Mini-review

Does metronomic chemotherapy induce tumor angiogenic dormancy? A review of available preclinical and clinical data

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Abstract

Tumor dormancy is the ability of cancer cells to survive in a non-proliferating state. This condition can depend on three main mechanisms: cell cycle arrest (quiescence or cell dormancy), immunosurveillance (immunologic dormancy), or lack of functional blood vessels (angiogenic dormancy). In particular, under angiogenic dormancy, cancer cell proliferation is counterbalanced by apoptosis owing to poor vascularization, impeding tumor mass expansion beyond a microscopic size, with an asymptomatic and non-metastatic state. Tumor vasculogenic or non-angiogenic switch is essential to promote escape from tumor dormancy, leading to tumor mass proliferation and metastasis. In avascular lesions angiogenesis process results blocked from the equilibrium between pro- and anti-angiogenic factors, such as vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1), respectively. The angiogenic switch mainly depends on the disruption of this balance, in favor of pro-angiogenic factors, and on the recruitment of circulating endothelial progenitors (CEPs) that promote the formation of new blood vessels. Metronomic chemotherapy, the regular intake of doses able to sustain low but active concentrations of chemotherapeutic drugs during protracted time periods, is an encouraging therapeutic approach that ~~have~~has shown to upregulate anti-angiogenic factors such as TSP-1 and decline pro-angiogenic factors such as VEGF, suppressing the proangiogenic cells such as CEPs. In this perspective, metronomic chemotherapy may be one of the available therapeutic approaches capable to modulate favorably the angiogenic tumor dormancy, but further research is essential to better define this particular characteristic.

Keywords: Angiogenic switch; Low dose chemotherapy; Thrombospondin-1; Vascular endothelial growth factor; Circulating endothelial progenitor

1 Introduction

Tumor dormancy is a condition characterized by the presence of non-symptomatic and non-invasive cancer cells which are diagnostically undetected for a long time. Accordingly, autopsies from people who did not die for tumors showed the accidental detection of non-expanding microscopic primary cancer, the occurrence of these non-invasive dormant cells being considered “normal”. This condition of hidden cancer represents the early stage of tumor development, but it can also account for tumor recurrence after a successful treatment, as well as micro-metastases. Both experimental and clinical studies suggest that tumor cell spreading can occur at very early stages and that these disseminated cells can remain dormant for decades. More in depth, the varying period of dormancy which characterizes the different cell lines has been termed *dormancy clock* by Naumov et al. [1]. Tumor dormancy is important in both primary and secondary tumors, but its particular features impede to have appropriate experimental models and clinical accessibility [2–4].

In this respect, tumor dormancy is clinically relevant when considering its involvement in the occurrence of non detectable primary tumors (*primary dormancy*) or metastatic recurrence after primary tumor excision (*metastatic dormancy*). Since dormancy just defines the condition of non symptomatic cancer lesions, autopsy investigations on subjects died after car accidents or trauma represent a useful approach to explore the rate of dormant tumors in apparently healthy population. These studies showed a surprisingly high number of microscopic clinically unapparent tumors. For instance, in the case of breast or prostate cancer, about 40% of autopsied subjects were found positive for an *in situ* carcinoma against about 1% of diagnosis during life in the same age range. This difference is particularly striking in the case of thyroid cancer, found in dormant state in quite all autopsied aged individuals who died of trauma, while only 0.1% were diagnosed in their age-compared lifetime. These shocking data claim a particular concern when considering transplantations. Tumor dormancy also accounts for long latency periods between treatment of primary tumors and occurrence of metastatic spreading. This would depend on tumor heterogeneity, including proliferation and apoptosis rates, epigenetic variability, adaptability to the environment or genetic mosaicism. Finally, it cannot be excluded that different growth laws underlie primary and metastatic dormancy [5,6].

Tumor dormancy may be referred to a single cancer cell (*tumor cell dormancy*) which lies in cell cycle arrest (G0-G1 arrest), or to a few proliferating cancer cells (*tumor mass dormancy* or *population-based dormancy*). These two types of dormancy have completely distinct conditions that significantly differ in their characteristics and regulatory mechanisms [4,7]. The tumor mass dormancy is modulated by immune system, with a dynamic equilibrium between cell proliferation and apoptotic death (*immunologic dormancy*), or insufficient blood supply (*angiogenic dormancy*), [8,9].

Accordingly, three mechanisms have been recognized as responsible factors for the occurrence of untraceable cancer cells: i) cellular dormancy (quiescence, mitotic arrest; intrinsic dormancy), ii) immunologic dormancy (immunosurveillance; equilibrium dormancy) and iii) angiogenic dormancy (limited tumor size; extrinsic dormancy) [7,10,11] (Fig. 1).

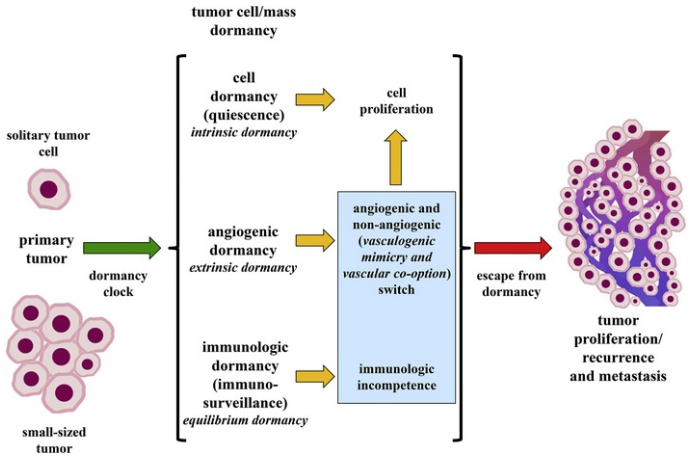

Fig. 1 Schematic illustration that shows three main mechanisms regulating tumor cell/mass dormancy and escape from dormancy, with particular reference to angiogenic and non-angiogenic switch. Tumor dormancy can result from cell cycle arrest (cell dormancy, quiescence), lack of functional blood vessels (angiogenic dormancy), or immunosurveillance (immunologic dormancy).

alt-text: Fig. 1

The understanding of the interactions occurring in the microenvironment, in particular with the extracellular matrix, is determinant in contrasting tumor growth. The tumor microenvironment includes all cellular and extracellular components surrounding cancer cells. The expression of some receptors, including those for urokinase and epidermal growth factor (EGF), has been associated to the regulation of tumor dormancy [12–14]. In this respect, the microenvironment has been classified as dormancy-permissive or dormancy-restrictive, this distinction accounting for the different incidence of metastases and disseminated tumor cells in the same organ (“seed and soil” theory) [8,15].

The immune system plays a fundamental role in suppressing cancer growth [16]. However, inflammation has been considered a promoting factor in tumor development, since it associates to the release of cytokines during some immune reactions, triggering angiogenesis-mediated escape from dormancy [17]. According to the immune editing hypothesis, tumors go through a phase of elimination, followed by a phase of equilibrium and a phase of escape, with the evolutionary pressure of immune mechanisms resulting in immune escape.

Other factors that favor the permanence of cancer in a dormant state include the hormonal withdrawal and the inhibition of angiogenesis [4,18,19]. Apart from chemotherapy, various dietary components, in terms of food intake, energy balance and physical activity, might also influence cancer cells and their microenvironment. Indeed, several dietary phytochemicals can affect the behavior and gene expression patterns of both tumor cells and host tissues [20]. Other mechanisms have been involved in tumor dormancy regulation, such as genetic and epigenetic changes, cancer stem cells, epithelial-mesenchymal transition, and non-coding RNA manipulation [10].

2 Angiogenic tumor dormancy

Angiogenesis is a process characterized by the formation of new capillaries from pre-existing blood vessels and occurs in both physiological (embryogenesis, ovulation, and wound healing and repair) and pathological conditions (arthritis, diabetic retinopathy and tumors). The development of solid tumors begins with a small non-angiogenic cellular aggregate which cannot grow until a vascular network is established. Indeed, tumor growth is thought to be angiogenesis-dependent and the inhibition of blood vessel formation is a possible mechanism for anticancer therapy [21]. Accordingly, failure in one or more of the angiogenic steps leads to tumor dormancy [4]. Abnormal angiogenesis is necessary to trigger tumor growing and expansion [22]. However, blood supply to the proliferating tumor can be provided also in a non-angiogenic manner. In this respect, two main mechanisms have been identified and described:

vessel co-option, consisting in cancer cells that infiltrate normal tissues to exploit pre-existing normal vessels; *vasculogenic mimicry*, consisting in cancer cells that can differentiate into structures that act like blood vessels, with the formation of intra-tumor perfused channels [23]. These two conditions can coexist in the same tumor, suggesting that cancer cells can readdress their proliferation program, according to the local environment or administered therapy. Then, tumor development and metastasis can recognize both angiogenic and non-angiogenic escape and this accounts for the failure of anti-angiogenic therapy when treating such tumors [23].

2.1 Pro-angiogenic and anti-angiogenic factors

Normal angiogenesis depends on the equilibrium between pro- and anti-angiogenic factors. The presence of a pro-angiogenic factor, named tumor angiogenic factor (TAF), was hypothesized more than 45 years ago by Folkman et al. [24,25], who also proposed anti-angiogenic therapy as a new strategy to treat cancer disease. Later, the scientists discovered that the factors able to activate and regulate angiogenesis were far more than just the one hypothesized by Folkman, including basic fibroblast growth factors-1 and -2 (bFGF-1 and 2) [26], the vascular endothelial growth factor (VEGF) family (from VEGF-A to -D) [27], platelet-derived growth factors (PDGFs) [28], placenta growth factor, insulin-like growth factors, angiopoietin-1 and -2 (Ang-1 and -2), ~~epidermal growth factor (~~EGF~~)~~, hepatocyte growth factor, hypoxia-inducible factor (HIF)-1 α and β, transforming growth factor (TGF) α and β, tumor necrosis factor (TNF) α, interleukins (IL)-1 β, −3, −6, −8, neuropilin 1 and 2, angiogenin, adrenomedullin, and stromal cell-derived factor-1 (SDF-1) [5,24,29–31].

Apart from hypoxia, other environmental stressors are able to induce the expression of pro-angiogenic factors, including glucose deprivation, accumulation of reactive oxygen species (ROS), cellular acidosis or iron deficiency, the activation of oncogenes, such as Ras and Myc, or the abnormal function of tumor suppressor genes [4].

The discovery of this heterogeneous population of pro-angiogenic growth factors, peptides, enzymes and cytokines stimulated the design of a large body of antagonistic therapeutic agents [30,32]. However, besides the development of several angiogenic inhibitors such as monoclonal antibodies and small molecule drugs, numerous endogenous inhibitors of angiogenesis were discovered and their list continues to grow because of their therapeutic potential [33]. Many proteins have been identified as endogenous angiogenesis inhibitors including thrombospondins (TSP)-1 and −2, chondromodulin, pigment epithelial derived factor (PEDF), platelet factor-4 (PF-4), and several members of the interleukin and interferon families [21,33]. Furthermore, also protein fragments such as endostatin (fragment of collagen XVIII), angiostatin (fragment of plasminogen), tumstatin (fragment of collagen IV), microRNAs and soluble VEGF receptors (sVEGFRs) have been identified as endogenous angiogenesis inhibitors [33].

2.2 Angiogenic switch

The angiogenic switch consists in the disruption of the balance between anti- and pro-angiogenic factors, in favor of the latter, with consequent recruitment of circulating endothelial progenitors (CEPs) that promote the formation of new blood vessels and the escape from tumor dormancy [34].

During the angiogenic switch, dormant cancer cells seem to undergo a genetic reprogramming process, leading to progress from non-angiogenic to angiogenic phenotype, with recruitment of new blood vessels. This event is considered an early marker of tumor transformation. Although irregularly shaped, tumor blood vessels are essential for the growth of malignant cancer cells. An essential concept is that angiogenic ability is required a long time before the emergence of an invasive malignancy [4].

Human tumors seem to contain cancer cell subpopulations with different angiogenic potential. In a human liposarcoma, three different clone patterns have been observed: highly angiogenic clones with rapid tumor growth; weakly angiogenic clones with slow tumor growth; non-angiogenic clones corresponding to vital but dormant tumors and also named “non-tumorigenic” or “no-take”. This pattern has been also examined in animal models of tumor dormancy, especially by inoculating human cancer cells in immunocompromised mice [22,35].

Hypoxia in cancer cell proliferation seems to be fundamental for the angiogenic switch, that is the transition from the non-angiogenic to the angiogenic tumor phenotype, with subsequent tumor growth. This condition might trigger compensatory mechanisms in hypoxic cells, with activation of HIF-1 pathway or HIF-1-independent pathways. Other pro-angiogenic factors are recruited, including VEGF, PDGF and nitric oxide synthase. The initial step of the angiogenic switch is represented by hyperemic reaction at the periphery of the tumor, with vasodilatation [17,18,36].

The angiogenic switch was associated with down-regulation of TSP-1 and decreased sensitivity to angiostatin. When levels of TSP-1 in non-angiogenic and angiogenic tumor cells isolated from the human breast cancer cell line MDAMB-436 were compared, angiogenic cancer cells contained significantly lower levels of TSP-1 with respect to non-angiogenic cells and the secretion of TSP-1 from non-angiogenic tumor cells was 20-fold higher than angiogenic cells. The decrease in TSP levels seems to be mediated by phosphatidylinositol 3-kinase [1,37].

It was shown that endothelial expression of angiogenic inducers epoxyeicosatrienoic acids (EETs) stimulated escape from tumor dormancy in mice. In this respect, EETs favored metastasis of various xenograft tumors, including Lewis lung carcinomas and B16-F10 melanomas [38].

Notch signaling pathway is largely used by endothelial cells during angiogenesis. Then, a novel interaction between cancer and endothelial cells has been shown to favor the escape from dormancy, this transition being

mediated by the Notch ligand Dll4 in endothelial cells and Notch 3 signaling in tumor cells, promoting a tumorigenic phenotype. Indeed, Notch 3 levels are low in dormant tumors. Metabolic features are also involved in the regulation of tumor dormancy. The activity of the Liver Kinase B1/AMP-activated protein kinase system, deputed to regulate cellular ATP levels, is increased by anti-VEGF therapy, leading to glucose depletion and reduction of ATP levels, with tumor regression [8,39,40].

Local traumas, injuries, wounds, burns and surgery are also able to favor tumor growth. They do not induce the onset of malignant cells, but promote the escape from tumor dormancy. An inflammatory state, the ability to attract circulating cancer cells or to mobilize circulating endothelial precursors, with an increase in VEGF plasma levels, might explain the transition to a non-dormant state [12,18].

Once recruited into tumor masses, distant bone marrow cells also participate to the induction of angiogenic switch. Stromal cells surrounding tumors mainly include fibroblasts, lymphocytes, neutrophils, macrophages and mast cells, which interact through intercellular signaling pathways, mediated by surface adhesion molecules, cytokines and their receptors. Paradoxically, cells of the immune system infiltrating tumors can be a fundamental source of growth stimulatory signals. Several bone marrow-derived cell types have been implicated in the escape from tumor dormancy, as well as in the metastatic dissemination. They include endothelial progenitor cells, Tie-2 expressing monocytes, the heterogeneous family of immature myeloid cells, hemangiocytes, M1 and M2 tumor associated macrophages, dendritic and mast cells. As in healing, infection, inflammation or ischemia, several cytokines and chemokines would be released by cancer cells to recruit many bone marrow-derived cell (BMDC) types which contribute to angiogenic switch. Circulating platelets seem to be involved in the transport and dissemination of pro-angiogenic factors [4,12,41]. These data suggest that many angiogenic factors are required to trigger tumor angiogenesis. On this basis, Indraccolo et al. [42] proposed the “spike hypothesis”, according to which a transient but consistent supply of angiogenic factors is able to promote the angiogenic switch.

A mathematical model of angiogenic escape has been proposed by Kareva [5], suggesting that, depending on the tissue of tumor origin, the time to escape from tumor dormancy may vary not only based on the degree of stromal stimulation, but also on the type of isoform of an angiogenesis regulator that is predominant in each tissue. In another work, Stéphanou et al. [43] developed a sophisticated computational model to describe the vascular changes induced by the development of a kidney tumor on the dorsal skinfold chamber implanted on the back of nude mice. The results showed that the tumor growth was strongly impaired by the constant vascular changes. Angiogenesis itself was affected by vascular changes occurring in bigger upstream vessels, resulting in a less efficient angiogenic network for oxygen delivery, and tumor cells were mostly kept in a non-proliferative hypoxic state. Thus, tumor dormancy seems to be the consequence of the intense vascular changes in the host tissue.

2.3 Molecular and genetic aspects

The angiogenic dormancy also depends on different genetic patterns and molecular characteristics which influence the expression of regulatory factors involved in the blood vessel remodeling. Indeed, some tumors can transform bone marrow cells into pro-tumorigenic cells even prior to their mobilization into the circulation, this process being defined systemic instigation. Certain breast tumors (instigators) release the cytokine osteopontin (OPN) into the circulation and tumor-derived OPN programs hematopoietic progenitor cells to adopt a pro-tumorigenic state, by inducing their over-expression of the secreted glycoprotein, granulin [44].

Exosomes from cancer cells contain soluble cytokines, growth factors, integrins, mRNA and microRNA which are able to reprogram bone marrow progenitor cells with pro-angiogenic and pro-metastatic activity [45]. Exosomes from renal carcinoma cells can activate an angiogenic phenotype in normal endothelial cells *in vitro* and tumor cell colonization of the lung and angiogenesis *in vivo* [46].

Considering that the transition to fast-growing tumor is angiogenesis-dependent and requires a stable transcriptional reprogramming, this event has been evaluated by genome analysis. Cancer cells expressing microRNA cluster 126 (miR-126) reduce the recruitment of endothelial cells to the tumor site by blocking GAS6/MER signaling [47]. Furthermore, suppression of the heat shock protein 27 associates with a non-angiogenic pattern, causing the inhibition of endothelial cell proliferation and leading to long-term dormancy in human breast cancer [48]. Almog et al. [49] evaluated 19 microRNAs dealing with the phenotypic switch to fast-growth of four human dormant tumors: breast carcinoma, glioblastoma, osteosarcoma, and liposarcoma. Loss of expression of dormancy-associated microRNAs was the prevailing regulation pattern correlating with the switch of dormant tumors to fast-growth. Reconstitution of a single dormant microRNA led to phenotypic reversal of fast-growing angiogenic tumors towards long-lasting tumor dormancy. Again, transcriptional reprogramming of tumors by means of dormant microRNAs over-expression led to down-regulation of pro-angiogenic factors, such as bFGF and TGF-α. Anti-angiogenic and dormancy promoting pathways such as ephrin type-A receptor 5 and angiomotin were up-regulated in dormant microRNA over-expressing tumors.

3 Metronomic chemotherapy

After almost two decades from its appearance in the field of cancer therapy, the metronomic chemotherapy concept still attracts the interest of researchers and clinicians for its various mechanisms of action, involving both tumor cells [50] and their microenvironment (i.e. the microvasculature and cells of immune system) [51], as well as for its interesting clinical activity, especially in older and frail patients [52,53]. The metronomic chemotherapy definition constantly evolved during these twenty years, based on the new discoveries and clinical trials, and recently it has been described as a schedule characterized by regular and frequent chemotherapeutic drug doses able to maintain low, but active, range of drug concentrations during prolonged periods of time without causing important toxicities [54]. Indeed, the metronomic chemotherapy have shown to be active through very different mechanisms such

as i) a direct cytotoxic effect on putative cancer stem cells, tumor-initiating cells [55,56] and cancer cells [50,57], ii) a stimulation of cytotoxic T cells by targeting T regulatory cells [58], iii) an inhibition of tumor angiogenesis [59] because of a preferential antiendothelial activity [60]. Moreover, the same drug (e.g. cyclophosphamide) may act metronomically through various mechanisms depending on its plasma concentrations [54]. In this context, the possible role of metronomic chemotherapy in the (re-) induction of tumor dormancy is still matter of research and discussion among scientists. In the present review we focus our interest on the particular aspect of *angiogenic tumor dormancy*, showing the available preclinical and clinical data that may support the hypothesis of an effect on this process by metronomic schedules.

3.1 Metronomic chemotherapy and the anti/pro-angiogenic growth factor balance

The asymmetric expression of endogenous inhibitors of angiogenesis (e.g., TSP-1 or PF-4) and pro-angiogenic factors (e.g., VEGF or bFGF), in favor of the first ones, is a key characteristic of the preservation of a dormant tumor angiogenesis [29]. Metronomic chemotherapy, as a therapeutic approach that can considerably modulate anti- and pro-angiogenic factors in preclinical and clinical settings, is a promising pharmacological tool to induce, or re-induce, the *angiogenic tumor dormancy* (Fig. 2).

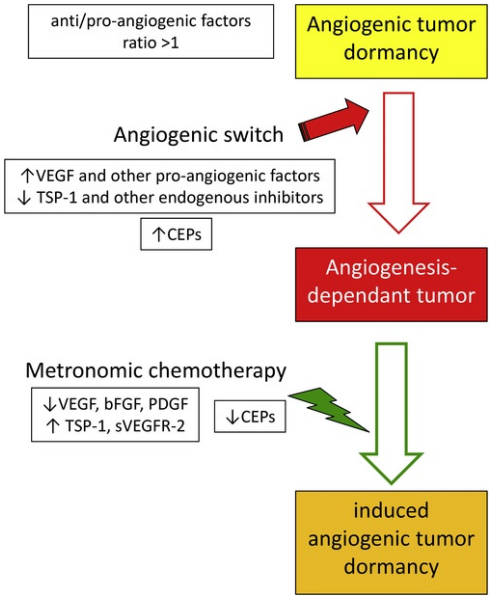

Fig. 2 Mechanisms influencing the angiogenic switch that leads to tumor dormancy escape. Metronomic chemotherapy modulates the equilibrium between anti- and pro-angiogenic factors in tumor microenvironment, decreasing the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and upregulating thrombospondin-1 (TSP-1), soluble VEGF receptor-2 (sVEGFR-2). Furthermore, low-dose chemotherapy blocks the recruitment of circulating endothelial progenitors (CEPs) in the tumor mass.

alt-text: Fig. 2

On the contrary, standard chemotherapy has been shown to induce the expression and secretion of pro-angiogenic factors such as VEGF and bFGF by tumor cells [61]. Indeed, these factors can support the angiogenesis process during the chemotherapy-free break period, causing a resistance of endothelial cells to the effects of chemotherapeutics such as taxanes, through the upregulation of survivin [62]. Robinson and colleagues [63], in a preclinical model of hepatocellular cancer, demonstrated a significant increase ~~of~~in VEGF-A and VEGF-C levels after the administration of the combination therapy FOLFOX (Folinic acid, 5-fluorouracil and Oxaliplatin). The boost of pro-angiogenic factors, such as VEGF-A and Ang-2, in serum after the chemotherapy intake has been also reported by Furstenberger et al. [64] in breast cancer patients and by Hanrahan et al. [65] in non-small cell lung cancer (NSCLC) patients. Interestingly, conventional concentrations of vinorelbine increased the pro-angiogenic factor IL-8 [66], but also reduced the number of TSP-1 receptors on endothelial cells.

3.1.1 Metronomic chemotherapy decreases the expression of pro-angiogenic factors in preclinical tumor models

The investigation of the antiangiogenic effects of the metronomic chemotherapy has been focused not only on the inhibition of microvessel formation and endothelial cell proliferation or migration [67] but also on the modulation of the balance between pro-angiogenic factors and natural inhibitors of angiogenesis.

Certain drugs such as topotecan and irinotecan, topoisomerase 1 inhibitors, or the anthracycline adriamycin are able to suppress the expression of HIF-1α [68,69~~]~~,70], a factor that stimulates the VEGF production and secretion by hypoxic tumor cells [71]. Indeed, low concentrations of topotecan, independently of topoisomerase I inhibition, reduced *in vitro* the VEGF-A levels in ovarian cancer cells [72], whereas the long term, continuous treatment with SN-38, the active metabolite of irinotecan, caused a significant decrease ~~of~~in secreted VEGF-A in cell media of colon cancer cells [73]. Moreover, Aktas et al. [74] analyzed the effects of lower doses of chemotherapeutic drugs such as irinotecan, 5-florouracil, oxaliplatin, paclitaxel and docetaxel in different tumor cell lines, demonstrating that these drugs were able to decline the VEGF secretion without causing cytotoxic effects. Metronomic docetaxel significantly decreased both the expression and secretion of VEGF also in gastric cancer cells [75].

The above mentioned *in vitro* data were confirmed *in vivo* as shown in colon cancer [76], and in gastric cancer [77] models by the 5-FU produg capecitabine which decreased the VEGF levels if metronomically administered. Moreover, Yuan and colleagues [77] also studied the PDGF levels in the gastric cancer cell culture supernatants, demonstrating that these concentrations were lower in the metronomic capecitabine than in the control group. These findings were also confirmed in the peripheral blood of tumor-bearing nude mice, where the PDGF levels decreased in the metronomic-treated group of animals [77]. Another nucleoside analog such as gemcitabine, when administered in a metronomic schedule, diminished the tumor levels of numerous proangiogenic molecules such as EGF, IL-1α, IL-8, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in pancreatic cancer xenografts [78]. It has been also shown that metronomic etoposide reversed the proangiogenic disequilibrium in tumors by inhibiting VEGF-A and FGF-2 secretion from tumor cells and by increasing endostatin plasma levels [79]. Furthermore, the metronomic administration of a chemotherapeutic drug affecting cellular energy metabolism, such as the GMX1777, in a mouse model of neuroblastoma significantly decreased the gene expression of VEGF-A and PDGF-B in the stromal tissue [80]. Interestingly, these effects were also achieved combining different metronomic chemotherapy schedules such as the one tested by Mainetti et al. [81], investigating the therapeutic efficacy of the association of cyclophosphamide and doxorubicin in mouse mammary adenocarcinoma models. Indeed, the combination was more effective than each monotherapy to decrease the VEGF serum levels and increase tumor apoptosis [81].

Interestingly, it has been also shown that VEGF-A has immunosuppressive properties, inducing the accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, and inhibiting the migration of T lymphocytes to the tumor [82]. Thus, it is conceivable that metronomic chemotherapy may enhance its antitumor immune stimulating effect also through the decrease of VEGF or other relevant pro-angiogenic factors such as TGF-β. Indeed, TGF-β enables tumors to evade immune surveillance and killing through the promotion of the generation of regulatory T cells [83]. Of note, in metastatic breast cancer patients treated with a metronomic-like schedule such as weekly docetaxel, the TGF-β mRNA levels decreased significantly upon treatment [84], whereas Ghiringhelli and colleagues [58] have demonstrated that metronomic cyclophoshamide strongly curtailed immunosuppressive regulatory T cells, favoring a better control of tumor progression. Therefore, these data seem to suggest that metronomic regimens, acting through the decrease of pro-angiogenic factors and the related induction *of angiogenic tumor dormancy*, may also be able to reduce the immune tolerance towards tumors.

3.1.2 Metronomic chemotherapy decreases the levels of pro-angiogenic factors in clinical trials

A constant evidence among the various phase I-II clinical studies, using metronomic chemotherapeutic drugs, was the decrease of plasma (or serum) VEGF and other pro-angiogenic factors during or after the schedule administration. These findings were mainly obtained and well described in metastatic breast and prostate cancer patients.

Breast cancer patients with lower VEGF and bFGF levels after 2 months of daily oral metronomic cyclophosphamide had higher progression-free survivals, while at the time of progression there was a significant increase of both VEGF and bFGF [85]. A similar decrease in serum VEGF was obtained after two months of metronomic cyclophosphamide plus methotrexate or thalidomide in 171 metastatic breast cancer patients [86], whereas the combination of metronomic capecitabine together with metronomic cyclophosphamide caused a significant drop of serum VEGF level not only after 2 months but also after a semester of therapy in patients with a complete/partial response and a stable disease [87]. Consistently, a significant suppression of VEGF-A expression was also found in primary breast cancer tissues of patients treated with a combination of letrozole/metronomic cyclophosphamide of patients if compared to the ones administered with letrozole alone [88~~]~~,89].

In metastatic castration-resistant prostate cancer patients responding to metronomic cyclophosphamide, celecoxib, and dexamethasone, the VEGF concentrations constantly declined for more than three months, whereas they significantly increased in non responder subjects [90]. In a similar subset of prostate cancer patients, the schedule including metronomic vinorelbine and dexamethasone ~~determined~~caused a plasma VEGF $AUC_{0-24day}$ significant decrease in responders subjects [91]. Of note, the *VEGF-A* genetic background of patients seems to predict progression-free survival among advanced castration-resistant prostate cancer patients treated with metronomic cyclophosphamide [92]. In particular, prostate cancer patients harboring the *VEGF* -634C C genotype - characterized by an increased secretion of VEGF in stimulated peripheral blood mononuclear cells [93] - had a significant lower median progression-free survival compared to patients with the *VEGF* genotype −634C G/GG [92].

A recent article by Zeng and colleagues [94] ~~suggested~~showed that, during the administration of a metronomic etoposide plus cyclophosphamide schedule also in relapsed or refractory non-Hodgkin's lymphoma patients with overall response and disease control, a significant decrease ~~of~~in serum VEGF levels was ~~shown~~reached. Guo and co-workers [95] investigated the effects of low-dose cyclophosphamide and prednisone on serum VEGF and PDGF-BB in multiple myeloma patients. After this metronomic schedule, there were significantly lower VEGF and PDGF-BB serum levels than pretreatment in the responder patients if compared to the non-responders [95]. The levels of both VEGF and PDGF-BB have been measured also in the serum of rectal carcinoma patients receiving a daily low dose of capecitabine in combination with pelvic irradiation, finding a significant decrease of both the pro-angiogenic factors during the treatment [96].

In advanced cancer patients, the combination of metronomic cyclophosphamide and methotrexate maintained stable the levels of both Ang-1 and matrix metalloproteinase-9 (MMP-9) in the group of patients with a stable disease whereas these pro-angiogenic factors increased in the progressive disease group only [97]. Interestingly, a significant decline in the in serum levels of Ang-1, as well as VEGF, was inducted in advanced ~~non-small-cell-lung-cancer~~NSCLC patients by metronomic cisplatin and

etoposide [98]. Finally, in castration resistant prostate cancer patients treated with metronomic taxotere and zoledronic acid combination, a decrease of serum levels of IL-8 and MMP-9 were detected [99].

However, although the phenomenon of the decrease of pro-angiogenic factors during or after the administration of metronomic schedules has been well described, some pilot clinical studies did not report this pharmacodynamic characteristic. This is the case of the trials by Kieran et al. [100] and Stempak et al. [101] in pediatric cancer patients, where plasma levels of both VEGF and bFGF were highly variable and no statistically significant relationships between them and disease progression or stable disease were found. In another small clinical trial by Ekinci and colleagues [102], eleven metastatic cancer patients, treated with daily metronomic cyclophosphamide and biweekly methotrexate, showed a no significant decrease of VEGF plasma levels. Finally, it has been also described that NSCLC elderly patients responding to metronomic vinorelbine maintained their baseline VEGF levels during the treatment whereas the non-responders showed a rapid increase [103].

3.1.3 Metronomic chemotherapy induces the expression of endogenous inhibitors of angiogenesis in preclinical and clinical settings

Besides the decreased levels of ~~the~~ pro-angiogenic factor~~s~~ ~~VEGF~~, metronomic chemotherapy determine, in parallel, a well-described increase of TSP-1. In 2003, Bocci et al. described that the *in vitro* administration of metronomic paclitaxel and 4-OH-cyclophosphamide (the active metabolite of cyclophosphamide) were able to significantly induce the expression of TSP-1 in endothelial cells [104] whereas a daily metronomic cyclophosphamide schedule *in vivo* increased circulating levels of plasma TSP-1 in human prostate tumor xenograft-bearing mice responding to the treatment [104]. Interestingly, the *in vivo* antiangiogenic and antitumor effects of metronomic cyclophosphamide were lost in TSP-1-*null* mice carrying Lewis Lung carcinomas [104]. The TSP-1 raise obtained with metronomic cyclophosphamide were confirmed in other *in vivo* models such as murine melanoma [105], rat prostate tumors [106], and human ovarian cancer [107]. Recently, Cicchelero and colleagues [108] showed that the co-treatment with metronomic cyclophosphamide and intratumoral interleukin-12 gene therapy in spontaneous canine cancers determined a continuous increase of interferon gamma and TSP-1 levels in tumor tissues, but not in serum, with significant antiangiogenic effects. Also other metronomic schedules, including widely used fluoronucleoside analogs, were capable to enhance both the gene expression and the protein secretion of TSP-1 determining a significant antiangiogenic effect in tumor tissues such as gemcitabine in human pancreatic adenocarcinomas xenografts [107,109], capecitabine in human colorectal cancer xenografts [110], [76], S-1 (alone and in combination with vandetanib) in hepatocellular carcinoma [111]. Moreover, taxanes, such as paclitaxel and docetaxel [112,113], at low doses caused antiangiogenic effects as a result of the marked increase of TSP-1 levels in tumor vascular endothelial cells [114] and in different tumor types such as ovarian [115], breast [116], colon [117], and a gastric cancer [75]. Analogous results were obtained with metronomic ceramide analogs (eg., C2 and AL6) that inhibited neovascularization and pancreatic cancer enlargement through up-regulation of TSP-1 and caveolin-1 [118], whereas protracted low concentrations of SN-38 (the active metabolite of irinotecan) augmented both TSP-1 gene expression and secretion by colon cancer cells [73].

The notable modulation of TSP-1 during metronomic chemotherapy was also found in patients enrolled in various phase II clinical trials, involving different types of cancer. In 2008, Allegrini et al. described an increment of TSP-1 plasma concentrations in metastatic colorectal cancer patients administered with a metronomic infusion of irinotecan at two different low dose levels [119]. The same group later confirmed these findings in metastatic gastrointestinal cancer patients treated with a combination of metronomic cyclophosphamide, UFT and celecoxib, showing that patients with a stable disease had higher values of TSP-1 AUCs [120]. Moreover, coherent results were retrieved in responding metastatic castration-resistant prostate cancer patients treated with metronomic vinorelbine and dexamethasone [91]. Interestingly, also a metronomic-like treatment such as the maintenance chemotherapy of acute lymphoblastic leukemia in children (i.e. daily administration of low-dose mercaptopurin and weekly low-dose methotrexate) determined a significant increase in TSP-1 plasma levels [121].

Despite the consistent preclinical and clinical studies showing an increase of TSP-1 during metronomic administration of chemotherapeutic drugs, some authors did not find such an increment of this endogenous inhibitor of angiogenesis. This is the case of Stempak et al. [101] who conducted a pilot pharmacokinetic and antiangiogenic biomarker study of celecoxib and low-dose metronomic vinblastine or cyclophosphamide in pediatric recurrent solid tumors. No variations of TSP-1 and endostatin plasma levels were retrieved and no statistically significant relationships between these concentrations and disease progression or stable disease were observed [101]. No changes of TSP-1 plasma concentrations were reported also by Camerini et al. [103] and by Tas et al. [122] in NSCLC patients during metronomic vinorelbine and metronomic cisplatin/docetaxel combination schedules, respectively; whereas Garcia and colleagues did not find any significant association between TSP-1 plasma levels and clinical outcome of patients affected with recurrent ovarian cancer and treated with a combination of bevacizumab and daily metronomic cyclophosphamide [123].

It has been described that endogenous inhibitors of angiogenesis, other than TSP-1, increased during and after metronomic treatments. Indeed, Jia and co-workers described in transfected colorectal cancer cells, overexpressing both TSP-1 and PEDF, that metronomic weekly cyclophosphamide caused an induction of expression of these endogenous inhibitors *in vitro* and *in vivo*, with a corresponding enhancement of responsiveness of primary tumors to the treatment [124]. Moreover, Perroud and colleagues have described in two different sets of advanced breast cancer patients, treated with metronomic cyclophosphamide and celecoxib, the statistical increase of the serum levels of sVEGFR-2, an inhibitor of angiogenesis, with a concomitant decrease of VEGF [125,126]. Indeed, the VEGF/sVEGFR-2 ratio significantly decreased during treatment and was associated with the time to progression of patients [125]. On the other hand, this increase of soluble forms of VEGF receptors (i.e. sVEGFR-1 and sVEGFR-2) was not reported in a phase I-II study in metastatic breast cancer patients treated with metronomic cyclophosphamide, methotrexate, daily dalteparin and prednisone [127]. Interestingly, the sVEGFR-1 serum levels were found not changed also during the metronomic administration of the cisplatin/docetaxel combination in patients with advanced NSCLC [122].

3.2 Metronomic chemotherapy and the circulating endothelial progenitors

In order to progress, microtumors have to activate and recruit normal cells such as circulating endothelial progenitors (CEPs) that promote the shift toward the genesis of new blood vessels [4]. The improvement of therapies that are able to inhibit the mobilization and viability of CEPs and other pro-angiogenic ~~bone-marrow-derived-cells~~BDMCs may maintain the tumor dormancy due to the angiogenesis blockage. In this perspective, the metronomic

chemotherapy could be a perfect therapeutic weapon to achieve this aim [51].

The effects of standard chemotherapy on endothelial cell precursors and other proangiogenic BDMCs has been well described during hematological recovery in the chemotherapy-free break period [64,128]. The mobilization of CEPs after standard chemotherapy appears to be VEGF-dependent and inducted by circulating SDF-1 [129]. Thus, both BMDCs and CEPs can amplify the ability of the drug-treated tumors to repopulate, at least in part, by stimulating tumor angiogenesis.

On the contrary, during the oral low-dose cyclophosphamide administration, a significant suppression of the number of CEPs in mice affected by lymphoma was retrieved, whereas at the end of the therapy the number of endothelial progenitors increased again and tumors started to grow [128]. Furthermore, a clear correlation between the minimum CEP's level and the maximum antiangiogenic effect was demonstrated in mice treated with metronomic cyclophosphamide, vinblastine, cisplatin, and vinorelbine [130,131]. Based on these data, it has been proposed that the decrease of CEP levels could be one of the mechanisms of metronomic chemotherapy [132] and a possible pharmacodynamic biomarker of therapeutic success [133]. Daenen and collaborators demonstrated that daily metronomic cyclophosphamide was able to impede the CEP peak and tumor invasion induced by a vascular disrupting agents [134]. Also metronomic topotecan in combination with the tyrosine kinase inhibitor pazopanib significantly reduced the number of viable CEPs as well as circulating endothelial cells, showing a parallel reduction in the tumor microvessel density of pediatric solid tumors [135]. Besides, the percentage of CEPs also decreased in the blood circulation of mice bearing gastric tumors after the treatment with metronomic 5-~~FU~~fluorouracil or capecitabine [77].

Clinically, low-dose metronomic trofosfamide stopped significantly the mobilization of circulating CEPs in the blood of tumor patients if compare with a standard trofosfamide schedule [136]. Moreover, Calleri and coworkers demonstrated that long-term responders to metronomic chemotherapy had significant lower levels of CEPs and circulating endothelial cells [85]. In a population of gastrointestinal cancer patients, the concentrations of CD133 (a progenitor or stem cell biomarker) mRNA were consistently lower in those subjects with stable disease during a combined metronomic schedule with UFT and cyclophosphamide [120].

4 Conclusions

Angiogenic tumor dormancy occurs as a result of a dynamic context in which antiangiogenic stimuli prevail on pro-angiogenic factors and, consequently, the neovascularization is inhibited. This process can occur at the primary site of cancer, but also in metastatic lesions. Accordingly, a therapeutic strategy that could determine an induction or a “re-induction” of the angiogenic tumor dormancy in primary and/or metastatic tumors may be more than an option into the clinic. Along this line, metronomic chemotherapy is surely able to decline VEGF and, simultaneously, increase the endogenous inhibitor TSP-1, probably playing a major role in the possible re-induction of the angiogenic tumor dormancy.

Conflicts of interest

None.

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Highlights

- Tumor dormancy consists in cancer cell surviving in a non-proliferating state.

- Tumor angiogenic switch is essential to promote escape from tumor dormancy.
- Angiogenic switch comes from the unbalance between pro and antiangiogenic factors.
- Metronomic chemotherapy upregulates TSP-1, declines VEGF and suppresses CEPs.
- Metronomic therapy acts as a promising tool in inducing tumor angiogenic dormancy.

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