TUMOR BLOOD VESSELS AND VASCULOGENIC MIMICRY – CURRENT KNOWLEDGE AND SEARCHING FOR NEW CELLULAR/MOLECULAR TARGETS OF ANTI-ANGIOGENIC THERAPY

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Summary: Blood vessel formation in tumor is defined as tumor angiogenesis. So far, the most known its mechanism is sprouting, which means formation of blood vessels from existing ones, as a result of the proliferation and migration of endothelial cells. The main mitogenic factor of these cells is vascular endothelial growth factor VEGF, acting by VEGFR-2 receptors. Recent studies have provided knowledge about the ability of tumors to form vessel-like structures. The phenomenon was called vascular mimicry. Tumor cells show a high plasticity and they can undergo differentiation to the ones with phenotype similar to endothelial cells. Each of the known tumor angiogenesis mechanisms is a result of many different factors and cell cooperation in tumor microenvironment. Tumor ability to the heterogeneous vascularization forces developing of complex, anti-angiogenic therapy directed to different molecular and cellular targets. Therapies, used so far, often lead to drug-induced hypoxia, which increases tumor cell aggressiveness and metastasis.

Keywords: tumor angiogenesis, vasculogenic mimicry, anti-angiogenic strategies, resistance to anti-angiogenic therapy

Abbreviations: **ALDH** – *aldehyde dehydrogenase*; **ANGPT** – *angiopoietin*; **α-SMA** – *alpha -smooth muscle actin*; **bFGF** – *basic fibroblast factor*; **BNIP3** – *Bcl-2 adenovirus E1B 19 kDa protein – interacting protein 3*; **CAFs** – *cancer-associated fibroblasts*; **CD31** – *cluster differentiation 31*; **CD44** – *cluster differentiation 44*; **CD105** – *cluster differentiation 105, endoglin*; **CD133** – *cluster differentiation 133*; **CD146** – *cluster differentiation 146*; **Cx43** – *connexin 43*; **CSCs** – *cancer stem cells*; **ECM** – *extracellular matrix*; **ECs** – *endothelial cells*; **EGF** – *epidermal growth factor*; **EGFR** – *epidermal*

growth factor receptor; **EMA** – *European Medicines Agency*; **EMMPRIN/CD147** – *extracellular matrix metalloproteinase inducer/cluster differentiation CD147*; **EMT** – *epithelial-mesenchymal transition*; **EPCs** – *endothelial progenitor cells*; **FDA** – *Food and Drug Administration;* **GC** – *gastric cancers*; **G-CSF** – *granulocyte colony-stimulating factor*; **GJ** – *gap junctions*; **HGF** – *hepatocyte growth factor*; **HIF-1** – *hypoxia-inducible factor-1*; **HSPs** – *heat shock proteins* ; **MACC 1** – *metastasis-associated in colon cancer -1*; **MDR1** – *multidrug resistance protein 1*; **MET** – *mesenchymal-epithelial transition*; **MVD** – *microvessel density*, **MMPs** – *matrix metalloproteinases*; **mTOR** – *mammalian target of rapamycin*; **Notch-1** – *Notch homolog 1 translocation-associated (Drosophila*); **NSCLC** – *non-small cell lung cancer*; **PAS** – *Periodic Schiff Reaction*; **PDGF** – *platelet-derived growth factor*; **PDGFRβ** – *platelet-derived growth factor beta receptor*; **PGCCs** – *polyploid giant cancer cells;* **SDF-1** – *stromal cell-derived factor-1;* **Sox-2** – *sex determining region Y- box2*; **TGF-β** – *transforming growth factor beta;* **TNBC** – *triple negative breast cancer*; **TNF-α** – *tumor necrosis factor alpha*; **TRAIL** – *TNF-related apoptosis-inducing ligand*; **TRC105** – *TRACON 105*; **Tregs** – *regulatory T cells*; **TWIST-1** – *TWIST family bHLH transcription factor 1*; **VEGF** – *vascular endothelial growth factor*; **VEGFR** – *vascular endothelial growth factor receptor* **VM** – *vasculogenic mimicry*; **VPF** – *vascular permeability factor*; **TAMs** – *tumor- associated macrophages*;

Dictionary of tumor angiogenesis mechanisms terms, used in the work:

Sprouting angiogenesis – formation via endothelial cells proliferation and migration towards avascular tumor area of new branches of blood vessels
Intussusception – formation of blood vessels as a result of intussusception (septum) inside existing blood vessels
Co-option – oxygen and nutrients acquire as a result of tumor cells migration along existing blood vessels
Vasculogenic mimicry – formation by tumor cells of vessel-like structures, without endothelial cells participation
Vasculogenesis – formation of tumor blood vessels *de novo*, as a result of endothelial progenitor cells recruitment

INTRODUCTION

One of the features of malignant tumors is their ability to vascularization. Blood vessels formation in tumor is called tumor angiogenesis. Sprouting angiogenesis, as a proliferation and migration of endothelial cells in existing blood vessels, is the most known mechanism. These changes occur under influence of pro-angiogenic factors, mainly vascular endothelial growth factor (VEGF), and are accompanied by extracellular matrix (ECM) remodeling, which takes place with contribution of metalloproteinases [20]. VEGF is a member of the vascular permeability factor family proteins (VPF), wich is a multifunctional cytokine responsible for proliferation and migration of endothelial cells as well as for increased permeability of the existing vessels [25]. Because of pro-angiogenic vs. anti-angiogenic factors advantage, angiogenic switch appears, and after that tumor passes from avascular to vascular phase. Tumor volume of about 2 mm^3 indicates a tumor growth limit without additional angiogenesis [29], and tumor microvessel density (MVD) indicates tumor vascularization degree [16, 86].

Inside growing tumor hypoxia has a direct effect on angiogenesis induction. In these conditions, activity of transcription factor HIF-1α, responsible for VEGF overproduction, is appeared [66]. In unfavourable conditions, including deficiency of pro-angiogenic factors, tumor vascularization can be achieved with other mechanisms like intussusception or co-option, without ECs proliferation [2]. A landmark discovery in tumor angiogenesis research was study of vasculogenic mimicry phenomenon (VM) [57]. The effect of VM are vessel-like structures, formed by tumor cells, which show a high plasticity and are able to differentiate towards cells resamble to ECs [10, 74].

Most of research on tumor vascularization is conducted with histological techniques, qualitative and quantitative analysis of ECs, as well as of VM antigens and receptors [9, 10, 27, 74].

Heterogeneity of tumors vascularization has serious implications in fast development of their resistance to current anti-angiogenic treatment, directed mainly to ECs. Inhibition of, dependent on ECs, blood vessels formation gives a risk of drug-induced hypoxia, which according to data contributes to higher tumor cells aggressiveness and metastasis [53, 64]. The main assumption of actual developed therapies is their action not only on ECs, but also on other cells of blood vessels – support cells (pericytes) [16]. Tumor cells are also important target of anti-angiogenic therapy because of their ability to differentiation towards cells with similar to ECs phenotype [11, 74].

In the present work, the most important discoveries in history of tumor angiogenesis and current data on heterogeneous tumor vascularization (mechanism of vessel formation, their cellular structure, molecular profile, as well as of search for new targets of effective anti-angiogenic therapy) were presented.

HISTORY OF RESEARCH AND DISCOVERIES OF TUMOR ANGIOGENESIS

Currently, tumor angiogenesis is in large interest. It is confirmed by the number of publications available in PubMed under term „tumor angiogenesis", reaching over 45 thousands. The scientific articles were published during last 40 years, including the works of Folkman about dependence of tumor progression on angiogenesis [29, 30], and the recent ones focused on searching for new targets of anti-angiogenic therapy [90]. A history of milestones in research of tumor angiogenesis is presented in figure 1. In the result of intensive studies, the role of VEGF in tumor ability to blood vessels formation, correlation between angiogenesis and tumor progression, as well as the mechanism of sprouting angiogenesis were known [29, 30]. This mechanism consists of new branch formation from existing host blood vessel network towards avascular region of tumor [5]. This process is similar to sprouting and requires of endothelial cells proliferation and migration.

A detailed description of this mechanism is presented in chapter Sprouting tumor angiogenesis.

The 90 years of XX century brought a real development of tumor angiogenesis research (fig. 1), when many scientific articles were published and described other strategies of tumor vascularization. One of them is co-option, for which typical is achieving of oxygen and nutrients by tumor cells migrating towards the nearest host blood vessels. Intussusception is based on septa formation in existing vessel. In contrast to sprouting angiogenesis, this mechanism is independent of high ECs proliferation [2]. Finally, in 1999, VM was discovered. This phenomenon was described for the first time on the basis of the results conducted *in vitro* and *in vivo* on human iris melanoma [57]. VM is completely different from previously known mechanisms and it involves formation of vessel-like structures, without ECs contribution. The research on human colon cancer xenografts provided data about mosaic vessels [9]. Most of cells in these vessels are typical ECs, and the other are tumor cells. The mechanism of vessel formation seems to be connected with tumor cell invasion and ECs displacement [9].

The most recent data on tumor angiogenesis show that in hypoxia some subpopulations of tumor cells are able to form polyploid giant tumor cells (PGCCs). The results from human breast cancer [94], as well as ovarian cancer [92], revealed that PGCCs produce discoid cells, filled with hemoglobin. These latter results complicate significantly possibility of effective anti-angiogenic therapy and certainly enforce searching for new solutions.

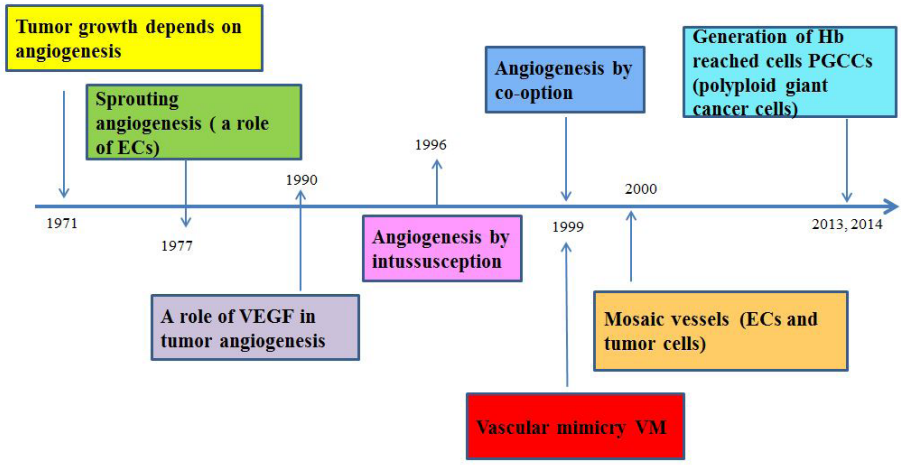

FIGURE 1. History of discoveries in tumor angiogenesis research. According to [2] changed

MECHANISMS OF TUMOR ANGIOGENESIS

So far, sprouting angiogenesis, where ECs are directly involved, is the best known mechanism of blood vessels formation [5]. This mechanism is typical in physiological conditions, as well as in some diseases [14].

If it is possible, sprouting angiogenesis is preferred by tumor. According to the results of Zhao and co-workers [95], co-option is a strategy providing survive of tumor in difficult conditions. This process is only seemingly more favourable than sprouting angiogenesis. The latter mechanism is more effective in oxygen and nutrients providing. The pro-angiogenic activity of tumor cells is determined by microenvironment conditions (including host blood vessels density), and also the number of tumor cells. From the results on *Danio rerio*, it was found that when tumor is composed of about 20-30 cells, a co-option is observed, without angiogenesis induction. However, when the amount of tumor cells is 200-300, almost immediately sprouting angiogenesis begins [95].

SPROUTING TUMOR ANGIOGENESIS

Sprouting angiogenesis is a result of many agents cooperation and cells in tumor microenvironment [20, 59]. The main of them were presented in figure 2. Endothelial cells of existing host blood vessels change their constitutive phenotype to proliferating one under microenvironment conditions. A high number of VEGF receptors, and of CD105 molecules occur on the surface of ECs [71]. For this reason, endoglin is considered to be a marker of endothelial activation towards proliferation and migration, while CD31 is a marker of constitutive ECs phenotype [19].

Hypoxia and its main product HIF-1 α, plays a crucial role in tumor angiogenesis initiation, and vessel formation [36]. The hypoxia is higher in larger tumor. In mouse xenografts of human glioblastoma, the hypoxia degree in 8 mm tumor increases about 5-fold, compared with the tumor of 2 mm^3 [36]. In the same manner, VEGF concentration is increased with the growth of tumor. For tumor of 2 mm^3 it equals to approximately 0.94 ng/mg, while in tumor of 10 mm^3 this concentration reaches 7.27 ng/mg. Among others, the results of Wang`s group [86] indicate the significance of HIF-1 in tumor blood vessel formation. The inhibition of HIF-1 α expression by connexin 43 (Cx43) leads to the decrease of VEGF level and in consequence to MVD limiting.

During sprouting angiogenesis, structural and functional heterogeneity of endothelial cells is strongly visible [45, 65]. For the first time, a concept of „tip" and „stalk" ECs was proposed by Gerhardt [33]. Heterogeneity of ECs is considered

by their differentiation in blood vessels of different organs, or in tumors with a high and low metastasis potential [38]. In the first group of tumors, ECs show higher proliferation degree and higher sensitivity to VEGF.

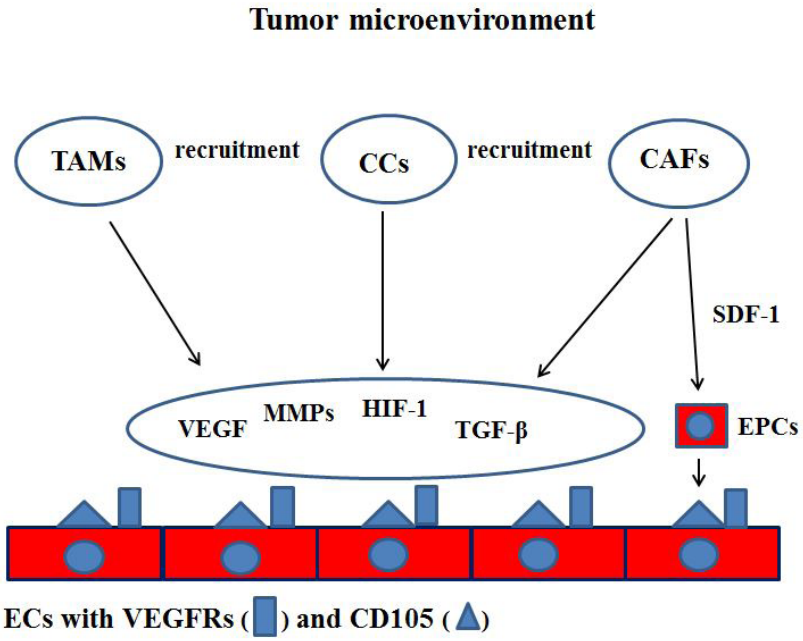

FIGURE 2. The main agents and cells involved in tumor sprouting angiogenesis. CAFs – cancer-associated fibroblasts, CCs – cancer cells, CD105 – endoglin, ECs – endothelial cells, EPCs – progenitor endothelial cells, HIF-1 – hypoxia inducible factor -1, MMPs – metalloproteinases, SDF-1 – stromal cell-derived factor-1, TAMs – tumor-associated macrophages, TGF-β – transforming growth factor beta, VEGF – vascular endothelial growth factor

„Tip" endothelial cells show ability to migration, while the second type "stalk" has a high proliferative potential and it contributes to lumen formation of a new vessel. Proliferating cells have VEGFR-1 receptors on their surface, while moving cells have VEGFR-2, which is responsible for their higher availability to VEGF and their dependence on VEGF concentration, than „tip" cells [65]. „Stalk" cells are also involved in formation of intercellular connections and synthesis of basement membrane components in a new vessel. It is worth emphasizing, that blood vessels formed in tumor as a result of sprouting angiogenesis show significant abnormalities of endothelium, as well as of basement membrane [24]. Mostly typical are relaxed intercellular connections and fenestrations in endothelial cells. The effect is chaotic vasculature, its leaking and slow blood flow (tab. 1).

TABLE 1. The main differences between tumor and physiological angiogenesis. According to [14] changed

ANGIOGENIC FACTOR/ FEATURE OF PROCESS	TUMOR ANGIOGENESIS	PHYSIOLOGICAL ANGIOGENESIS
VEGF	angiogenesis induction; recruitment of immune cells in suppressing anti-tumor immune response; recruitment of immune cells in promoting tumor growth and angiogenesis	induces proliferation, sprouting and tube formation of ECs; increase vascular permeability; suppress apoptosis for vessel stabilization
ANGPT1	a role dependent on cell type and ANGPT2 level	contribution in interaction ECs-ECM; inhibition of ECs apoptosis
ANGPT-2	TAMs recruitment; promotion of VEGF-mediated tumor vascularization	induces ECs apoptosis in absence of VEGF
duration of process	sustained process	short term process
structure of vessels	abnormalities of endothelium and basement membrane; relaxed intercellular connections, fenestrations in ECs; not clear function of pericytes; chaotic vasculature; leaking, slow blood flow	no abnormalities in endothelium, basement membrane; contribution of pericytes in vessel stabilization; vasculature without leaking and slow blood flow

bFGF and angiopoietin are also strongly involved in tumor angiogenesis. These factors stimulate endothelial cell growth and migration. FGF can exert its angiogenic mechanism of action via release by tumor and stromal cells and then paracrine signaling, or through its mobilization from ECM [49]. bFGF may also act via autocrine signaling in endothelial cells, stimulating the proliferation of new capillary endothelial cells. FGF may increase the expression of VEGF, as well as angiopoietin-2, a potent regulator of vascular branching and angiogenesis [49]. Interestingly, FGF-2 is ale to be twice as potent as VEGF in inducing formation of new vessels.

In tumor angiogenesis, similar to physiological, VEGF plays a pivotal role in inducing of proliferation and migration of ECs. However, in tumor VEGF, as well as other pro-angiogenic factors, may change their function in specific microenvironment [14] (tab. 1). Another difference between tumor and physiological angiogenesis is that the latter process is activated for short periods and then completely inhibited. Tumor angiogenesis is sustained process.

Before maturation and stabilization of a new vessel, a degradation of basement membrane with contribution of MMPs in existing one occurs [20]. MMPs action in angiogenesis can be defined as pleiotropic. It includes modifications of extracellular matrix, remodeling of vessel basement membrane, modifications of ECs surface molecules, and also releasing from ECM pro-angiogenic agents, mainly VEGF [20]. A lot of tumor microenvironment cells, including recruited by tumor CAFs, TAMs, pericytes, neutrophils, mast cells, lymphocytes, and ECs are the source of MMPs. The most active distinguished MMPs in tumor angiogenesis is MMP-2, MMP-9 and MMP-14 [20]. The role of the agent which induces angiogenic switch is attributed to MMP-9, the essential point for sprouting angiogenesis beginning. TAMs are considered as main cells releasing MMP-9. Currently it is known, that TAMs are multi-tasking cells, also able to discharge of chemokines, VEGF, and TGF-β [59]. In the same manner, CAFs, which are the source not only of MMPs, but also of VEGF, produce SDF-1, which plays a role of factor recruiting progenitor endothelial cells (EPCs) from bone marrow (fig. 2) [4]. The studies on tumor vascularization proved that blood vessel formation can take place with contribution of EPCs [19], and it can be defined as tumor vasculogenesis. Not only VEGF, SDF-1, but also G-CSF, that shows overexpression in the presence of HIF-1, plays the main role in EPCs recruitment [42].

MMPs, including MMP-9, elastase, cathepsin G also mediate in EPCs recruitment. The process, where EPCs contribute to blood vessel formation, is named vascularization. Asahara with his group [3] first described EPCs ability to incorporation between mature endothelial cells of blood vessels, also their presence outside tumor blood vessels, and pro-angiogenic agents release. Compared to glioblastoma, or stomach cancer, breast cancer, lymphoma and NSCLC are tumors with high contribution of EPCs in blood vessel formation [39, 42].

Pericytes are involved in maturation and stabilization of a new blood vessel. α-actin of smooth muscles (α-SMA), desmin, CD146, PDGFRβ receptor are markers of these cells. The latter is a target of PDGFβ, which is responsible for pericyte sending from tumor stroma to blood vessels [67].

Current data on pericyte role in tumor angiogenesis are not clear (tab. 1). A quantitative contribution of these cells in vessels of different tumor types varies from low to high. For example, a hyperplasia of pericytes is typical in glioblastoma. The amount of $\alpha\text{-SMA}^+$ cells in tumor blood vessels is increased with the stage of tumor progression [80]. For the III tumor grade, the amount of pericytes is about 49%, while for the IV grade is almost 80%. Tumor aggressiveness and resistance to therapy is higher with the increase of pericytes contribution in blood vessels of melanoma or kidney cancer [32]. However, the results of Cooke and co-workers [16] show that low amount of pericytes in tumor vessels correlates with low survival of patients with invasive breast cancer. Tumor MVD is decreased, and tumor vessels become unstable, leaking, which is a reason of higher hypoxia. It is provided by an increase of HIF-1α expression. In these conditions, induction of epithelial-mesenchymal transition, crucial for tumor progression, appears.

VASCULOGENIC MIMICRY OF TUMORS

VM was found 15 years ago [57]. Despite that, it is now one of the most intensive investigated phenomenons in tumor angiogenesis [51, 63]. As a result of mimicry, vessel-like structures appear, with tumor cells involved. VM mechanism is completely independent of endothelial cells proliferation and migration [57, 76]. The main features of VM structures and of mechanism of their formation are presented in table 2.

Immunohistochemical studies conducted on melanoma [57], ovarian cancer [76], or on glioblastoma [26], showed the presence of structures with tumor cells lying on basement membrane. A positive PAS reaction (Periodic Schiff Reaction) proves the basement membrane within VM structures. Erythrocytes were found in the lumen of VM, which means that these structures may be connected with near located blood vessels [57], and tumor cells engaged in VM formation are able to cooperate with endothelial cells [45]. Tumor cells, with features of PGCCs subpopulation, may be the source of erythrocytes in the lumen of VM structures [92, 94]. Most of PGCCs are located around necrotic areas of tumor, which show high hypoxia. The amount of PGCCs is increased with increase of tumor progression. In primary human ovarian cancer without metastasis this amount is 18.12 ±8.70, while in metastatic cancer this number equals to 57.21 ±24.88. In comparison, PGCCs amount in patients with cystadenoma is 5.80 ±1.89 [92]. An increase of VM density is connected with the increase of PGCCs amount.

TABLE 2. Characteristics of vessel-like structures VM. According to [63] changed

VM FEATURES	VM CHARACTERISTICS	LITERATURE
structure	the walls formed by tumor cells, basement membrane, erythrocytes in the lumen, ability to form connections with blood vessels	[10, 57, 92]
mechanism of formation	independent of VEGF, EMT contribution	[52, 54, 57, 81]
interaction with ECM	increase of MMPs activity and ECM remodeling	[50, 60]
phenotype/ cell plasticity	expression of stem cell multipotent phenotype (Sox-2, TWIST-1, CD133/CD44) with ability to ECs differentiation at the same time	[46, 68, 74, 84, 85]
tumor invasiveness and metastasis	high tumor progresion and aggressiveness	[50, 63]
prognosis for patient	adverse prognostic factor, shorter survival of patient	[8, 50, 51]

Final classification of vessel-like structures needs usually more studies towards amplification of EGFR gene, which is often observed in tumor cells derived from epithelial tissue [26].

Unexpected were the results that VM tumor cells are able to differentiate towards ECs [11]. Thus the cells in vessel-like structures were described as positive to CD31 [74]. There were reports about tumor cells, which *in vitro*, in the presence of ECs, acquire some of endothelial cell markers, as a result of fusion with ECs, not of their differentiation [27].

A strong expression of genes determining stem cells may indicate the ability of tumor cells to differentiate (tab. 2) [74]. Glioblastoma cells from C6 line may be the example [11]. These cells under HIF-1, Notch-1 undergo differentiation towards ECs with CD31 positive reaction. As was calculated, about 31% of ECs are cells derived from C6 cell line.

The results of Wang and co-workers [84], obtained from patients with metastatic gastric cancer (GC), as well as in cell lines and xenografts with GC, show a high expression of TWIST-1 gene in VM tumor cells. MACC1, which expression is correlated with VM density, is a factor responsible for this situation. EMT is initiated under MACC1-indued changes, which is important for VM expansion [84]. An essential increase of EMT-connected agents, including TWIST-1 and VE-cadherin has been shown in ovarian cancer [23]. The latter agent is not the only adhesive molecule, but it may lead to induction of vascular cascade [23]. Earlier, Sun and co-workers [81] and Liu and co-workers [52] indicated a strong connection between EMT and VM. It is suggested that as a result of VM connection with EMT, tumor cells acquire features typical for stem cells phenotype (CSCs) with ability to invasion and migration [28]. A high expression of CD44 is characteristic for breast CSCs. Recent results of Liu and co-workers [51] indicate a contribution exactly of these cells to VM promotion and aggressive tumor progression.

The research on melanoma [46], as well as on glioblastoma [68, 85], showed the presence of $CD133^+$ tumor cells with stem cell phenotype in VM structures (tab. 2). The authors also pointed to ability of these cells to differentiate towards ECs phenotype. The results of Cheng and co-workers [12] prove remarkable plasticity of CSCs. Most of pericytes in glioblastoma blood vessels are the cells derived from CSCs. It is assumed that these cells protect ECs in vessels and make them unrecognized by drugs used in therapy directed to ECs [12].

Hypoxia plays a crucial role in promotion of stem cell phenotype in tumor cells, as well as in EMT promotion [23, 75, 79, and 86]. In these conditions, transdifferentiating of tumor cells to ECs occurs [68, 74]. In this approach, VM phenomenon may be considered as incomplete differentiation of tumor cells with stem cell phenotype to endothelial cells [23].

Tumor cells of different types of cancer may also demonstrate CD105 expression [43]. The discovery of tumor cells ability to endoglin expression was unexpected, and now this antigen is regarded as a very important therapeutic target. The research on Ewing sarcoma or on melanoma cells [61] showed that these cells have a high CD105 expression and that there is close correlation between

CD105 and VM formation. However, the study on oesophagus cancer provided that CD105 expression is low [88]. The effect of endoglin is not the same in different types of tumors [43].

An intensive remodeling of ECM occurs at the same time as VM structures formation. Degradation of extracellular matrix with MMPs contribution allows microenvironment reorganization and tumor cells migration. The MMPs contribution to VM phenomenon was well documented [60, 63]. MMP-2, MMP-9 and CD147, also called EMMPRIN play a major role. The study on ovarian cancer shows a high correlation between MMPs expression, CD147 and VM formation [60]. $CD147^+$ tumor cells stimulate fibroblasts of tumor microenvironment to MMPs expression.

According to data in table 2, the presence of VM in tumor vascularization is unfavourable prognostic factor for patient survival. Tumor cells forming VM are able to metastasis easier and with much higher rate, because they are as close to the lumen of vessel-like structure as possible. From this point, VM is more dangerous than sprouting angiogenesis, where tumor cells reaching blood vessels in tumor stroma and intravasation need more time [64]. The connection of VM with blood vessels may influence on high ability and contribution of these structures to metastasis.

Meta-analysis results of Cao and co-workers [8] indicate that VM phenomenon is present in different degree in certain types of tumors. Percentage contribution of VM in different tumors is summarized in the figure 3. The highest contribution of VM structures was observed among patients with ovarian cancer, NSCLC, melanoma. Prostate cancer, stomach cancer or glioblastoma, are also tumors with

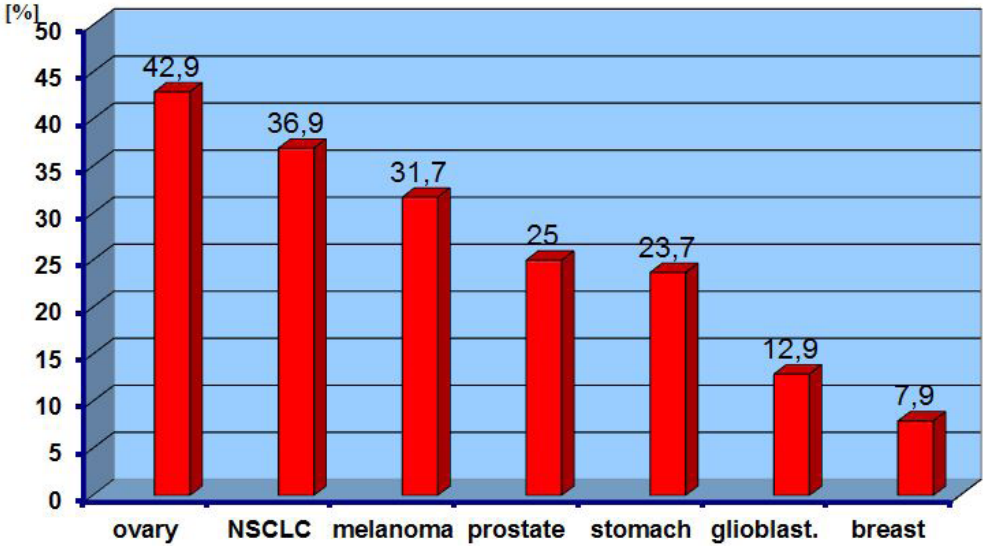

FIGURE 3. The percentage participation of VM in vasculature of different tumor types. According to [8] changed

high vasculogenic mimicry, while in patients with breast cancer VM percentage is low. The contribution of VM is dependent also on tumor stage. In NSCLC, vasculogenic mimicry is characteristic for early stages of tumor (T1 7.1%, T2 57.1%), and with tumor progression, the percentage of VM is decreased (T3 19.1%, T4 16.7%) [55]. However, from meta-analysis of Cao and co-workers [8] it is known that metastatic tumors show higher VM content (45.3%), in comparison to non-invasive (22.6%). Independently of these differences, VM is always connected with high potential to metastasis and is a poor prognostic factor [6].

TARGETS AND MECHANISMS OF ANTI-ANGIOGENIC THERAPY

The studies directed to develop effective anti-angiogenic therapies have been conducted for several decades. Data on the vascularization of tumors and VEGF decisive role in this phenomenon allowed fairly quickly find the main drug candidate. It was Bevacizumab, a humanized monoclonal antibody, which acts as VEGF-A blocker. This drug was approved by FDA in 2004 for colon cancer treatment. Bevacizumab is effective in inhibition of ECs-dependent angiogenesis. Unfortunately, its action leads to hypoxia increase in tumor and to VM induction [89]. According to Qu and co-workers hypothesis [64], these conditions enhance invasiveness and metastasis of tumor cells.

Current knowledge of tumor vascularization clearly indicates that effective anti-angiogenic therapy must be multidimensional, with using of different targets not only in ECs, but also in other cells of tumor microenvironment, including tumor cells. The main targets of anti-angiogenic therapy, with the agents influencing them, and the effect of some of them on VM are presented in table 3.

Similar to Bevacizumab, Aflibercept is also used as VEGF inhibitor. It is active against not only VEGF-A, but also VEGF-B, and even PIGF [69]. The latter is mainly derived from tumor stroma. Probably, this drug action against several agents, at the same time, is responsible for its higher anti-angiogenic effect, compared to Bevacizumab. Such data were provided, among others, by the results of Chiron and co-workers [13] on human model of colon cancer.

An important group of drugs with anti-angiogenic activity are those addressed to VEGF receptors. Currently, about 7 drugs are admitted to trading by FDA, among them Sunitinib and

Sorafenib [18]. Thyrosine kinase of VEGFR is their directed target. From research on triple negative breast cancer is known that Sunitinib, decreasing MVD, contributes to VM induction via increase of tumor stem cell population with $CD133^+$ phenotype [91]. Potential, negative effect of Sunitinib therapy is

supported by the results of Cooke and co-workers [16]. This drug acts on ECs, as well as on pericytes. The decrease of the latter is a poor prognostic factor because it is correlated with higher ability to metastasis.

Sorafenib action is not limited only to VEGFR inhibition (tab. 3). The drug blocks also EMT, dependent on TGF-β [73]. However, no inhibiting influence on mesenchymal-epithelial transition (MET) was found, which is also, similar to EMT, crucial stage of tumor metastasis. This may be the explanation why Sorafenib does not give good results in highly advanced ovarian cancer [73]. Great expectancy is connected with another VEGFR inhibitor, Imatinib, which in Paulis and co-workers [62] studies on Ewing sarcoma, showed blocking effect on VM by limiting of VE-cadherin and MMP-2 expression.

TABLE 3. Targets of anti-angiogenic agents and influence of some of them on VM

TARGET	ANTI-ANGIOGENIC AGENT	INFLUENCE ON VM
VEGF-A	Bevacizumab	↑ [89]
VEGF-A, VEGF-B, PIGF	Aflibercept	ND
VEGFR	Sunitinib	↑ [91]
VEGFR	Sorafenib	ND
VEGFR	Imatinib	↓ [62]
FGF	FP-1039	ND
FGFR	TKI258, BIBF 1120, AZD4547	ND
CD105 (ECs/CCs)	TRC105, DNA vaccine	ND
HIF-1α	Sirolimus	↓ [41]
TNF-α	Thalidomide	↓ [93]
CCL-2	Carlumab	ND
caspase-8/TRAIL (TAMs) MDR1, HSPs (CCs)	Trabectedin	ND
signaling pathways engaged in VM (HIF-1, p53, p21, caspase-3)	CVM-1118	↓[37]

↑ – VM induction, ↓ –VM inhibition, ND – no data, ECs – endothelial cells, CCs – cancer cells, CCL-2 – chemokine 2, HSPs – heat shock proteins, MDR1 – multidrug resistance protein 1, TAMs – tumor associated macrophages

With regard to contribution of FGF in tumor angiogenesis, this molecule as well as its receptors are also potential targets in angiogenesis inhibition (tab. 3). Several FGF pathway inhibitors are in preclinical or clinical trials [49].

In anti-angiogenic therapy, also CD105 can be classified as a strategic molecular target. The antigen which plays a role of TGF-β in ECs is engaged in proliferation signal transmission. Endoglin inhibition on ECs surface by chimeric TRC105 antibodies induces antibody-dependent cytotoxicity, and in consequence apoptosis [70]. TRC105 combinations with anti-angiogenic agents, e.g. Bevacizumab or Sorafenib [71] are also in research.

Gene therapy with oral DNA vaccine focused on CD105 is an important component in fight against tumor vascularization [43, 47]. Attenuated Salmonella bacteria, with CD105 gene introduced into their genomes, were used to construct the vaccine. The vaccine acts as an inductor of host immunological system (T lymphocytes, dendritic cells) towards elimination of ECs with CD105 overexpression. Its effect is positive in endothelial cells. Endoglin is present also on the cells of different tumor types (chapter Vasculogenic mimicry of tumors). In melanoma, or Ewing sarcoma, endoglin leads to the increase of tumor cell migration and metastasis, and this is a reason for the vaccine application. However, in breast, oesophagus, or prostate cancer, endoglin is responsible for limiting of tumor cell migration and metastasis. In this situation, the decrease of CD105 expression can be very negative [43]. The results of other effective immunotherapy were also published [44]. Its components, CAMEL peptide and the IL-12 gene, are extracted from melanoma cells. The results are promising and indicate both anti-angiogenic and immunomodulatory action by suppression of regulatory T lymphocytes (Tregs).

Wang`s group [87] focused on developing gene therapy with using of polymer-modified Salmonella as a Cx43 gene carrier. The decrease of VEGF expression, and, in consequence tumor growth inhibition, is the effect of gene delivery to the tumor [86]. Antitumor activity of Cx43 may be directly connected with the presence and the number of GJ between tumor cells. The results of Tittarelli and co-workers [82] show that. Cx43 overexpression and high GJ number in melanoma xenografts protects against tumor cells proliferation and metastasis.

Sirolimus (rapamycin) can be another therapeutic tool, which as an inhibitor of HIF-1 α, contributes to blocking of signalling pathway mTOR, and in consequence of VM [41]. Thalidomide, with characteristic for tumor microenvironment TNF-α as its target, proved to be also effective inhibitor of vasculogenic mimicry [93]. Studies on mice with developed melanoma after inoculation cell line B16F10 demonstrated that administration of thalidomide, at a dose corresponding to safe in humans, leads to more than 3-times decrease of vessel-like structures, compared to their number in untreated mice. The expression of MMP-2 and MMP-9 was decreased twice. Worth noting are the latest reports on CVM-1118

agent, which inhibit activity of human melanoma cells able to vessel-like structure formation [37]. This small molecule (molecular weight of about 360) effectively blocks VM by influence on many signalling pathways in melanoma cells (tab. 3).

TAMs, recruited from the bone marrow, by tumor cells with CCL-2 contribution, are the other important target of therapy [58]. Monoclonal antibody, Carlumab (CNTO888), directed to this target is currently in clinical trials, and its action is reduction/inhibition of TAMs influx to tumor [72]. In this way, limiting of MMPs production and ECM remodeling is possible. The combinations of Carlumab with drugs used in conventional chemotherapy, e.g. docetaxel, carboplatin, are also tested [7]. However, in the beginning the drug provides low level of CCL-2 in patients, but at later stage an increase of the chemokine appears. With regard to that, Carlumab combination with the drugs mentioned above is not recommended.

Trabectedin, from tunicate *Ecteinascidia turbinate*, approved by EMA, has a direct action on TAMs. The drug induces dependent on caspase-8 apoptosis of TAMs via pathway connected with TRAIL receptors. It leads to inhibition of tumor progression, including inhibition of angiogenesis. It is proved by lover level of VEGF, CD31, and also CD105 in immunohistochemical studies, conducted on slices from tumor xenografts [34]. Simultaneously, Trabectedin shows the action towards tumor cells, where it plays a role of MDR1, or HSPs gene inhibitor [22].

TUMOR RESISTANCE TO ANTI-ANGIOGENIC THERAPY

Previous studies on tumor angiogenesis showed clearly that this process takes place with contribution of many agents, with different mechanism of action. For example, it is known that more than one pro-angiogenic factor is involved in tumor angiogenesis [31]. VEGF inhibitors or VEGFR blockers application in therapy is not sufficient, and even is dangerous. Currently, the progress of tumor resistance to therapy is a huge challenge. According to the data presented in figure 4, there are many mechanisms responsible for tumor progression even though used anti-angiogenic therapy [53, 83].

Involvement of different pro-angiogenic agents is one of the resistance mechanisms. Pro-angiogenic pathways, in which EGF, PDGF, bFGF, HGF, or IGF is involved, can be alternative to VEGF pathway [35]. It is worth noting that these agents can be produced not only by ECs, but also for example by CAFs [53, 83]. Recently, ECs resistance as a result of VEGFR2 activity, without VEGF contribution was focused [17]. Galectin-1 is responsible for this, and the process is the effect of VEGFR2 glycosylation.

Mobilization of EPCs from the bone marrow may be responsible for progression of tumor resistance to therapy. CAFs have a large contribution to EPCs recruitment.

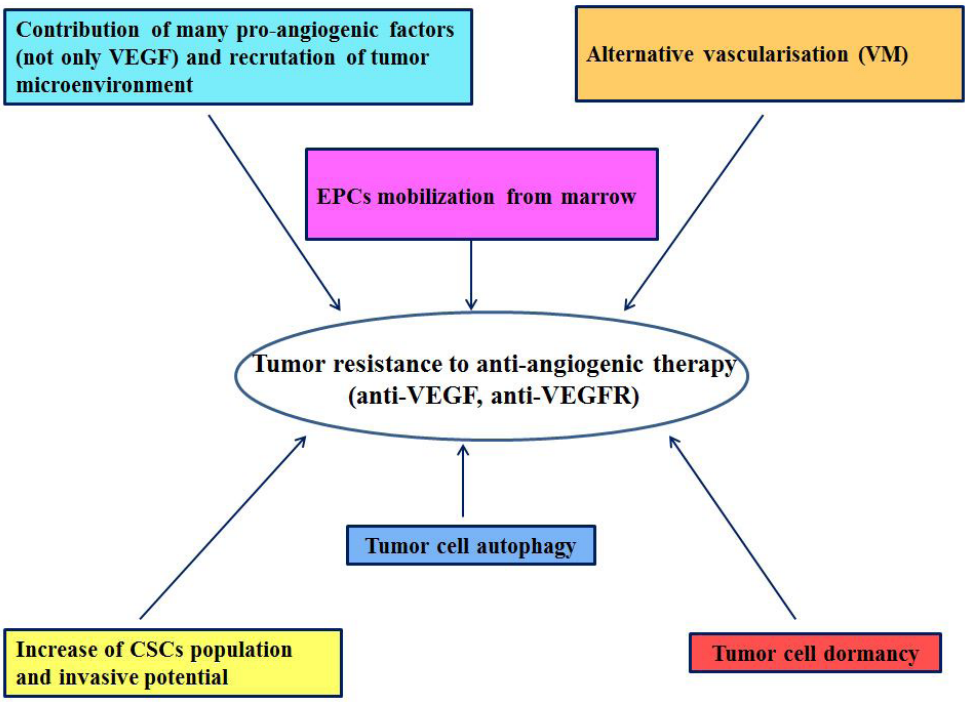

FIGURE 4. Mechanisms of tumor resistance to anti-angiogenic therapies

The resistance to anti-angiogenic therapy may be the effect of cytoprotective mechanisms. Autophagy and tumor cells dormancy belong to them. The dormancy of tumour cells undergo chemotherapy allows to survive, and when treatment is finished the tumor is developed. The research of Li and co-workers [48], conducted *in vitro* on breast cancer (SUM159 line), and on prostate cancer (DU145 line), indicate that short-termed exposure to docetaxel, as well as to doxorubicin, leads to dormant cells appearance. After chemotherapy, these cells show a high proliferative status, measured by uptake of labelled thymidine. Their presence can explain tumor recurrence in patients, which responded positively to earlier treatment.

The mechanism of autophagy can be engaged in dormancy regulation, because dormant cells are still alive [77]. Autophagy provides cell ability to adaptation and it protects the cell from apoptosis. In this way, autophagy shows the action promoting tumor metastasis [78]. From the studies on ovarian cancer xenografts, it is found that ARH1 overexpression promotes cell dormancy, which is correlated with higher level of autophagosome formation [54]. The resistance of breast cancer cells to TRAIL-dependent apoptosis is induced by autophagy as a result of death receptor expression decrease [21]. In the result of Bevacizumab-induced hypoxia, glioblastoma cells also trigger autophagy mechanism, which is provided

by the increase of expression of BNIP3, a marker of autophagy mitochondrial degradation [40], and tumor growth. This process is limited in the presence of chloroquine, BNIP3 inhibitor.

Drug-induced hypoxia contributes to the increase of CSCs number in tumor. According to the results of Allies and Weismann [1], the amount of CSCs in solid tumors is relatively low, compared to other tumor cells without ability to invasion. In breast cancer, about 200 CSCs is per 500 thousand of cancer cells, and in glioblastoma this proportion is 100 CSCs per 100 thousand tumor cells. Conley and co-workers [15] show that Bevacizumab, as well as Sunitinib induces the increase of CSCs amount in xenografts of breast cancer, via signalling pathway Akt/β-catenin, which is responsible for CSCs self-renewal. The increase of aldehyde dehydrogenase (ALDH) expression (marked by Aldefluor$^+$) proves the increase of CSCs. This enzyme, similar to Sox-2, or c-Met is accepted as CSCs markers [56].

SUMMARY

Years of study in tumor angiogenesis provided data that tumors are able to heterogeneous vascularization. Among mechanisms alternative to sprouting angiogenesis, intussusception, co-option, or vasculogenic mimicry may be mentioned. Many factors and conditions within tumor decide which mechanism is triggered. The cells of tumor microenvironment play a crucial role in formation of blood vessels, as well as vessel-like structures, and they undergo recruitment and adaptation to pro-angiogenic action induced by tumor cells. A special concern is aroused by VM mechanism, because of high plasticity ability and high metastatic potential of tumor cells forming vessel-like structures. The discovery of this phenomenon was one of the milestones in a history of tumor angiogenesis and opened completely new part in its research.

Nowadays, it is known that effective anti-angiogenic therapy should be multidirectional, addressed not only to ECs, but also to EPCs, CCs, as well as to TAMs, or CAFs. This approach gives a chance to limit or eliminate tumor resistance to previously used therapies. Is this chance will exploited, probably the results of further research will show.

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