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BIOIMMUNOTHERAPEUTIC TARGETS ON ANGIOGENETIC BLOOD VESSELS IN SOLID MALIGNANGIES

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1. ABSTRACT

Physiological angiogenesis is a tightly regulated process that occurs mainly during reproduction, development and wound healing. Although angiogenesis is a continuous process, different consecutive steps can be identified, including: i) release of pro-angiogenetic factors; ii) release of proteolytic enzymes; iii) endothelial cell migration, morphogenesis and proliferation. Angiogenesis is also a hallmark of malignant diseases, and an inverse correlation between tumor vascularity and survival was demonstrated. Thus, strategies aimed at interfering with tumor blood supply by targeting tumor vasculature, presently represent promising new approaches for the treatment of solid malignancies. In fact, at least 30 angiogenetic inhibitors, utilized alone or in combination with other therapeutic agents, are currently being tested in clinical trials in humans. In this paper, we will review current knowledges on selected molecules expressed by endothelial cells and involved in distinct steps of the angiogenetic process, that represent potential targets for bioimmunotherapeutic approaches in human malignancies.

2. INTRODUCTION

Angiogenesis is a complex process that leads to new blood vessels development from pre-existing microvessels, and involves sequential events including proteolysis and remodeling of the extracellular matrix, as well as proliferation and migration of endothelial cells (1). In the adult, with the exception of the reproductive cycle in women, angiogenesis occurs in response to pathological conditions such as inflammation, wound healing and hypoxia (2). Furthermore, excessive or insufficient vascularization has been associated with several non-malignant diseases (2-6), and it has long been established that angiogenesis plays a crucial role in tumor growth and metastasis (7). In this regard, it has been demonstrated that microvascular density correlates with distant metastasis and prognosis in solid malignancies of different histotype (8-13), and in hematological malignancies (14-15). Recent progresses in identifying and characterizing physiological regulators of blood vessels development, prompted several pre-clinical studies designed to block tumor vessel growth in order to interrupt blood supply to neoplastic cells. In light of these pre-clinical data, a variety of angiogenetic inhibitors are currently being tested in clinical trials aiming to target specific molecules involved in blood vessel neoformation, or to directly inhibit specific biologic functions of endothelial cells or their response to angiogenetic stimuli (16).

Due to their active involvement in angiogenesis, targeting of proliferating endothelial cells presents several advantages compared to conventional treatment of human malignancies; in fact, it allows: i) easy accessibility of therapeutic agents to endothelial cells through the blood stream; ii) suitability of this therapeutic strategy to solid tumors of different histotype; iii) targeting of a genetically stable cell population, thereby reducing the possibility of acquiring drug resistance. In addition, targeting of proliferating endothelia potentially amplifies the killing of

transformed cells since each blood capillary sustains the growth of a great number of malignant cells (17).

Although anti-angiogenetic therapy currently represents one of the most promising approaches for cancer treatment, a number of limitations must be taken into account when anti-angiogenetic therapies are carried out in humans. In fact, angiogenesis is highly regulated by a balance between positive and negative stimuli, that are tightly coordinated (1). Additionally, the mechanism of action of several angiogenetic inhibitors is poorly understood yet (1). Furthermore, cytokines and pro-angiogenetic molecules secreted by cancer and immune cells can modulate the phenotypic profile of tumor endothelia (1). Finally, the quantification of angiogenesis in response to angiogenetic inhibitors remains, to date, impractical in metastatic diseases; thus, the identification of reliable soluble markers of angiogenesis is required to monitor the effectiveness of anti-vascular therapies. In this regard, recent findings suggested that measurement of serum vascular cell adhesion molecule (VCAM)-1 might help in the assessment of anti-angiogenetic drugs currently in clinical trials (18).

3. VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF is a disulphide-linked dimeric glycoprotein, that represents a key mediator of vasculogenesis and angiogenesis (19-21), and presents at least 5 isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) generated by alternative splicing of a single gene (19-20). These different isoforms show similar biological activities, but differ for their binding to heparin and to the extracellular matrix (22). The smaller isoforms are secreted in a soluble form, whereas the larger ones remain cell-associated and their availability is regulated by proteolysis (22). Many different cell types, including cancer cells, are able to produce VEGF that exerts its biological activity predominantly on endothelial cells (19-20). *In vivo*, it induces both vascular permeability and angiogenesis, and contributes to vasculature maintenance (20, 23). *In vitro*, VEGF promotes endothelial cell proliferation and it modulates the expression of adhesion molecules such as VCAM-1 and ICAM-1 on endothelial cells (20). Additionally, it has been recently demonstrated that VEGF prolongs the survival of human dermal microvascular endothelial cells by inducing the expression of the anti-apoptotic protein Bcl-2 (24).

Increased levels of serum VEGF and of VEGF expression have been found in different angiogenesis-related diseases including malignancies of different histotype (25-26), and anti-VEGF monoclonal antibodies (mAb) strongly inhibited the growth of human tumor xenografts transplanted subcutaneously in nude or SCID mice (27-30). Taken together, these studies demonstrated that treatment with anti-VEGF mAb inhibited tumor neovascularization in animal models, and interfered with tumor vasculature maintenance, malignant ascites fluid formation, and metastatic spreading (27-30). However, tumor growth resumed upon cessation of the mAb treatment, suggesting that it may not be sufficient for complete tumor eradication (27, 31). Thus, curative therapy in cancer patients may necessitate a combination of both anti-angiogenetic agents such as anti-VEGF mAb and cytotoxic agents, to disrupt both tumor and endothelial cells (27). Humanized forms of anti-VEGF mAb, which retain the same affinity and efficacy of murine mAb, have been generated and are being tested in humans (16, 32-34, URL: http://cancertrials.nci.nih.gov). Results emerging from Phase I clinical trials with anti-VEGF mAb, administered alone or in association with chemotherapy, showed that these treatments are well tolerated; thus, human anti-VEGF mAb can be safely combined with chemotherapy without apparent synergistic toxicity (33-34). Phase II clinical trials showed objective responses, including one complete response, in breast cancer patients treated with anti-VEGF mAb (33, 35). In addition, treatment of patients affected by advanced non-small cell lung carcinoma or colorectal cancer with anti-VEGF mAb in combination with chemotherapy, increased the clinical response rate and prolonged the time-to-disease progression compared to chemotherapy alone (33, 36-37).

4. VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTORS (VEGFR)

The main receptors that initiate signal transduction cascades in response to VEGF comprise a family of closely related receptor tyrosine-kinases VEGFR-1, VEGFR-2 and VEGFR-3. Among these, VEGFR-1 and VEGFR-2 expression is largely restricted to the vascular endothelium, and both receptors bind VEGF with high affinity (19-21). VEGFR-2 seems to mediate the major growth and permeability actions of VEGF, whereas VEGFR-1 may have a negative role, either by acting as a decoy receptor or by suppressing signaling through VEGFR-2 (19-21). In adult human tissues, VEGFR-3 is mainly expressed in the lymphatic endothelia and in some high endothelial venules (38). Noteworthy, the mRNA for VEGFR-1 and -2 was found to be up-regulated in tumor-associated endothelial cells (26, 39); thus, VEGF receptors represent attractive targets in the aim to effectively block VEGF activity. Opposite to anti-VEGF mAb, the efficacy of SU5416, an inhibitor of the tyrosine-kinase activity of VEGFR-2, was reported to be best against slow-growing tumors, and more variable against fast-growing tumors (27). In addition, it was demonstrated that SU5416 has long-lasting effects on VEGFR-2 phosphorylation and function (40), and that it reverts tumor resistance to radiotherapy (41). Results from Phase I clinical studies indicated that anti-VEGF therapy with antibodies (Ab) or receptor kinase inhibitors is well tolerated; moreover, patients with advanced disease appeared to respond to therapy with disease stabilization or tumor shrinkage (27). In addition, among 28 patients with metastatic colorectal cancer enrolled in a Phase I/II clinical study, designed to investigate the safety of SU5416 in combination with 5-fluoruracil (FU)/leucovorin, 15 patients showed a clinical response (i.e., 1 complete response, 5 partial responses, 9 stable diseases) (42). According to these results, SU5416 is currently in Phase III clinical trials for advanced malignancies.

5. MATRIX METALLOPROTEINASES (MMP)

The MMP are a family of secreted and membrane-associated endopeptidases that selectively degrade components of the extracellular matrix and basement membrane, allowing endothelial cells migration and metastatic spread of cancer cells (43). These enzymes are produced by a variety of cell types, including endothelial and epithelial cells, fibroblasts, and inflammatory cells (43).

The identification of natural tissue inhibitors of MMP (TIMP), that are primarily secreted by endothelial cells, has stimulated studies focused on MMP inhibition to reduce the metastatic spreading of neoplastic cells. Among TIMP, TIMP-1, which is mainly released by endothelial cells (44), was shown to inhibit angiogenesis both *in vitro* and *in vivo* (45-46). Furthermore, TIMP-1 over-expression induced on endothelial cells by gene transfer, strongly decreased their migration and invasion of the extracellular matrix (47); furthermore, levels of TIMP-1 expression correlated with prognosis in patients with gastric carcinoma (48).

Extensive pre-clinical data generated in animal models have shown that the administration of synthetic MMP inhibitors (MMPI) reduces primary tumor growth as well as the number and size of metastatic lesions. Based on these promising results, synthetic MMPI have been developed and taken into clinical trials (49). Among these, Marimastat, BAY- 129566, CGS-27023A, Prinomastat (AG-3340), BMS-275291 and Metastat (COL-3) are in different stages of clinical development, ranging from Phase I to Phase III trials (50). Furthermore, with the aim to potentiate tumor cytotoxicity, as well as to reduce the size and number of metastatic lesions, several MMPI are being administered in clinical trials in combination with chemotherapy (49-51).

6. ALPHA V BETA 3 INTEGRIN (CD51/CD61)

The integrin family member alpha v beta 3 is an adhesion receptor, strongly implicated in the response of endothelial cells to angiogenetic stimuli. Its expression on angiogenetic endothelial cells is thought to facilitate their adhesion to the extracellular matrix during migration; in fact, alpha v beta 3 integrin was shown to bind directly to the MMP-2 on the surface of vascular endothelial cells during angiogenesis, suggesting a possible functional link between these endothelial cells surface proteins (52).

Furthermore, alpha v beta 3 integrin has been described as a marker for angiogenetic blood vessels, as it has been found predominantly expressed in wound healing and in tumor-associated blood vessels (53-54). Although the vasculature within apparently normal tissues also stained for alpha v beta 3 integrin, the percentage of stained vessels and their staining intensity were lower compared to neoplastic tissues (55). The relevance of this integrin in neovascularization was strongly supported by the ability of the anti-alpha v beta 3 integrin mAb LM609 to induce endothelial cells apoptosis within angiogenetic blood vessels (56), and to promote tumor regression by inhibiting tumor angiogenesis (57).

Clinical trials utilizing a humanized version of mAb LM609 (Vitaxin) have been initiated, to evaluate its safety and pharmacokinetics in late stage cancer patients (58). Results emerging from a pilot study have shown that Vitaxin was generally well tolerated; however, no objective regressions or significant stabilizations of disease were observed in 15 patients with advanced leiomyosarcomas (59).

7. ENDOSTATIN

Endostatin is a 20 kDa terminal fragment of collagen XVIII, that was originally isolated as an inhibitor of endothelial cells proliferation from the culture medium of the EOMA hemangioendothelioma cell line (60). Endostatin shows a widespread distribution in blood vessel walls and basement membrane zones, and a strong association with elastic fibers of aorta and with large arteries was found in adult mouse tissues (61).

Functional studies demonstrated that Endostatin inhibits endothelial cell proliferation (60) and migration (62), and that it induces endothelial cell apoptosis (63). The action of Endostatin seems to be endothelium-specific since it has no activity on fibroblasts and smooth muscle cells (60, 63-64); however, its mechanism(s) of action remain to be elucidated. It has been suggested that Endostatin inhibits the proteolytic activation of pro-MMP-2 and the catalytic activities of Membrane Type (MT)1-MMP and MMP-2 (65). In addition, most recent findings indicated that Endostatin down-regulates many genes involved in proliferation, apoptosis and migration of growing endothelial cells, resulting in a potent anti-migratory effect (66).

In vitro, Endostatin significantly reduced endothelial and malignant cells invasion into reconstituted basement membrane (65), while *in vivo*, it regressed established syngeneic Lewis lung carcinoma, T241 fibrosarcoma, and B16 melanoma tumors in xenograft models (60). Moreover, repeated cycles of Endostatin therapy prolonged tumor dormancy in mice, suggesting that it does not generate drug resistance (67); however, anti-angiogenetic therapy with Endostatin in tumor-bearing mice required prolonged administration and high doses of protein (60, 64). Further support to the potential usefulness of Endostatin for cancer therapy, has recently derived from the demonstration that intratumoral delivery of the Endostatin gene efficiently suppressed MCa-4 murine mammary carcinoma growth in immunodeficient mice (68). In this study, it was also demonstrated that the observed reduction of tumor growth was associated with a marked reduction in vascular density as assessed by CD31, CD105 and $DiOC_7$ staining. Noteworthy, radiation has been shown to increase the production of Endostatin; in fact, plasma levels of Endostatin were twice as high in mice that underwent tumor irradiation as compared to mice that underwent tumor resection (69). In addition, a significant tumor growth inhibition was observed in mice bearing radio-resistant tumors following combined treatment with

Endostatin and radiotherapy, compared to mice treated with irradiation alone (70). Altogether, these findings suggest that the efficacy of combined anti-angiogenetic and conventional anti-cancer therapies should be further investigated for their potential implications in the treatment of human cancer. Interestingly, Ab to Endostatin were detected in the serum and in the tumor tissue of a patient with a multifocal glioblastoma, suggesting that Endostatin over-expression might induce a humoral immune response (71).

At present, Phase I clinical trials are ongoing to test the efficacy and toxicity of Endostatin in patients with advanced solid tumors (i.e., breast cancer, melanoma, head and neck cancer, colon cancer, renal carcinoma and sarcoma) for which no other standard therapy exists (URL: http://cancertrials.nci.nih.gov).

8. PLATELET ENDOTHELIAL CELL ADHESION MOLECULE-1 (PECAM-1/CD31)

CD31 is a 130 kDa glycoprotein that belongs to the immunoglobulin (Ig) superfamily (72), and that is mainly expressed on endothelial cells of large and small vessels (73). In cultured endothelial cells, and in continuous endothelia of blood vessels in human tissues, CD31 was found predominantly localized at intercellular junctions (73-74); additionally, CD31 is constitutively expressed on platelets, monocytes and leukocytes (75).

The role of CD31 in angiogenesis has not been fully clarified yet, however, several experimental findings suggest that it is involved in neovascularization. In this respect, CD31 was found to play a role in endothelial cell migration (76), endothelial cell-cell adhesion (77), and in the development of the cardiovascular system (72). Additionally, it was reported that high levels of CD31 inhibited endothelial cells morphogenesis (78), and anti-CD31 Ab inhibited tube formation in Matrigel by human umbilical vein endothelial cells (HUVEC) (79-80*). In vivo*, CD31 has proven to represent an useful immunohistochemical marker of blood vessels, and it is currently considered as the "golden standard" for the assessment of angiogenetic activity in tumors (81); however, it was recently demonstrated that opposite to Endoglin, levels of CD31 expression inversely correlate with HUVEC proliferation (82).

9. ENDOGLIN (CD105)

CD105 is a homodimeric cell membrane glycoprotein of approximately 180 kDa, composed of disulphide-linked subunits of 95 kDa (83), which has limited species-specificity (84-85). Two different isoforms of CD105, L-CD105 and S-CD105 have been characterized (86-87). L-CD105 is predominantly expressed on endothelial cells and shares regions of sequence identity with betaglycan, a component of the Transforming Growth Factor (TGF)-beta receptor complex, that is weakly expressed or absent on endothelial cells (88).

CD105 is an accessory component of the TGF-beta receptor complex (89-90), and it binds several factors of the TGF-beta superfamily including TGF-beta 1 and -beta 3 (90-91), activin-A, BMP-7, and BMP-2 (90). The exact role of CD105 in TGF-beta signaling remains unclear. However, CD105 over-expression on different cell types modulates several cellular responses to TGF-beta 1, including inhibition of cellular proliferation and down-regulation of c-myc mRNA, stimulation of fibronectin synthesis, cellular adhesion, platelet-endothelial cell adhesion molecule-1 phosphorylation, and homotypic aggregation (89, 92-93). On the contrary, using an antisense approach, it was shown that the inhibition of CD105 expression in cultured endothelial cells enhanced the ability of TGF-beta 1 to suppress their growth and migration (93).

Concerning its tissue distribution, CD105 was found mostly expressed on cellular lineages within the vascular system, and preferentially and strongly expressed on endothelial cells (83, 94-95). Noteworthy, highest levels of CD105 expression were identified on cultured endothelial cells with protein, RNA, and DNA levels consistent with cellular activation and proliferation (96). In agreement with this observation, a significant correlation was found between levels of CD105 expression and endothelial cells proliferation and density in culture (82, 97), as well as with markers of cell proliferation (i.e., cyclin A and Ki-67) in tumor endothelia (98). Consistently, a stronger intensity of staining for CD105 was detected on vascular endothelial cells in tissues undergoing active angiogenesis, such as regenerating and inflamed tissues or tumors (96, 98-99), compared to normal tissues. In solid malignancies of different histotype investigated, anti-CD105 mAb reacted almost exclusively with venous and arterial endothelium of both peritumoral and intratumoral vessels (96-97, 100). Additional support to the involvement of CD105 in angiogenesis derives by the demonstration that mutations in the coding region of CD105 gene are associated with hereditary hemorrhagic telangiectasia type 1 (HHT), a dominantly inherited vascular disorder characterized by multisystemic vascular dysplasia and recurrent hemorrhage (101). In addition, mice heterozygous for CD105 showed signs of HHT (102), and CD105 knockout mice died of defective vascular development at gestational day 10-11 (102-103).

The identification of CD105 as an optimal marker of endothelial cells proliferation has encouraged studies designed to test the clinical usefulness of anti-CD105 mAb for the *in vivo* diagnosis and treatment of malignant diseases. Consistently, CD105 was shown to represent an ideal marker to quantify tumor angiogenesis (104); furthermore, microvessel density assessed by using an anti-CD105 mAb, was found to be an independent prognostic factor in breast cancer patients (104). Additionally, using *in vivo* models of spontaneous canine mammary adenocarcinoma (82) or human melanoma xenografts in C57BL/6 mice (105), it has been recently demonstrated that targeting of endothelial CD105 by radiolabeled mAb is an efficient procedure to image solid malignancies, regardless of their histological origin. Most interestingly, *in vivo* studies conducted in SCID mice bearing human breast carcinomas, demonstrated that

radiolabeled or immunotoxin-coniugated anti-CD105 mAb had a highly effective anti-tumor efficacy (106-108). In light of these findings, Phase I clinical trials have been initiated to evaluate the therapeutic efficacy and toxicity of anti-CD105 mAb in cancer patients (109).

10. CONCLUSIONS AND FUTURE DIRECTIONS

Agents that target the tumor vasculature by killing and/or interfering with biological functions of endothelial cells (i.e., proliferation, migration and differentiation), represent promising candidates to set up new therapeutic approaches in solid malignancies, regardless of their histotype. The pre-clinical and clinical experiences so far obtained demonstrate that a more in-depth knowledge of the endothelial cell molecules playing a role in angiogenesis, and of the molecular mechanism(s) regulating angiogenesis in tumors, may allow to design more specific and eventually more effective therapeutic approaches to cancer. Furthermore, these anti-vascular therapeutic strategies, that potentially do not induce drug resistance, might represent useful approaches for the long-term maintenance of cancer treatment, following or in association with conventional therapeutic strategies such as surgery, chemotherapy, radiotherapy and immunotherapy.

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