determinants of radiation therapy [48], and tumor oxygenation levels can be measured using a sterile polarographic needle electrode [51]. Alternatively, dynamic contrast-enhanced magnetic resonance imaging (MRI) is used to assess tissue microcirculation. Tumor perfusion imaging is noninvasive, potentially easy to implement clinically, and reproducible for therapeutic monitoring [52,53]. Dynamic contrast MRI is based on the combination of rapid dynamic MRI techniques and bolus injection of gadolinium (Gd) contrast agent. In the first-pass (bolus injection) method, contrast enhancement patterns are used to assess tissue microcirculation [52]. The equilibrium method is based on the tracer-kinetic model to measure tissue perfusion [53]. There is reportedly a significant correlation between tumor oxygenation measured on a pO_2 histograph and maximal tumor enhancement in dynamic contrast-enhanced MRI. However, intratumor MVD and MRI parameters are not

significantly correlated [51]. Results from Mayr et al further suggest that MR microcirculation parameters do not always correlate with histomorphometric parameters, while there is evidence that MR parameters predict treatment outcome [48]. It can be interpreted that MR microcirculation assessment may reflect the "dynamic" angiogenic and metabolic status of a tumor, rather than the "static" single time point provided by histomorphologic parameters.

Various *in vivo* and *in vitro* models have been developed to test the effects of angiogenic agonists or antagonists. In developing an animal model for angiogenesis study, Guedez et al described the directed *in vivo* angiogenesis assay (DIVAA) to overcome difficulties in quantifying the angiogenic response [54]. The assay consists of subcutaneous implantation of semi-closed silicone cylinders (angio-reactors) into nude mice, and the results seem reproducible and quantitative in dose–response analysis; DIVAA can identify the effective doses of angiogenesis-modulating factors *in vivo*. Alternatively, the Matrigel-perfusion assay is widely used to measure angiogenesis [55]. Matrigel, an ECM gel-like plug, containing angiogenic factors and/or an experimental inhibitor is implanted into the skin of mice. The new blood vessels that grow into the plug can be quantified by measuring perfusion by hemoglobin or large fluorescently tagged molecules (such as intravenously administered dextran) into the plugs. Subsequently, Guidolin et al described an automatic image analysis method to evaluate angiostatic activity using the *in vitro* Matrigel assay [56]. This method allows several parameters to be established, such as dimensional (percentage area covered by endothelial cells and total length of the cellular network per field), topologic (number of meshes and number of branching points per field), and fractal (fractal dimension, lacunarity) parameters of the capillary-like network. The results indicate that both topologic and fractal parameters allow characterization of the spatial texture generated by endothelial cells during *in vitro* angiogenesis, although topographic parameters exhibit a wider dynamic range than fractal ones [56]. The analysis can be implemented in a computer program to facilitate calculation.

How Does the Angiogenesis Inhibitor TSP1 Regulate Angiogenesis in Cervical Neoplasm?

Role of angiogenesis activators in cervical neoplasm

VEGF is a disulfide-bonded glycoprotein whose transcription and expression are upregulated with hypoxia and appear to play an important role in the development and growth of tumors [57]. The tumor microvasculature, accompanied by overexpression of VEGF, is progressively upregulated during cervical carcinogenesis. VEGF is significantly increased in HSIL compared with LSIL and benign epithelium [58]. Dobbs et al found progressive increases in MVD and VEGF expression from CIN I through CIN III to invasive SCC [59]. There is a strong correlation between MVD and VEGF expression, and both are associated with histologic grade of CIN. The onset of angiogenesis is an early event in premalignant changes in the cervix due, in part, to enhanced expression of VEGF by the abnormal epithelium. Cheng et al found that cytosolic VEGF might be a biomarker for pelvic lymph node status in early cervical carcinoma and an independent prognostic indicator of overall survival [60]. On the contrary, Raleigh et al found no relationship between hypoxia and VEGF expression in cervical cancer [61]. In summary, it appears that hypoxia is important in the response of cervical tumors to treatment, but the actual angiogenic factors that play a role in the development and progression of cervical tumors are not yet clear [62].

TSP1 may play a comparative role to VEGF

TSP1, a 450-kDa glycoprotein expressed in platelets and ECM, was first discovered by Baenziger et al in 1970 as a large glycoprotein released from platelet alpha granules upon activation [63]. The TSPs are a family of extracellular proteins that participate in cell-to-cell and cell-to-matrix communication. Five family members have been identified [64]. TSP1 and TSP2 can inhibit angiogenesis in response to many angiogenic cytokines and growth factors such as basic fibroblast growth factor (bFGF) and VEGF [65]. Evidence suggests that TSP1 is involved in angiogenesis. However, the mechanisms by which TSP1 regulates angiogenesis are not well known, and the exact role of TSP1 in angiogenesis has been controversial; both stimulatory [66] and inhibitory effects [67] have been reported.

Upregulation of angiogenesis activators alone may not be enough for the angiogenic switch; it is accompanied by downregulation of some angiogenesis inhibitors [68]. Rastinejad et al demonstrated that tumor cells do not become angiogenic until they have significantly reduced their production of TSP1 [16]. VEGF is significantly increased in HSIL compared with LSIL and benign epithelium [58]. TSP1, as an endogenous angiogenesis inhibitor, may play a comparable role to other angiogenesis activators such as VEGF in angiogenesis balance during cervical carcinogenesis. In the skin cancer model, downregulation of TSP1 and upregulation of VEGF are coincident and are spatially correlated throughout the consecutive stages of

tumorigenesis [69]. Additionally, Kwak et al showed higher VEGF and lower TSP1 expression in prostate cancer than in non-cancerous tissue [70]. Genetic evidence also implies that TSP1 and VEGF play a comparative role to each other; overexpression of VEGF and downregulation of TSP1 can be triggered by the same oncogene, *ras*, and the loss of function of the tumor suppressor gene *p53*. Activating mutations in *K-ras* and *H-ras* upregulate VEGF expression and downregulate TSP1 expression [8,71]. Wild-type *p53* normally suppresses tumor angiogenesis by upregulating TSP1 [72] and suppressing transcription of VEGF [73]. TSP1 and VEGF appear to be the constituents of a "switch" that regulates, in concert, many components of the angiogenic and differentiated phenotypes of endothelial cells.

TSP1 acts as an "angiogenic fence" during cervical carcinogenesis

In bladder cancer, downregulation of TSP1 secretion is a key event in the switch from an anti-angiogenic to an angiogenic phenotype, while VEGF seems to play little part [74]. Recent work has identified TSP1 as an endogenous angiogenesis inhibitor that plays an important role in the angiogenic switch in skin, prostate, and bladder cancers [69,74,75]. Wu et al examined the spatial and temporal expression of TSP1 in patients with pre-invasive and invasive SCC of the uterine cervix [37]. TSP1 decreased significantly during the transition from LSIL to HSIL, with a concomitant increase in MVD. TSP1 was mainly localized on basal cervical epithelial cells, and arrayed like a barrier, and was therefore proposed as the "TSP1 fence". The temporal and spatial concordance of TSP1 downregulation and the emergence of angiogenesis, which occurs in the early phase of cervical carcinogenesis, imply that the TSP1 fence may act as an angiogenic barrier, inhibiting angiogenesis. The disappearance of the angiogenic barrier may induce a vigorous angiogenic response for tumor growth and, perhaps, tumor metastasis [76].

The origin of TSP1 production is currently unknown, with two origins, tumor cells themselves and host cells (e.g. stromal, endothelial cells, etc), possibly responsible [6]. Data suggest that basal epithelial cells contribute to the production of TSP1 under physiologic conditions but lose the ability to secrete TSP1 during the transition from LSIL to HSIL [37]. Kodama et al showed that TSP1 mRNA expression is significantly lower in advanced-stage cervical cancer and that its expression is of value as a prognostic factor in cervical cancer [77]. The inverse correlation between TSP expression and MVD also indicates that decreased TSP expression may be associated with an angiogenic phenotype in this class of neoplasm.

Do TSP1 or other angiogenesis inhibitors play a physiologic gatekeeper role in cancer prevention?

It has been suggested that TSP1 acts as an "angiogenic fence" to inhibit angiogenesis during cervical carcinogenesis [37]. The loss of the TSP1 barrier in early cervical cancer lesions leads to a more aggressive and more vascular cancer phenotype. In a related situation, elevated levels of the angiogenesis inhibitor endostatin in conjunction with elevated VEGF are associated with either a more aggressive phenotype or with metastasis or shortened survival in renal cell cancer, colon cancer, and soft tissue sarcoma [78–80]. These observations show that the angiogenesis inhibitors can be modulated as a result of changes in the tumor environment or in tumor disease burden [81,82]. Although circulating angiogenesis activators such as bFGF, VEGF, and angiogenin have been evaluated as diagnostic and/or prognostic factors in cancer patients, little is known about the clinical significance of angiogenesis inhibitors. Neither the source of TSP1 nor its mechanism of protein externalization has been clarified in detail [83].

The cause–effect relationship of TSP1 as a gatekeeper during cervical carcinoma development has not been clearly established. Although TSP1 levels decrease with the increasing malignancy potential of lesions, these determinations are only semi-quantitative and may be influenced by the degree of antibody reactivity and variability in the samples [84]. Meanwhile, the inverse relationship between TSP1 staining and severity of tumor lesions may be influenced directly or indirectly by other processes, e.g. angiogenic factors. Similarly, a cause–effect relationship has never been established between increasing MVD and decreasing TSP1 level [84]. The prevention of blood vessel development appears to be the mechanism of action of many successful chemopreventive drugs of natural or synthetic origin, which is termed "angioprevention". The hypothesis that anti-angiogenesis is the basis of tumor prevention also suggests that many anti-angiogenic drugs could be used for chemoprevention in higher-risk populations or in early intervention. There is a growing body of experimental evidence that anti-angiogenic strategies will contribute to the future therapy of cancer [85]. Further work on the therapeutic implications of angiogenesis inhibitors includes elucidation of the cause–effect relationship between changes in these inhibitors and tumor progression. Proof of such a relationship would provide a rationale for the use of angiogenesis inhibitors as preventive agents in patients at high risk for developing cancer.

TSP1 may influence angiogenesis by changing the ECM

The disappearance of the "TSP1 fence" modulates the

pericellular environment and potentially changes the cell–matrix interactions associated with cell movement and further progression. The basement membrane, a specialized form of the ECM, is involved in the regulation of tumor angiogenesis [86]. TSP1 does not appear to contribute directly to the structural integrity of connective tissue elements. Instead, it acts by modulating the activity and bioavailability of protease and growth factors and by interaction with cell-surface receptors [87,88]. Matrix metalloproteinases (MMPs) play an active role in the neovascularization of tumors through their ability to degrade the ECM [89,90]. Bergers et al showed that the switch from vascular quiescence to angiogenesis involves MMP9, which is upregulated in angiogenic islets and tumors, rendering VEGF more available to its receptors [91]. Notably, MMP9 is negatively modulated by TSP1. Thus, TSP1 acts as a multifunctional modulator of angiogenesis by modulating the activity and bioavailability of MMP9 [66,92]. How TSP1 modulates MMPs in cervical carcinogenesis deserves further study.

Anti-angiogenic Therapy Offers a Paradigm Shift for Anticancer Therapy

Tumor vasculature as a therapeutic target

There are some limitations to conventional chemotherapy, e.g. tumor cells easily develop resistance to cytotoxic agents that cause DNA damage or disrupt DNA replication, a phenomenon related to their genomic instability after varying periods of sensitivity [93]. Conventional chemotherapy is normally given on one or more consecutive days, followed by 3- to 4-week periods of rest, to allow recovery of normal proliferating cells, mainly bone marrow progenitors. The applied clinical strategy involves multidrug regimens designed to kill as many tumor cells as possible by administering combined cytotoxic agents at the maximum tolerated dose (MTD). The goal is to obtain total eradication of cancer cells [94]. However, most solid neoplasms are the result of multiple genetic abnormalities and may contain heterogeneous subpopulations of cells with different cell kinetics and invasive and metastatic properties [95].

Increasingly, research highlights the importance of tumor–host interactions and of the surrounding microenvironment in tumor development, invasiveness, metastasis, and responsiveness to therapy. There are some advantages to regarding the tumor vasculature as a therapeutic target. It is composed of more genetically stable cells than tumor cells and is less likely to acquire chemoresistance. There are fewer systemic side-effects and less toxicity with angiogenesis inhibitors than with other cytostatic agents. They offer more feasibility of long-term administration and can be combined with other cytostatic and/or molecularly targeted therapy [94]. Furthermore, the tumor endothelium is qualitatively distinct from normal endothelium at the molecular level, so it offers a specific and selective target [96]. Systemic administration of inhibitors can easily reach the target at the concentration of drug needed [94]. The tumor endothelium may proliferate under the stimulus of known growth factors, so therapeutic neutralization of endothelial cell growth factors can also be used as a therapeutic modality [97]. The proliferation and migration of tumor endothelium can be inhibited by naturally occurring angiogenesis inhibitors, e.g. endostatin, angiostatin, TSP1, etc. [13,98]. Endothelial cells do not appear to acquire resistance to some anti-angiogenic agents, which offers the possibility of re-inducing a response after interruption of therapy [99].

Low-dose "metronomic" chemotherapy is anti-angiogenic

Surprisingly, cytotoxic chemotherapy has anti-angiogenic effects, particularly when administered at low and frequent doses. This scheduling is more effective in targeting the tumor endothelium than large single bolus doses followed by long rest periods [98]. Conventional cytotoxic chemotherapeutic drugs were designed to treat cancer by directly killing or inhibiting the proliferation of rapidly dividing tumor cells. However, recent studies have highlighted the possibility that cytotoxic agents might reasonably be considered to have meaningful anti-angiogenic activity as a secondary mechanism [100]. Browder et al developed an alternative anti-angiogenic schedule for administration of cyclophosphamide that provided more sustained apoptosis of endothelial cells within the vascular bed of a tumor [98]. The use of chronically administered chemotherapeutic agents in a frequent, even daily, schedule with no prolonged drug-free breaks at low doses significantly below the MTD is called "anti-angiogenic" or "metronomic" chemotherapy [101]. Studies of the anti-angiogenic activity of metronomic chemotherapy have been conducted *in vivo* in a mouse corneal model [102], disc model [103], and chorioallantoic membrane model [103]. The sustained and potent anti-angiogenic effects are based on targeting the endothelial cells of newly growing tumor blood vessels [97,98]. Activated, differentiated endothelial cells, as well as circulating endothelial progenitor cells, are sensitive to low-dose chemotherapy [104,105].

The potential advantages of metronomic chemotherapy include: significant delay in the onset of mutation-dependent mechanisms of acquired drug

resistance, because the target of the therapy is presumed to be genetically stable, activated endothelial cells rather than genetically unstable, highly mutable cancer cells [106]; facilitation of the efficacy and durability of long-term integration of chemotherapy drugs with targeted anti-angiogenic agents [97]; reduction or loss of traditional toxic side effects due to the high sensitivity and selectivity [104,107]; and induction of an anti-angiogenic effect by decreasing the mobilization and/or viability of circulating bone marrow-derived endothelial precursor cells [108].

TSP1 plays angio-inhibitory roles by direct targeting or as a mediator of low-dose metronomic chemotherapy

TSP1 has potent angio-inhibitory effects in epithelial tumor development. Human SCC cell lines, stably transfected to overexpress human TSP1, exhibit inhibited tumor growth or completely abolished tumor formation in xenotransplants [67]. In addition to the direct targeting effects, Bocci et al reported that protracted exposure of endothelial cells *in vitro* to low concentrations of cytotoxic chemotherapeutic drugs caused marked induction of gene and protein expression of TSP1 [106]. Increases in circulating TSP1 were also detected in the plasma of human tumor-bearing severe combined immunodeficient mice treated with metronomic low-dose cyclophosphamide. The induced angiogenesis inhibitor can cause further growth arrest or apoptosis of endothelial cells. The induction of TSP1, as a secondary mediator of anti-angiogenic effects, in low-dose metronomic chemotherapy regimens can explain the "indirect" pathway to induce growth arrest or apoptosis of endothelial cells [55]. In summary, TSP1 may exhibit its anti-angiogenic effects in two ways: direct targeting of the endothelium and as a mediator of metronomic chemotherapy.

Paradigm shift from conventional dose-density chemotherapy to metronomic scheduling

Significant anti-angiogenic and antitumor effects are unlikely to be achieved in the clinical setting with a single chemotherapeutic agent at metronomic doses. Pioneering studies by Kakeji and Teicher showed potentiality or synergism when angiogenesis inhibitors were combined with standard schedules of certain cytotoxic agents [109]. The efficacy of metronomic chemotherapy can be significantly increased when administered in combination with anti-angiogenic drugs, such as antibodies against VEGF or VEGF receptor 2 [97]. Browder et al used cyclophosphamide and TNP-470 to reveal the ability of cancer chemotherapy to eradicate chemoresistant tumors [98]. The proposed rationale for the beneficial effect of such combinations was based on their ability to target both the parenchymal and stromal components of neoplasia [110]. Tumor endothelial targeting and tumor cell targeting should not be thought of as mutually exclusive. Anti-angiogenic therapy can be added to chemotherapy, radiotherapy, immunotherapy, gene therapy, or any other traditional cancer cell-directed modality [19].

A "new" paradigm for chemotherapeutic dosing is theoretically based on targeting the vascular system of the tumor rather than the tumor itself by using low-dose continuous chemotherapy [111]. As angiogenesis inhibitors become more widely used in anticancer therapy, it will be important to reduce the harsh side-effects and risk of drug resistance with conventional chemotherapy [9]. The paradigm of anticancer treatment may shift from cancer-centered to epigenic, endothelium-centered therapy [19]. The focus is not on the gene alterations within tumor cells, but on physiologic constraints imposed on the overall tumor system [111]. Conventional anticancer therapy uses cytotoxic drugs to kill tumor cells and to achieve the goal of cancer eradication. Homogeneous groups of patients are treated with the same chemotherapeutic schedule [94]. Individual tailored therapy uses metronomic therapy and/or molecular target treatments (e.g. angiogenesis inhibitors) to target the molecular abnormalities involved in tumor and endothelial cells to achieve the goal of cancer control in each patient [94]. The final goals of anti-angiogenesis therapy are not to cure cancer, but to make cancer more survivable and controllable, and eventually to be converted to a chronic manageable disease, like heart disease or diabetes, especially in conjunction with radiation, chemotherapy, and other treatments [112].

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References

1. Risau W. Mechanisms of angiogenesis. *Nature* 1997;386: 671–4.
2. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86: 353–64.
3. Bussolino F, Mantovani A, Persico G. Molecular mechanisms of blood vessel formation. *Trends Biochem Sci* 1997;22:251–6.
4. Jendraschak E, Sage EH. Regulation of angiogenesis by SPARC and angiostatin: implications for tumor cell biology. *Semin*

Cancer Biol 1996;7:139–46.
5. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;285:1182–6.
6. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992;3:65–71.
7. Watnick RS, Cheng YN, Rangarajan A, Ince TA, Weinberg RA. *Ras* modulates *Myc* activity to repress thrombospondin-1 expression and increase tumor angiogenesis. *Cancer Cell* 2003; 3:219–31.
8. Udagawa T, Fernandez A, Achilles EG, Folkman J, D'Amato RJ. Persistence of microscopic human cancers in mice: alterations in the angiogenic balance accompanies loss of tumor dormancy. *FASEB J* 2002;16:1361–70.
9. Folkman J. Fundamental concepts of the angiogenic process. *Curr Mol Med* 2003;3:643–51.
10. Achilles EG, Fernandez A, Allred EN, et al. Heterogeneity of angiogenic activity in a human liposarcoma: a proposed mechanism for "no take" of human tumors in mice. *J Natl Cancer Inst* 2001;93:1075–81.
11. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* 1984; 223:1296–9.
12. O'Reilly MS, Holmgren L, Shing Y, et al. Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. *Cold Spring Harb Symp Quant Biol* 1994;59:471–82.
13. O'Reilly MS, Boehm T, Shing Y, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277–85.
14. Maeshima Y, Sudhakar A, Lively JC, et al. Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. *Science* 2002;295:140–3.
15. Bouck N. Tumor angiogenesis: the role of oncogenes and tumor suppressor genes. *Cancer Cells* 1990;2:179–85.
16. Rastinejad F, Polverini PJ, Bouck NP. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell* 1989;56:345–55.
17. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002;2:727–39.
18. Rice A, Quinn CM. Angiogenesis, thrombospondin, and ductal carcinoma *in situ* of the breast. *J Clin Pathol* 2002;55: 569–74.
19. Folkman J, Hahnfeldt P, Hlatky L. Cancer: looking outside the genome. *Nat Rev Mol Cell Biol* 2000;1:76–9.
20. Franck-Lissbrant I, Haggstrom S, Damber JE, Bergh A. Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. *Endocrinology* 1998;139:451–6.
21. Folkman J. Is tissue mass regulated by vascular endothelial cells? Prostate as the first evidence. *Endocrinology* 1998;139: 441–2.
22. Folkman J. Tumor angiogenesis and tissue factor. *Nat Med* 1996;2:167–8.
23. Hahnfeldt P, Panigrahy D, Folkman J, Hlatky L. Tumor development under angiogenic signaling: a dynamical theory of tumor growth, treatment response, and postvascular dormancy. *Cancer Res* 1999;59:4770–5.
24. Folkman J. Angiogenesis and apoptosis. *Semin Cancer Biol* 2003;13:159–67.
25. Ellenson LH, Wu TC. Focus on endometrial and cervical cancer. *Cancer Cell* 2004;5:533–8.
26. Pinto AP, Crum CP. Natural history of cervical neoplasia: defining progression and its consequence. *Clin Obstet Gynecol* 2000;43:352–62.
27. Bremer GL, Tiebosch AT, van der Putten HW, Schouten HJ, de Haan J, Arends JW. Tumor angiogenesis: an independent prognostic parameter in cervical cancer. *Am J Obstet Gynecol* 1996;174:126–31.
28. Sotiropoulou M, Diakomanolis E, Elsheikh A, Loutradis D, Markaki S, Michalas S. Angiogenic properties of carcinoma *in situ* and microinvasive carcinoma of the uterine cervix. *Eur J Gynaecol Oncol* 2004;25:219–21.
29. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996;56:4509–15.
30. West CM, Cooper RA, Loncaster JA, Wilks DP, Bromley M. Tumor vascularity: a histological measure of angiogenesis and hypoxia. *Cancer Res* 2001;61:2907–10.
31. Cantu De Leon D, Lopez-Graniel C, Frias Mendivil M, Chanona Vilchis G, Gomez C, De La Garza Salazar J. Significance of microvascular density (MVD) in cervical cancer recurrence. *Int J Gynecol Cancer* 2003;13:856–62.
32. Siracha E, Sirachy J, Pappova N. Vascularization and radiocurability in cancer of the uterine cervix. *Neoplasma* 1994; 29:183–8.
33. Kohno Y, Iwanari O, Kitao M. Prognostic importance of histologic vascular density in cervical cancer treated with hypertensive intraarterial chemotherapy. *Cancer* 1993;72: 2394–400.
34. Smith-McCune KK, Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. *Cancer Res* 1994;54:800–4.
35. Abulafia O, Triest WE, Sherer DM. Angiogenesis in malignancies of the female genital tract. *Gynecol Oncol* 1999;72: 220–31.
36. Abulafia O, Triest WE, Sherer DM. Angiogenesis in squamous cell carcinoma *in situ* and microinvasive carcinoma of the uterine cervix. *Obstet Gynecol* 1996;88:927–32.
37. Wu MP, Tzeng CC, Wu LW, Huang KF, Chou CY. Thrombospondin-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis. *Cancer J* 2004;10: 27–32.
38. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;84: 1875–87.
39. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 2002;94:883–93.
40. Weber WA, Haubner R, Vabuliene E, Kuhnast B, Wester HJ, Schwaiger M. Tumor angiogenesis targeting using imaging agents. *Q J Nucl Med* 2001;45:179–82.
41. Di Leo S, Caschetto S, Garozzo G, et al. Angiogenesis as a prognostic factor in cervical carcinoma. *Eur J Gynaecol Oncol* 1998;19:158–62.
42. Dellas A, Moch H, Schultheiss E, et al. Angiogenesis in cervical neoplasia: microvessel quantitation in precancerous lesions and invasive carcinomas with clinicopathological

correlations. *Gynecol Oncol* 1997;67:27–33.
43. Rutgers JL, Mattox TF, Vargas MP. Angiogenesis in uterine cervical squamous cell carcinoma. *Int J Gynecol Pathol* 1995; 14:114–8.
44. Graflund M, Sorbe B, Hussein A, Bryne M, Karlsson M. The prognostic value of histopathologic grading parameters and microvessel density in patients with early squamous cell carcinoma of the uterine cervix. *Int J Gynecol Cancer* 2002;12: 32–41.
45. Vieira SC, Zeferino LC, Da Silva BB, et al. Quantification of angiogenesis in cervical cancer: a comparison among three endothelial cell markers. *Gynecol Oncol* 2004;93:121–4.
46. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. *Cancer Res* 2000;60:1388–93.
47. Hlatky L, Tsionou C, Hahnfeldt P, Coleman CN. Mammary fibroblasts may influence breast tumor angiogenesis via hypoxia-induced vascular endothelial growth factor up-regulation and protein expression. *Cancer Res* 1994;54: 6083–6.
48. Mayr NA, Hawighorst H, Yuh WT, Essig M, Magnotta VA, Knopp MV. MR microcirculation assessment in cervical cancer: correlations with histomorphological tumor markers and clinical outcome. *J Magn Reson Imaging* 1999;10: 267–76.
49. Cheng WF, Lee CN, Chu JS, et al. Vascularity index as a novel parameter for the *in vivo* assessment of angiogenesis in patients with cervical carcinoma. *Cancer* 1999;85:651–7.
50. Hsu KF, Su JM, Huang SC, et al. Three-dimensional power Doppler imaging of early-stage cervical cancer. *Ultrasound Obstet Gynecol* 2004;24:664–71.
51. Cooper RA, Carrington BM, Loncaster JA, et al. Tumour oxygenation levels correlate with dynamic contrast-enhanced magnetic resonance imaging parameters in carcinoma of the cervix. *Radiother Oncol* 2000;57:53–9.
52. Ueda T, Yuh WT, Taoka T. Clinical application of perfusion and diffusion MR imaging in acute ischemic stroke. *J Magn Reson Imaging* 1999;10:305–9.
53. Hawighorst H. Dynamic MR imaging in cervical carcinoma. *Radiology* 1999;213:617–8.
54. Guedez L, Rivera AM, Salloum R, et al. Quantitative assessment of angiogenic responses by the directed *in vivo* angiogenesis assay. *Am J Pathol* 2003;162:1431–9.
55. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* 2004;4:423–36.
56. Guidolin D, Vacca A, Nussdorfer GG, Ribatti D. A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay *in vitro*. *Microvasc Res* 2004;67:117–24.
57. Senger DR, Brown LF, Claffey KP, Dvorak HF. Vascular permeability factor, tumor angiogenesis and stroma generation. *Invasion Metastasis* 1994;14:385–94.
58. Guidi AJ, Abu-Jawdeh G, Berse B, et al. Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. *J Natl Cancer Inst* 1995;87: 1237–45.
59. Dobbs SP, Hewett PW, Johnson IR, Carmichael J, Murray JC. Angiogenesis is associated with vascular endothelial growth factor expression in cervical intraepithelial neoplasia. *Br J Cancer* 1997;76:1410–5.
60. Cheng WF, Chen CA, Lee CN, Wei LH, Hsieh FJ, Hsieh CY. Vascular endothelial growth factor and prognosis of cervical carcinoma. *Obstet Gynecol* 2000;96:721–6.
61. Raleigh JA, Calkins-Adams DP, Rinker LH, et al. Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. *Cancer Res* 1998;58:3765–8.
62. Wolf JK, Ramirez PT. The molecular biology of cervical cancer. *Cancer Invest* 2001;19621–9.
63. Baenziger NL, Brodie GN, Majerus PW. A thrombin-sensitive protein of human platelet membranes. *Proc Natl Acad Sci USA* 1971;68:240–3.
64. Lawler J. The functions of thrombospondin-1 and -2. *Curr Opin Cell Biol* 2000;12:634–40.
65. Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J Cell Biol* 1993;122:497–511.
66. Qian X, Wang TN, Rothman VL, Nicosia RF, Tuszynski GP. Thrombospondin-1 modulates angiogenesis *in vitro* by up-regulation of matrix metalloproteinase-9 in endothelial cells. *Exp Cell Res* 1997;235:403–12.
67. Streit M, Velasco P, Brown LF, et al. Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. *Am J Pathol* 1999;155:441–52.
68. Folkman J. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. *N Engl J Med* 1995;333:1757–63.
69. Hawighorst T, Velasco P, Streit M, et al. Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism. *EMBO J* 2001;20: 2631–40.
70. Kwak C, Jin RJ, Lee C, Park MS, Lee SE. Thrombospondin-1, vascular endothelial growth factor expression and their relationship with *p53* status in prostate cancer and benign prostatic hyperplasia. *BJU Int* 2002;89:303–9.
71. Rak J, Yu JL, Klement G, Kerbel RS. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. *J Investig Dermatol Symp Proc* 2000;5:24–33.
72. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by *p53* regulation of thrombospondin-1. *Science* 1994;265:1582–4.
73. Zhang L, Yu D, Hu M, et al. Wild-type *p53* suppresses angiogenesis in human leiomyosarcoma and synovial sarcoma by transcriptional suppression of vascular endothelial growth factor expression. *Cancer Res* 2000;60:3655–61.
74. Campbell SC, Volpert OV, Ivanovich M, Bouck NP. Molecular mediators of angiogenesis in bladder cancer. *Cancer Res* 1998;58:1298–304.
75. Jin RJ, Kwak C, Lee SG, et al. The application of an anti-angiogenic gene (thrombospondin-1) in the treatment of human prostate cancer xenografts. *Cancer Gene Ther* 2000;7: 1537–42.
76. Sheibani N, Frazier WA. Thrombospondin-1, PECAM-1, and regulation of angiogenesis. *Histol Histopathol* 1999;14: 285–94.
77. Kodama J, Hashimoto I, Seki N, et al. Thrombospondin-1 and -2 messenger RNA expression in invasive cervical cancer:

correlation with angiogenesis and prognosis. *Clin Cancer Res* 2001;7:2826–31.
78. Feldman AL, Pak H, Yang JC, Alexander HR Jr, Libutti SK. Serum endostatin levels are elevated in patients with soft tissue sarcoma. *Cancer* 2001;91:1525–9.
79. Feldman AL, Alexander HR Jr, Bartlett DL, et al. A prospective analysis of plasma endostatin levels in colorectal cancer patients with liver metastases. *Ann Surg Oncol* 2001;8:741–5.
80. Feldman AL, Alexander HR Jr, Yang JC, et al. Prospective analysis of circulating endostatin levels in patients with renal cell carcinoma. *Cancer* 2002;95:1637–43.
81. Feldman AL, Tamarkin L, Paciotti GF, et al. Serum endostatin levels are elevated and correlate with serum vascular endothelial growth factor levels in patients with stage IV clear cell renal cancer. *Clin Cancer Res* 2000;6:4628–34.
82. Ozatli D, Kocoglu H, Haznedaroglu IC, et al. Circulating thrombomodulin, thrombospondin, and fibronectin in acute myeloblastic leukemias. *Haematologia (Budap)* 1999;29: 277–83.
83. Kuroi K, Toi M. Circulating angiogenesis regulators in cancer patients. *Int J Biol Markers* 2001;16:5–26.
84. Libutti SK. Do angiogenesis inhibitors perform a physiologic gatekeeper role in cancer prevention? *Cancer J* 2004;10:12–4.
85. Bisacchi D, Benelli R, Vanzetto C, Ferrari N, Tosetti F, Albini A. Anti-angiogenesis and angioprevention: mechanisms, problems and perspectives. *Cancer Detect Prev* 2003;27: 229–38.
86. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer* 2003;3:422–33.
87. Bornstein P, Kyriakides TR, Yang Z, Armstrong LC, Birk DE. Thrombospondin 2 modulates collagen fibrillogenesis and angiogenesis. *J Investig Dermatol Symp Proc* 2000;5:61–6.
88. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest* 1999;103:1237–41.
89. Zetter BR. Cell motility in angiogenesis and tumor metastasis. *Cancer Invest* 1990;8:669–71.
90. Liotta LA, Thorgeirsson UP, Garbisa S. Role of collagenases in tumor cell invasion. *Cancer Metastasis Rev* 1982;1:277–88.
91. Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2000;2:737–44.
92. Albo D, Shinohara T, Tuszynski GP. Up-regulation of matrix metalloproteinase 9 by thrombospondin 1 in gastric cancer. *J Surg Res* 2002;108:51–60.
93. Kerbel RS, Yu J, Tran J, et al. Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. *Cancer Metastasis Rev* 2001;20:79–86.
94. Gasparini G. Metronomic scheduling: the future of chemotherapy? *Lancet Oncol* 2001;2:733–40.
95. Fidler IJ, Ellis LM. Chemotherapeutic drugs – more really is not better. *Nat Med* 2000;6:500–2.
96. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. *Science* 2000;289:1197–202.
97. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000;105:R15–24.
98. Browder T, Butterfield CE, Kraling BM, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000;60: 1878–86.
99. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 1997;390:404–7.
100. Miller KD, Sweeney CJ, Sledge GW Jr. Redefining the target: chemotherapeutics as antiangiogenics. *J Clin Oncol* 2001; 19:1195–206.
101. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *J Clin Invest* 2000;105:1045–7.
102. O'Leary JJ, Shapiro RL, Ren CJ, Chuang N, Cohen HW, Potmesil M. Antiangiogenic effects of camptothecin analogues 9-amino-20(S)-camptothecin, topotecan, and CPT-11 studied in the mouse cornea model. *Clin Cancer Res* 1999;5:181–7.
103. Presta M, Rusnati M, Belleri M, Morbidelli L, Ziche M, Ribatti D. Purine analogue 6-methylmercaptopurine riboside inhibits early and late phases of the angiogenesis process. *Cancer Res* 1999;59:2417–24.
104. Wang J, Lou P, Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs* 2003;14: 13–9.
105. Bertolini F, Paul S, Mancuso P, et al. Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. *Cancer Res* 2003; 63:4342–6.
106. Bocci G, Francia G, Man S, Lawler J, Kerbel RS. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci USA* 2003;100:12917–22.
107. Bocci G, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival *in vitro* reveal a selective antiangiogenic window for various chemotherapeutic drugs. *Cancer Res* 2002;62:6938–43.
108. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001;7:1194–201.
109. Kakeji Y, Teicher BA. Preclinical studies of the combination of angiogenic inhibitors with cytotoxic agents. *Invest New Drugs* 1997;15:39–48.
110. Gasparini G, Harris AL. Does improved control of tumour growth require an anti-cancer therapy targeting both neoplastic and intratumoral endothelial cells? *Eur J Cancer* 1994;30A:201–6.
111. Kamen BA, Rubin E, Aisner J, Glatstein E. High-time chemotherapy or high time for low dose. *J Clin Oncol* 2000; 18:2935–7.
112. Ezzell C. Starving tumors of their lifeblood. *Sci Am* 1998; 279:33–4.