Review Article

Metronomic chemotherapy and anti-angiogenesis: can upgraded pre-clinical assays improve clinical trials aimed at controlling tumor growth?

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Metronomic chemotherapy, which is continuously administered systemically at close to non-toxic doses, targets the endothelial cells (ECs) that are proliferating during tumor angiogenesis. This leads to harmful effects of an even greatly increased number contiguous tumor cells. Although pre-clinical studies of angiogenesis-related EC features in vitro and of the anti-angiogenic and anti-tumor effects in vivo of metronomic chemotherapy have provided valuable insights, clinical trials with this type of therapy have been less successful in inhibiting tumor growth. One possible reason for the apparent disconnect between the pre-clinical and clinical outcomes is that most of the currently used experimental angiogenesis assays and tumor models are incapable of yielding data that can be translated readily into the clinical setting. Many of the assays used suffer from unintentional artifactual effects, e.g., oxidative stress in vitro, and inflammation in vivo, which reduces the sensitivity and discriminatory power of the assays. Co-treatment with an antioxidant or the inclusion of antioxidants in the vehicle often significantly affects the angiogenesis-modulating outcome of metronomic mono-chemotherapy in vivo. This ‘metronomic chemotherapy vehicle factor’ merits further study, as do the observations of antagonistic effects following metronomic treatment with a combination of standard chemotherapeutic drugs in vivo.

Key words: Metronomic chemotherapy; angiogenesis assays; in vitro; in vivo; autochthonous tumors; reactive oxygen species; inflammation.

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The major obstacles to devising effective treatments for cancer are the heterogeneity and genetic instability of tumors, as a heterogeneous population of tumor cells contains many genetic and epigenetic variations. Tumor growth and spread are angiogenesis-dependent processes, which makes the endothelial cells (EC) in tumor vasculature a desirable target for anti-tumor agents. Metronomic chemotherapy is a promising treatment modality, in that it primarily targets the proliferating microvascular ECs that participate in tumor angiogenesis. The basis for this therapy is the frequent administration of low, close to non-toxic doses of cytotoxic agents.

For several cytotoxic agents, metronomic chemotherapy has been shown to suppress angiogenesis and tumor growth in pre-clinical studies without causing severe side effects, which are often problematic in conventional chemotherapy. Thus, metronomic chemotherapy has the potential to improve considerably the quality of life of cancer patients.

The results of clinical trials conducted to date with metronomic chemotherapy do not satisfactorily match the expectations raised by the compelling pre-clinical data, obtained for this regimen. One may assume that the better the pre-clinical data are from a biological point of view, the higher the probability that their clinical application will improve the outcome.

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Pre-clinical studies on the effect of metronomic chemotherapy on angiogenesis are performed in vitro, ex vivo, and in vivo. In vitro, cultured macro- or micro-vascular ECs, as well as tumor cells, are used. Studies conducted in vivo involve a variety of assays in tumor-free tissues and also look at the effects of low-dosage chemotherapy on tumor growth. The present paper discusses certain aspects of currently used pre-clinical models encompassing cell culture, angiogenesis assays, and tumors in the perspective of metronomic chemotherapy.

METRONOMIC CHEMOTHERAPY AND ANGIOGENESIS

Chemotherapy exerts anti-mitotic and anti-proliferative activities on dividing cells, primarily by increasing the intracellular production of reactive oxygen species (ROS) rather than through discrete pharmacologic mechanisms, as discussed previously (1, 2). In 2000, the laboratories of Folkman and Kerbel showed that by altering the dosing regimen to one of regular inoculations without rest periods using low, minimally toxic concentrations of the drugs, which is known as ‘metronomic’ chemotherapy dosing, certain commonly used cytotoxic drugs produced anti-angiogenic effects in xenotransplant models, even against drug-resistant tumors (3–5). This type of treatment schedule differs from conventional chemotherapy, which uses the maximum tolerated dosages of drugs (directed against rapidly dividing tumor cells) and which through targeting of normal tissues and cells causes toxic effects in the patient and makes it necessary to have a resting period of ~3 weeks per treatment cycle. During the resting periods, the damaged microvasculature can recover, resulting in an insufficient anti-angiogenic effect over the whole treatment cycle of conventional chemotherapy.

How normal are the endothelial cells and the vasculature in tumors?

Although tumor-associated angiogenesis has traditionally been defined as the sprouting of new microvessels from pre-existing microvessels, it is maintained that the blood vessels that support tumor growth or tumor rebound from therapy-induced trauma may originate from cells that are recruited from the bone marrow or that differentiate from tumor stem cells, in a process termed ‘vascular mimicry’ (6). However, considerable controversy surrounds the nature and function of bone marrow-derived circulating EC progenitors (7, 8). Furthermore, the tumor vasculature may, in many instances, contain cytogenetically abnormal ECs, which may even harbor tumor-specific genetic material. Although the biological features of these genetically abnormal EC-like cells have not been fully elucidated, it is clear that these cells can mutate to acquire drug resistance and generate drug-resistant populations more readily than normal ECs (9–14). Nevertheless, the phenotypes of genetically abnormal tumor-associated ECs are reported to be highly stable both in vitro, in human xenografts, and following serial in vivo passage (15).

It is not only the vascular endothelial cells in tumors that are affected by metronomic chemotherapy

In general, tumors are made up of neoplastic cells, ECs, smooth muscle pericytes, other perivascular cells, stromal cells, and various inflammatory cells. In a hypoxic environment, which is characteristic of most tumors, all these cell types are able to produce and release pro-angiogenic (and proliferative) factors, including vascular endothelial growth factor-A (VEGF-A), platelet derived growth factor, acid fibroblast growth factor, base fibroblast growth factor (bFGF), angiopoietins, and stromal cell-derived factor 1 (6, 16, 17). Moreover, tumor-associated macrophages, mast cells (MCs), and neutrophils are recruited and generate proliferative signals that act on fibroblasts and perivascular cells, as well as pro-angiogenic signals, all of which affect the extracellular matrix. Although ECs and platelets are exposed extensively to systemically administered drugs, all the other intra-tumoral and extra-tumoral proliferating cells in the body are affected to various extents by metronomic chemotherapy.

A complicating factor in these studies is that there is accumulating evidence to suggest that the efficacy of metronomic chemotherapy does not rely exclusively on its anti-angiogenic activities (via direct effects on ECs). Several complementary activities, such as the restoration of various anti-cancer immune responses and the induction of tumor dormancy, have been described for metronomic chemotherapy (11). Metronomic chemotherapy can also stimulate the production of endogenous anti-angiogenic factors. One example is thrombospondin, which is produced in diverse cells, including platelets, and acts as a potent and EC-specific inhibitor of angiogenesis (18–20).

As noted above, metronomic chemotherapy is now considered as a form of multitargeted therapy, which can impose long-term adverse effects, including a high incidence of secondary leukemia in children and young adults (21).
Moderately successful clinical outcomes for metronomic chemotherapy

Pre-clinical data have confirmed anti-angiogenic and/or anti-tumor effects for metronomic chemotherapy with several cytotoxic agents. Numerous clinical trials have been performed in which metronomic chemotherapies, often using a combination of two or more cytotoxic agents and including specific anti-angiogenic agents such as anti-VEGF-A antibodies, have been tested (21–24).

Even though promising results have emerged from a number of clinical trials, it is generally considered that metronomic chemotherapy has not lived up to the expectations raised by the promising anti-angiogenic and anti-tumor pre-clinical testing. In several cases, these agents have proved disappointing in phase II/III clinical studies (11, 25–28). This lack of consistency between the pre-clinical and clinical results raises questions as to whether the current pre-clinical angiogenesis and tumor models are adequate in providing useful data for clinical applications concerning metronomic chemotherapy.

One of the main limitations in this field is the current lack of relevant and reliable biomarkers (i.e., diagnostic, predictive, and surrogate markers) to enable identification of those patients who are most likely to benefit from metronomic chemotherapy (21). Furthermore, it appears that a single metronomic regimen is unlikely to have universal efficacy; the optimal combination regimens for metronomic chemotherapy remain to be determined for any given tumor type in patients (11, 21).

Drug-specific and unexpected effects of drug combinations in vivo

In dose–response experiments, several metronomically administered standard chemotherapeutic drugs significantly suppress angiogenesis, while other drugs show no effect and several drugs significantly stimulate VEGF-A-mediated angiogenesis, as observed using one and the same in vivo model, i.e., the tumor-free rat mesentery assay (29–31). In fact, low-dosage metronomic monotherapy can have dramatically different outcomes depending on the drug used: paclitaxel, vinblastine, and cyclophosphamide have anti-angiogenic effects; doxorubicin and epirubicin exert no effects on angiogenesis; and cisplatin, 5-fluorouracil, irinotecan, and mitoxantrone stimulate angiogenesis (29–33). Even at higher doses, using bolus scheduling, significant and distinct drug-specific angiogenesis-modulating effects are observed (34, 35). Clearly, there are dose-related effects with these regimens.

It is noteworthy that the angiogenesis-modulating effects of many metronomically administered drugs are significantly influenced by antioxidants, given either as a co-treatment or present as components of the vehicle, resulting in either reduction or enhancement of the drug-specific effect (29, 32). Therefore, there is a 'metronomic chemotherapy vehicle factor', which is related to the presence of antioxidants and the redox balance with the potential to affect significantly the outcome, as first observed by Albertsson et al. (29).

The finding that the administration of low-dosage 5-fluorouracil or cisplatin promoted VEGF-A-mediated angiogenesis, an effect that was not observed when 5-fluorouracil or cisplatin compounds were administered at higher doses (30, 31), might be attributed to the fact that angiogenic response to 5-fluorouracil or cisplatin is Bell-shaped (27). An alternative explanation is that the pro-angiogenic effect of metronomic mono-chemotherapy with irinotecan, mitoxantrone, cisplatin, or 5-fluorouracil is due mainly to a moderate increase in intracellular ROS, which triggers angiogenic responses through the production of pro-angiogenic factors, including VEGF-A, in the targeted ECs (and possibly other cells), as discussed below. It could alternatively relate to the drug-specific effects on platelets, which are able to release either pro- or antiangiogenic factors, as discussed below.

The original suggestion (3–5) that metronomic scheduling of almost any standard chemotherapeutic drug should suppress angiogenesis is also called into question by the finding that treatment combinations may strongly influence the efficacies of individual cytotoxic agents. In a xenograft tumor model in mice, metronomic irinotecan monotherapy suppressed tumor growth and tumor vascularity, whereas metronomic monotherapy with 5-fluorouracil or oxaliplatin had no effect on the tumor (36). Moreover, metronomic therapy with irinotecan, 5-fluorouracil, and oxaliplatin in combination had no effect on tumor vascularity (36). In a study that used the rat mesentery assay, epirubicin monotherapy did not influence VEGF-A-induced angiogenesis, whereas monotherapy with the low-molecular-weight heparin dalteparin (an anti-coagulant and antioxidant) acted to promote angiogenesis; interestingly, the treatment with a combination of these two agents significantly inhibited angiogenesis (33). Thus, combination regimens can yield surprising outcomes and they may not always be more effective than single-drug therapy (36). A possible explanation of these findings is that the combined effects of chemotherapy on individual signaling pathways are additive, synergistic, or antagonistic, and depend not only on the
oxidative anti-tumor agent/cytotoxic drug examined but also the dosages employed (1).

The extent of which these chemotherapeutic agents with or without pro-angiogenic effects pertain to tumor angiogenesis is difficult to corroborate because, as noted above, several anti-tumor modes unrelated to angiogenesis may operate in parallel during metronomic chemotherapy (21, 32).

**CELL CULTURE**

*In vitro* angiogenesis assays are useful for screening potential targets and provide an early validation step in the process of testing a new drug, owing to their rapid implementation and ease of quantitation. Extracellular matrix substitutes, such as collagen gel and Matrigel, are popular components of *in vitro* 3-D angiogenesis assays because they enable tubule formation by cultured ECs. However, these assays are usually used with a single cell type, which lacks the complex multicellular interactions that are essential for angiogenesis.

**Cell selection, phenotypic alteration, and oxidative stress**

Cell culture studies are used to investigate the molecular effects and mechanisms associated with ECs or tumor cells following exposure to low doses of a chemotherapeutic agent(s) in the growth medium. It should be remembered that the establishment of an EC line necessarily entails an initial selection of the EC population from the site of isolation, with additional rounds of selection occurring during subsequent subculturing. It should also be noted that ECs, as is the case for all cells, undergo phenotypic alterations *in vitro* when incubated in media that contain growth factors and other components, to which the cells adapt.

Multiple enzymes that use molecular oxygen as a substrate generate ROS. In cells, there is a fluctuating redox balance between the effects of ROS and enzymatic or non-enzymatic antioxidant systems. Various non-enzymatic molecules (e.g., glutathione, vitamins A, C, and E, and flavonoids), as well as enzymatic scavengers of ROS (e.g., superoxide dismutase, catalase, and glutathione peroxide) act to reduce or balance the intracellular levels of ROS (1).

For conventional culturing techniques, there is an increase in the level of ROS because the O$_2$ pressure is dramatically increased: normal culture conditions are basically a state of hyperoxia. Most cells in the human body are exposed to O$_2$ pressure in the range of 1–10 mmHg, whereas for conventional culture conditions with 95% air and 5% CO$_2$, the O$_2$ pressure is dramatically increased to 150 mmHg (37).

The largest proportion of intracellular ROS production occurs in the mitochondria (38, 39). Various exogenous agents, including chemotherapy, trigger intracellular ROS generation, thereby disrupting the cellular redox balance homeostasis with consequences for cellular functions, as ROS act as intracellular mediators (see below). An excess of ROS causes oxidative stress, and a high level of oxidative stress harms cells. Accumulation of ROS within cells and/or their release into the culture medium is highly specific for the cell type being examined (40).

**Culture medium-specific effects on ROS production**

In general, basic cell growth media are deficient in antioxidants. The amount of ROS produced during growth in culture medium depends not only on the cell type but also on the composition of the medium and whether the culture is incubated in light or (to a lesser extent) in the dark (41–43). The buffering capacity and composition of the medium are important with regard to the behavior of the cultured cells and may affect significantly the results (44). In fact, it has been suggested that cell studies using different cell growth media are scarcely comparable (43).

**ROS act as intracellular mediators**

At physiologically low or moderate levels, ROS act as signaling molecules in essential metabolic pathways, including the induction of gene expression (1, 45) of various growth factors, including VEGF-A, which is a key pro-angiogenic and cell survival factor (45–50). Notably, the combined effects of individual metabolic pathways can be additive, synergistic, or antagonistic, and depend not only on the oxidative agent/anti-tumor agent examined but also the dose employed and the cell type in which they are analyzed, which might explain the pre-clinical finding of antagonism in relation to angiogenesis reported by Fioravanti et al. (36), as discussed above.

Typically, low levels of ROS, particularly those of hydrogen peroxide (H$_2$O$_2$), promote cellular proliferation, whereas ROS levels reduced with the aid of potent antioxidants to below the homeostatic set point may inhibit the physiologic role of oxidants in cellular proliferation (1, 39). ROS at high levels have a propensity to push the cell to the brink of toxicity and even to eventual apoptosis and cell death (51, 52).
Cell types and variables that are commonly studied in relation to angiogenesis

In cultures of ECs, the variables of cell proliferation, differentiation, migration, tube formation, growth factor production, altered gene expression, and apoptosis are commonly studied. The interactions of ECs with the surrounding supporting cells, such as fibroblasts, and extracellular matrix substitutes have been studied (28). The microvascular EC is often the preferred cell type for these studies. However, it should be remembered that there is genetic heterogeneity among the microvasculature across different sites (53), which may complicate comparisons between different microvascular cell lines.

Cultured tumor cells are used to investigate proliferation and the extent to which low doses of chemotherapeutic agents affect the receptor repertoire and production of growth factors, particularly VEGF-A. Moreover, the results of such experiments are often uncertain as tumor cells basically have markedly increased oxidative stress levels compared with tumor cells usually seen in vivo (39, 54).

Can the in vitro procedures be improved?

Modification in simple experimental variables, such as the choice of medium buffer, the control of exposure to light, the pH of the medium, and the amount of ROS in the medium (40, 43), as well as oxygen-controlled cultures (55) could arguably improve the outcomes from a biological point of view. If all of these factors were controlled, the results obtained from in vitro studies on angiogenesis would be more reproducible and thereby more useful for the design and interpretation of animal experiments and clinical trials.

EX VIVO ASSAYS (ORGAN CULTURE)

The ex vivo assays are all similar in that segments, disks, or sections of a specific tissue type are cultured in a 3-D matrix in vitro and are monitored for microvessel outgrowth over a period that can extend up to a couple of weeks (28). A major problem with all these assays is that they employ non-human tissues, which raises questions as to their applicability as pre-clinical screening assays, given that the responses to various drugs or test substances may be species-specific (28).

Although not a traditional ex vivo assay, the exteriorized rat mesentery has been used for real-time observations of the mesentery; this model has proven to be extremely useful in identifying novel cellular events in angiogenesis (56, 57).

INFLAMMATION INTERFERES WITH ANGIOGENESIS AND ANTI-ANGIOGENESIS RESPONSES TO DRUGS IN VIVO

Angiogenesis is a hallmark of both hypoxia and inflammation. Inflammation occurs in various forms in response to different host tissue injuries, such as hypoxia, tissue trauma (including implantation of foreign material) that induces wound healing reactions, infections, and tumor development (58). In reaction to tissue injury, ROS are generated and a multifactorial network of chemical signals initiates and maintains host responses that are designed to 'heal' the affected tissue. This process involves the activation and directed migration of inflammatory cells, which include neutrophils, monocytes, and eosinophils, from the venous system to the sites of damage; monocytes differentiate into macrophages after extravasation from the circulation into tissues.

Among the innate immune cells, macrophages, MCs, and dendritic cells (DCs) serve as sentinel cells, residing in tissues and continuously monitoring the cellular microenvironment for signs of distress. When tissue homeostasis is disturbed, the sentinel cells release soluble mediators that act singly or in combination to induce the mobilization and infiltration of neutrophils and other leukocytes into the damaged tissue. Macrophages and MCs also induce metabolic and proliferative fibroblast responses and are able to initiate and sustain potent angiogenic activities (59–63). Once activated, macrophages are considered to be the main source of growth factors and cytokines, which exert profound effects on ECs and other cells in the local microenvironment. Plasmacytoid DCs are able to support angiogenesis (64), whereas tumor angiogenesis is reported to depend largely on the activities of immature DCs (65). In certain tumors, MCs are essential for the development of angiogenesis (66, 67). MC-mediated angiogenesis was discovered using the rat mesentery angiogenesis assay (61, 68).

In phylogenetic terms, the MC represents an ancient cell type found in all species that have a blood circulatory system, and it precedes lymphocytes and other cells of the immune system (69). It has been suggested that the MC is essential for promoting and orchestrating inflammation (69), so it may also be involved in orchestrating inflammation-induced angiogenesis. MCs are important cells owing to their release of stored and newly synthesized inflammatory mediators and growth factors, which include histamine (and serotonin in certain species), cytokines, such as VEGF, bFGF, IL-1, IL-6, IL-8, and TNF, proteases, lipid mediators, and heparin, many of which can individually promote angiogenesis (61, 66, 70).
Clearly, using inflammatory activity-inducing experimental procedures, the effect of any given test agent or treatment regimen is distorted to an unknown degree by the artifactually induced angiogenesis. Unfortunately, most mammalian in vivo angiogenesis assays employ procedures that cause cellular damage to the test tissue, thereby initiating some level of non-specific angiogenesis, which diminishes the sensitivity of the assay.

ANALYSIS OF ANTI-ANGIOGENESIS IN TUMORS IN VIVO IS PARTICULARLY CHALLENGING

To date, no method has been developed that accurately assesses the anti-angiogenic effect per se in a tumor, as inhibition of angiogenesis limits tumor growth and vice versa, which is a major impediment to the clinical development of many anti-angiogenic drugs. Furthermore, there is a severe lack of reliable biological surrogate markers of anti-angiogenic effects in tumors. Assessing angiogenesis in cancer patients is currently limited to the use of functional measures derived using various imaging modalities (6, 71).

An often-used method for quantifying tumor vascularity, and purportedly angiogenesis, involves the measurement of microvessel density (MVD) based on immunohistochemical visualization of EC epitopes, such as CD34 or CD105, in excised tissue samples or in superficially located tumors. Although tumor MVD analysis of biopsies is a useful prognostic indicator for patients with most types of cancer, it is not suitable for measuring angiogenic activity or assessing the angiogenic dependence of a tumor (72). Therefore, the possibilities to assess angiogenesis and anti-angiogenic effects in patients are currently very limited.

Rodent studies are vital for the development of novel anti-cancer therapeutics and are used in pharmacokinetic, pharmacodynamic, toxicology, and efficacy studies. The mouse is undoubtedly the most widely used species in these experiments, primarily because various inbred and genetically engineered or manipulated strains are available.

Syngeneic mouse tumor models

Tumor cell lines, which are propagated in culture for a long period of time before being injected into the mouse, are frequently used. As a rule, these tumors grow very rapidly and are highly aneuploid, which makes them quite dissimilar to the types of tumors generally seen in clinical practice.

Xenograft tumors

Using immunocompromised animals, non-syngeneic tumor cells are injected as a bolus into a tissue that is disrupted and disorganized by the ectopic transplantation of a high number of foreign cells. As the tumor transplant may initially be severely hypoxic, angiogenesis is highly dependent on expression of the Id gene, which exerts a selection pressure on the tumor cell population. When human tumor cells or tumor tissue pieces are implanted into immunocompromised animals, the developing xenograft tumor stromal cells, extracellular matrix, blood components, sentinel immune cells, and any EC progenitors recruited from the bone marrow all originate from the host. The tumor endothelium is probably mainly of host origin.

Orthotopic tumors

Implantation of cultured tumor cells into the organ of origin is thought to allow organotypic interactions between the tumor cells and surrounding stroma. Intratumoral lymphangiogenesis is absent from orthotopic and xenograft tumors, which may affect experimental end-points, such as the occurrence/timing of metastasis, tumor progression, and survival.

Autochthonous tumors

Autochthonous tumors originate in the place where they are found and feature a physiologic stroma–tumor relationship, as in human cancer. There are major differences in angiogenesis between transplanted or xenograft tumors and autochthonous tumors (25, 66, 73). The usefulness of xenograft models for efficacy testing has been questioned, whereas tumors in genetically engineered mouse models (GEMMs) may offer advantages with respect to efficacy assessments (74, 75), as these latter tumors arise autochthonously. GEMM tumors occur in animals that have an intact immune system and unperturbed DNA repair mechanisms. Moreover, differences in the gene expression of tumor-associated immune sentinel cells, such as macrophages, between xenografts and GEMMs have been observed (75).

Metastasis

Tumor cell spread to and tumor growth at secondary sites are extremely important clinical events, as the majority of cancer-related mortality is associated with metastatic tumors, rather than the primary tumor. Unfortunately, there is a lack of reliable pre-clinical metastasis models, although in many
orthotopically implanted models, metastasis occurs but it is very heterogeneous and not detectable in all animals after implantation. Metastasis models involving autochthonous tumors are eagerly awaited.

Mouse strain-specific characteristics
In addition to species-specific features, strain-specific differences with respect to various features that influence angiogenesis have been reported in mice, which can complicate comparisons between experiments. There are significant differences in the responses to pro-angiogenic factors among inbred mouse strains (76). There is a striking correlation between highly genetically heterogeneous bFGF- or VEGF-A-induced angiogenesis and the intrinsic levels of circulating EC progenitors among different inbred mouse strains (7). In addition, the genetic backgrounds of inbred strains determine the inflammatory-induced angiogenesis responses in these mice (77, 78). Similar strain-specific differences can be expected in other species.

COMPARING AND EVALUATING PRE-ClinICAL IN VIVO ANGIOGENESIS ASSAYS IN TERMS OF THEIR USEFULNESS IN GUIDING CLINICAL TRIALS

The key to generating accurate dose–response curves is having knowledge of the release rate and the spatial and temporal concentration distributions of the exogenously administered pro-angiogenic or anti-angiogenic test agent(s). However, this is very difficult to control in any of the currently used models. A complicating fact is that the dose–response curves can be Bell-shaped or U-shaped, as discussed elsewhere (27). Thus, unambiguous dose-effect studies are very rare, if they exist at all, and stringently controlled dose-effect studies that compare two or more assays in parallel have not been published to our knowledge. Each experimental approach offers specific advantages and suffers from certain limitations.

Administration routes and scheduling of test agents
The simplest way to administer a test agent is via the oral route, whether this is performed experimentally or in the clinical setting. If oral delivery works, it becomes the favored route. However, the injection of test agents subcutaneously, intravascularly, or intraperitoneally is more common. Through injection, a controlled rate and dosage of the test solution can be achieved over a relatively long time period per setting; osmotic minipumps that release test solution at a constant rate are used extensively in mice and rats. Dosing scheduling of chemotherapeutics can thus vary considerably across experiments. The perfect scheduling regimen for each test agent relates to its toxicity, pharmacokinetic, and pharmacodynamic properties. Conclusive studies on the influence of scheduling on angiogenesis-modulating effects of metronomic chemotherapy remain to be performed.

Experimentally related disarray of the model
The impact of unintentional inflammation-induced angiogenesis, which may confound the results of metronomic chemotherapy, should be minimized or at least taken into account when the data are interpreted. Furthermore, disruption of the microvascular basal membranes during tissue trauma is a pro-angiogenic cue. The toxic effects of drugs should be kept under strict control, as toxicity may mask specific angiogenesis-modulating drug effects. However, toxic effects in terms of retarded or decreased body weight are easily overlooked in species that exhibit very slow physiologic growth during adulthood (e.g., mice), whereas these effects are more noticeable in rats, which exhibit robust physiologic growth in adulthood.

Data acquisition and verification
It is important to be able to assess the combined effect on angiogenesis of two or more drugs that are administered simultaneously or sequentially systemically, not only for the evaluation of scheduling of combination therapies but also because strong antagonism may emerge from metronomic chemotherapy (36). For molecular-structure analyses, truly quantitative and sensitive assays are required. The value of data obtained in one species is greatly increased if the data are confirmed in a second mammalian species. In this regard, the current predominant use of a single species (i.e., mice) in angiogenesis studies is a matter of concern.

Use of vascularized test tissues to take into account effects of circulating cells and platelets
A major obstacle to the progress of research on angiogenesis in vivo is that measurements of neoangiogenesis are confounded by the presence of well-developed vascular networks in virtually all mammalian tissues. Nevertheless, assays of vascularized tissues are needed to take into account the contributions of platelets and other circulating cells, including EC precursors from the bone marrow (7),
which promote angiogenesis. Platelets accumulate angiogenesis-regulatory proteins in two sets of alpha-granules, with positive regulators in one set and negative regulators in the other set, which may be released separately (79, 80), depending on the type of therapeutic molecule used. These two populations of alpha-granules are distinct in terms of pharmacologic and morphologic features (79). The overall effect on tumors of platelet–endothelium interactions is thought to stimulate tumor angiogenesis (81).

**IN VIVO ANGIOGENESIS ASSAYS**

Is it conceivable that tumor-free models can provide pertinent information regarding tumor anti-angiogenic and tumor-suppressing effects in the clinical setting?

Almost all current assays are based on the analysis of tumor-free tissues. As the inhibition of angiogenesis limits tumor growth and vice versa, it can be argued that studies on the angiogenesis-modulating effects of any potent anti-tumor therapy should be conducted in a tumor-free tissue. It is notable that the ECs in the human tumor vasculature have a gene expression pattern that resembles that of angiogenically activated ECs in normal human tissues (82).

In vivo angiogenesis reflects the complex, stepwise, multifactorial, multicellular process that leads to the formation of new blood microvessels, which cannot be mimicked by in vitro studies. Although there are numerous in vivo angiogenesis assays and models, their relevance from a clinical perspective remains to be demonstrated unequivocally; thus, no currently used in vivo assay is optimal. The merits and limitations of the current in vivo angiogenesis assays have been reviewed repeatedly (28, 70, 83, 84), and it would appear that the criteria listed in Table 1 define high-quality assays from the biological standpoint.

In Table 2, some examples from the literature are given of important mammalian models, as well as the embryonic avian chorioallantoic membrane (CAM) assay. The microsurgical corneal, CAM, and Matrigel assays are probably the most widely used angiogenesis models, and the results obtained using these models have been extensively tested in clinical trials. There is also an interesting model, which is adapted from the Matrigel plug assay, in which human blood-derived endothelial progenitor cells and mature smooth muscle cells are delivered subcutaneously into immunodeficient mice, resulting in the de novo formation of a vascular network and the development of functional anastomoses with the host circulatory system (85).

**Table 1. Desired features of biologically appropriate in vivo angiogenesis assays**

| Feature                                                                 | Requirement                                                                 |
|------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| Mammalian-based, so as to capture responses to mammalian proteins (100) | Natively vascularized, to incorporate the effects of circulating endothelial progenitor cells and platelets |
| No significant angiogenesis occurring in the adult test tissue if de novo angiogenesis is to be studied |
| Displaying minimal non-specific, artifactually induced inflammation, as inflammation induces angiogenesis, which may interfere with the results |
| Truly quantitative in terms of microvessel formation, which is a prerequisite for molecular-activity and dose-effect studies, although analyses of dose-effect responses in a strict sense are probably not yet achievable, as discussed in the text |
| Allowing the recording of concurrent influences of two or more test agents administered systemically in parallel or sequentially, given that antagonistic or unforeseeable effects may occur (33, 36) |
| Test animals should have mature liver function for metabolizing drugs |
| Any significant toxic influence of the treatment should be measurable |
| High-quality animal facilities and experimental procedures are highly recommended |
| Ethically acceptable |

The rat mesentery angiogenesis assay (Table 2) distinguishes itself from the other mammalian assays in that it is non-surgical (non-traumatic with minimal or no inflammation induced), the test tissue is naively vascularized (albeit sparsely), and it lacks physiologic angiogenesis in adulthood (70, 86). This assay allows the quantitation (in exceptional detail) of angiogenesis with respect to a number of objective parameters, owing to the fact that the membranous test tissue is extremely thin and can be analyzed microscopically in intact form. Signaling events that are measured in the mesentery after stimulation with VEGF-A can be attributed with confidence to events that take place in the ECs (87). Moreover, when syngeneic ascitic tumor cells are transplanted into the mouse peritoneal cavity, the mesentery exhibits a complete repertoire of biological responses that can be attributed to VEGF-A, including sprouting angiogenesis (87).

A case study: iron-unsaturated bovine lactoferrin inhibits angiogenesis in rats, mice, and chicken embryos, inhibits tumor angiogenesis, carcinogenesis, and metastasis in mice and rats, and it retards the growth of precancerous adenomatous colonic polyps in humans

Lactoferrin (LF) is an iron- and heparin-binding glycoprotein that is present in most biological fluids of mammals, including milk (colostral and mature),

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| Assay                  | Advantage(s)                                                                                           | Disadvantage(s)                                                                                     |
|-----------------------|--------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
| CAM                   | Technically simple to conduct both in ovo and in vitro                                                | Avian embryonic tissue, in which all tissue cells proliferate                                        |
|                       | Test agent applied via a carrier onto the CAM surface                                                  | Sometimes difficult to distinguish angiogenesis from artifactual increases in blood vessel density linked to tissue contraction caused by the applied carrier |
|                       | Suitable for large-scale screening experiments                                                         | Very sensitive to increases in oxygen tension; production of ROS                                     |
|                       | Permits non-invasive observations                                                                    | Sprouting angiogenesis is followed by intussusceptive microvessel growth                            |
|                       | Permits biochemical and genetic analyses                                                              | Pro-angiogenic treatment accelerates and anti-angiogenic treatment suppresses constant organogenic angiogenesis |
|                       | Suitable for mammalian xenografts until EDD 15–18                                                   | Induction of (de novo) angiogenesis is possible only after EDD 12–13                                |
|                       | Inexpensive                                                                                            | The tissue is overly sensitive to inflammatory angiogenesis                                          |
|                       | Drugs can be administered topically onto the CAM, injected i.v. (difficult), injected i.p. into the body of the embryo or injected into the yolk sac and amnion | Drugs that require metabolic activation cannot be assessed due to liver immaturity                    |
|                       | Lack of excretion from the CAM allows test agents to be maintained in the circulation for extended periods | The relevance to human angiogenic diseases is limited; non-mammalian species, which may respond differently to mammalian proteins (100) |
| Corneal micropocket²,³ | New vessels, except the smallest microvessels, are, in principle, easily identifiable                   | Atypical angiogenesis, as the normal cornea, is avascular because of the trapping of soluble VEGFR-1 |
|                       | Mammalian model: mice, rats, and rabbits are used                                                      | Technically demanding, especially in animals with small eyes, as in mice                            |
|                       | Permits non-invasive observation                                                                     | The surgery- or suture-induced lesion causes inflammation and angiogenesis                           |
|                       | Quantitative assessment is reported                                                                   | Toxicity within the micropocket area is difficult to assess                                        |
|                       | Immuno logically privileged site before vascularization, allowing tumor implantation                   | Expensive                                                                                           |
|                       | Angiogenesis by sprouting                                                                             | Ethically questionable as the cornea is a sensory organ                                            |
| Mesentery¹,²           | Mammalian adult tissue; natively vascularized; lacks significant physiologic angiogenesis              | The cornea is not a highly relevant site for tumor growth                                           |
|                       | The test tissue is visceral; visceral organs are common sites of primary cancers and metastasis        | Only a few substances can be tested in one setting                                                  |
|                       | Minimal trauma, if any, is inflicted upon the test tissue by i.p. injection of the test solution       | Exposure to oxygen via the corneal surface causes angiogenesis through the generation of ROS        |
|                       | Truly quantitative, allowing dose–response and molecular-activity studies⁴ with respect to spatial extension, density, length of individual microvessel segments, and sprouts in situ⁵ | Time-consuming, especially when assessing the numbers and lengths of individual microvessel segments and sprouts Does not allow real-time observations⁶ |
|                       |                                                                                                       | Mice are much less suitable than rats for quantitative angiogenesis                                 |

Table 2. Critical synopsis of a selection of the main currently used mammalian in vivo angiogenesis assays, as well as the chick chorioallantoic membrane (CAM) assay, listed in chronologic order of their introduction.
Table 2. (continued)

| Assay               | Advantage(s)                                                                 | Disadvantage(s)                                                                                           |
|---------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| Matrigel plug²,³    | Technically simple when used s.c.                                            | Matrigel is not chemically defined and contains growth factors (even in the growth factor-reduced form) |
|                     | Rapid quantitative analysis assessing vascular-specific tissues in chambers  | Difficult to make plugs in a uniform 3-D shape (except in chambers)                                      |
|                     | with 3-D defined plugs                                                        | No angiogenic response to VEGF in chambers                                                                |
|                     | Suitable for large-scale screening                                           | Analysis of plugs is time-consuming for tissues other than vascular-specific tissues                     |
|                     |                                                                               | Does not allow real-time observations                                                                   |
|                     |                                                                               | The s.c. tissue is not highly relevant for tumor growth                                                  |
|                     |                                                                               | Plugs lack cells that are able to produce endogenous pro- and anti-angiogenic factors, which would affect |
|                     |                                                                               | vascular responses                                                                                       |
|                     |                                                                               | Expensive                                                                                                 |

Compiled from the following references: (28, 70, 83, 84).

EDD, Embryonic development day; s.c., subcutaneous; i.p., intraperitoneal; i.v., intravascular; VEGF, vascular endothelial growth factor.

The CAM assay was introduced by Auerbach et al. in 1974 (101), the corneal micropocket assay by Gimbrone et al. in 1974 (102), the rat mesentery assay by Norrby et al. in 1986 (68) [the assay has recently been demonstrated and discussed in a DVD movie with added detailed protocols in an Open Access journal (86)], and the Matrigel assay was introduced by Passaniti et al. in 1992 (103).

¹These analyses can also readily be performed using the mesentery.
²Inbred and outbred mouse and rat strains are available.
³Genetically engineered or immunocompromised mice are available.
⁴As with all current angiogenesis assays, the release rate and the spatial and temporal distributions of exogenous pro- and anti-angiogenic test factors are not fully known, so uncontestable analyses of dose-effects are not feasible.
⁵This enables for the first time the large-scale accurate counting and measurement of representative populations of individual sprouts and individual microvessel segments in any tissue.
⁶Can be used for real-time observations following exteriorization of the mesentery; the model has been proven extremely useful in identifying novel cellular events in angiogenesis (57, 58, 104). However, the homeostasis of the tissue is rapidly disturbed outside the body, as discussed previously (70).

saliva, tears, and mucous, and it is released from activated neutrophils during inflammatory responses. The iron-unsaturated form of LF, termed apoLF (aLF), appears to be the dominant form of LF in vivo. ApoLF exerts antioxidant activity, is multifunctional and immunostimulatory, and exerts an antimicrobial activity thanks to a specific affinity for bacterial cell membranes (88). When LF becomes iron-saturated, remarkable changes occur in the 3-D structure of the molecule, its molecular flexibility and activities (89), including loss of antimicrobial action. Bovine and human LF have been shown to be non-toxic even after long-term oral administration to rats (90, 91).
Bovine HαLF (abLF) and lactoferricin, a basic peptide of LF, although non-cytotoxic, inhibit the in vitro proliferation of human macrovascular ECs (HUVECs) in terms of both basal proliferation and proliferation in response to bFGF or VEGF165 (92, 93).

Metronomically scheduled ingestion of natural bovine HαLF (abLF) has been shown to suppress significantly VEGF-A-mediated angiogenesis in the rat mesentry assay (92). Angiogenesis in the CAM assay was inhibited by local application of abLF, whereas metronomic oral or intraperitoneal administration of abLF inhibited cancer cell-induced angiogenesis in a murine dorsal air sac model (94).

Following oral intake, pepsin degradation of LF yields lactoferricin, LFcin. Bovine lactoferricin, bLFcin, inhibits bFGF- and VEGF-A-induced angiogenesis in mice by competing for heparin-like binding sites on ECs, as assessed using the subcutaneous Matrigel plug assay (93). Systemic administration of abLF or bLFcin suppresses carcinogenesis in many organs, and inhibits metastasis formation in mice and rats (95–97). Metronomic ingestion of bLF by transgenic mice carrying the human VEGF-A165 gene, which spontaneously develop autochthonous pulmonary tumors, suppresses the expression of VEGF-A and tumor development (98). Moreover, the ingestion of HβLF (abLF) significantly delays the growth of precancerous adenomatous colonic polyps in patients (99).

These results confirm that the anti-angiogenic effects recorded in tumor-free models, such as the rat mesentery assay or the subcutaneous mouse Matrigel assay, can, under certain conditions, be valid for tumor angiogenesis and tumor growth suppression in several species, including humans.

CONCLUDING REMARKS

Based on the available experimental and clinical data, it seems worthwhile to attempt to exploit the full potential of metronomic chemotherapy for clinical use. An initial requirement for reaching this goal is the introduction of biologically upgraded pre-clinical assays for both in vitro and in vivo analyses. These assays should have the ability to provide data that can be used with confidence to guide the design of more efficacious clinical trials. A second requirement is the introduction of reliable biomarkers for the assessment of anti-angiogenic responses in patients.

With respect to in vitro studies, it appears that reproducible and biologically more reliable results could be achieved by rectifying the culturing procedures that result in artifactual effects in the cells. Critical choices are needed to be made regarding the growth medium buffer, optimal control of the pH of the medium, and control of the oxygen pressure, which would reduce both the risk of super-physiologic intracellular ROS generation and the abnormal level of ROS in the medium. These adjustments could be achieved by employing techniques that already exist but which are not commonly employed collectively. An increased level of ROS, i.e., oxidative stress, activates numerous major signaling pathways and changes the gene expression profile of the cells. Adjustments along these lines in relation to in vitro procedures would facilitate comparisons between experiments and arguably strengthen the rationale for further animal and clinical studies. In particular, the issue of redox balance is bothersome when chemotherapeutic agents are involved, as these agents potently trigger ROS production, thereby further modifying the functions of the cultured cells. Co-cultures of ECs and other cell types, particularly perivascular cells and fibroblasts, are important in that they imitate the complex intercellular activities of angiogenesis in vivo.

Characteristics that, in our view, exemplify high-quality in vivo angiogenesis assays are listed in Table 1. This list should probably be extended. Novel or upgraded assays lacking injury-induced wound healing and inflammatory responses, which blur results concerning anti-angiogenic processes, are eagerly awaited. It is likely, we believe, that mammalian high-quality in vivo assays will generate biologically pertinent information, which will assist in the design of successful clinical trials.

Improved pre-clinical in vivo models might be helpful in identifying bona fide biomarkers that are useful for the appraisal of anti-angiogenic responses in patients. The challenging pre-clinical findings of strict drug-specific effects (anti-angiogenic or no effect on angiogenesis), and in several cases even pro-angiogenic effects, for standard cytotoxic drugs, as well as the antagonistic and unforeseeable effects of drug combinations following metronomic chemotherapy require further study. Although the experience from clinical trials speaks for the use of combination therapies, one may wonder if there is a need to re-examine the rationale behind metronomic combination drug therapies, which hitherto have not been especially successful in the clinic.

The recently reported effect of antioxidants on the angiogenesis-modulating outcome of metronomic chemotherapy, including the significance of the ‘metronomic chemotherapy vehicle factor’, is also a topic that needs further consideration.

As the focus is on the anti-angiogenic and consequential anti-tumor effects of metronomic
chemotherapy, appropriate pre-clinical tumor models are required. The widely used syngeneic (non-orthotopic or orthotopic) and xenograft tumors are considered to be of questionable value, as they differ substantially in many critical biological respects from the majority of tumors seen in clinical practice. Spontaneously developing autochthonous tumors in genetically engineered mice, which exhibit unperturbed DNA repair mechanisms, seem to be considerably more appropriate models of human cancers, as they exhibit a natural stroma–tumor relationship. Moreover, these tumors allow important studies on the influence of stem cells on angiogenesis, and as they develop de novo, they are less artificial in terms of growth characteristics and genetic abnormalities than other currently used pre-clinical tumor models.

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