Angiogenesis as a therapeutic target in urothelial carcinoma

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In the last few years, angiogenesis has confirmed its critical role in the development of malignant neoplasms. Antiangiogenic drugs, mainly bevacizumab, sorafenib, or sunitinib, are currently approved in a wide number of tumor types, such as breast, colorectal, liver, or kidney cancer, and have changed dramatically the evolution of our patients. Unfortunately, in urothelial carcinoma, which is a very common neoplasm, antiangiogenic agents are still in a very preliminary phase of clinical research. In this study, we focus on the biological basis of angiogenesis in urothelial tumors, its influence in the prognosis of these malignancies, and the available evidence about the use of antiangiogenic drugs in urothelial carcinoma.

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Introduction

Urothelial carcinoma, mainly bladder cancer, accounted for more than 70 000 cases in 2008 in the US, becoming the fourth most frequent cancer in male patients and the ninth leading cause of death among male cancer patients (more than 10 000 deaths) [1]. There are very limited therapeutic strategies with proven efficacy in advanced disease, with 5-year survival rates of approximately 4–5%, so better knowledge of molecular biology of these tumors may lead to the discovery of new potential therapeutic targets for this disease.

Angiogenesis is known to play a leading role in the survival, proliferation, and metastatic potential of malignant tumors. Markers of angiogenesis and expression of angiogenic factors are associated with adverse outcomes in urothelial carcinoma [2]. Microvessel density (MVD) can be measured either as an average of counts over a number of randomly selected areas, also called the mean MVD, or quantified in the most dense areas of vascularization, termed hotspots [3]. There is a high variability in the techniques used to measure MVD, and this can be a reason for the inconsistent results observed among different investigators [4]. MVD has been correlated with the prognosis of urothelial carcinoma, and we will discuss this issue further in this study.

In addition, levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and matrix-metalloproteinases (MMPs), thrombospondin-1 (TSP1), or interleukin (IL)-8, have been shown to influence recurrence and survival [2]. We will analyze the available data regarding this matter.

These and other findings make angiogenesis a suitable therapeutic target for urothelial carcinoma, a disease in which targeted therapy has not achieved the same results as seen in other tumors. Clinical development of anti-angiogenic agents such as bevacizumab, sunitinib, or sorafenib, is currently under way, and results of the different trials with these drugs are eagerly awaited.

Angiogenesis in urothelial carcinoma

Angiogenesis is defined as the development of new vessels from existing vasculature. It is a normal physiological process in fetal development, menstrual cycle, and wound healing. But in cancer, it is essential for tumor growth and metastatic spread. This process is strictly regulated, and there are different mechanisms involved.

Angiogenesis depends mainly on endothelial cell migration and proliferation [2]. It is known that VEGF and other proangiogenic factors recruit circulating endothelial progenitors derived from bone marrow to sites of active angiogenesis [5]. These create a first group of migrating cells, which further develop new capillary sprouts and finally, through recruitment of pericytes and smooth muscle cells and organization of the endothelial cells, capillary stabilization [6]. There is a fine balance between all the involved factors, and in normal conditions angiogenesis remains strictly controlled. During tumorigenesis, the so called 'angiogenic switch' is activated, and the whole process of angiogenesis becomes deregulated resulting in enhanced neovascularization [7].

Hypoxia as an angiogenesis regulator

Variations in oxygen tension may result in the activation of different pathways, therefore producing numerous transcriptional factors [8]. The most important ones are hypoxia-inducible factor (HIF)-1 and HIF-2. In normoxia conditions, HIF interacts with the Von Hippel–Lindau

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protein, an ubiquitin ligase. Owing to ubiquitinization, HIF is degraded by the proteasome. Conversely, in hypoxia, HIF is not ubiquitinized, and the two subunits of HIF (α and β) bind and activate the expression of numerous genes involved in these processes.

These genes are mainly those involved in angiogenesis, such as *VEGF*, VEGF receptor 1 (*VEGFR-1*), and *ANGPT2*, but there are also genes that participate in glucose metabolism (glucose transporter 1), adhesion (e-cadherin, vimentin), migration (*c-Met*, *TGF-α*), proteolysis (cathepsin D, MMP-2), pH regulation (carbonic anhydrase 9), and proliferation (insulin-like growth factor 2) [2,9]. In addition, loss of function of Von Hippel–Lindau protein leads to the absence of HIF-1α degradation, and therefore to constitutive activation of the above-mentioned target genes, which are finally responsible for angiogenesis, proliferation, and survival. This feature has been mainly observed in kidney cancer.

Major proangiogenic factors

Vascular endothelial growth factor

VEGF is the most important of the angiogenic stimulators. There are four main forms of this factor, each one with a variety of functions, including recruitment and mitogenic stimulation of endothelial cells. The main ligand for tumor angiogenesis is VEGF-A that binds to VEGFR-1 and VEGFR-2, transducing major signals for angiogenesis [10]. Other factors, such as VEGF-C and VEGF-D, bind to VEGFR-3, which is mainly involved in lymphangiogenesis.

These factors are proven to act in a paracrine manner, as the tumor cells and their supporting macrophages and mesenchymal cells have been shown to secrete VEGF-A, which subsequently activates its receptors on endothelial cells, to promote angiogenesis [11]. VEGF initially interacts with VEGFR-2 to enhance endothelial cell proliferation, migration, and vascular permeability, and then with VEGFR-1 to assist the organization of new capillary tubes [12].

Matrix metalloproteinases

MMPs are a big family of proteolytic enzymes that are involved in the breakdown of the extracellular matrix (ECM). The four major groups are gelatinases, collagenases, stromelysins, and membrane-associated proteases [13]. They are activated by proenzymes, hypoxia, and acidosis, and contribute to release many proangiogenic factors, such as bFGF. In addition, through the degradation of the basement membrane of the vascular endothelium and the ECM, they create a passage in this barrier toward new capillary formation [14].

There is a huge amount of evidence about the role of MMPs in the development of malignancies. The first MMP to be isolated was that of the rat homolog, MMP-3, also known as transin, which was found to be induced by oncogenes and growth factors [15]. This was found to be homologous to interstitial collagenase, considered to be the prototypic MMP family member, and was itself observed to encode a protease expressed in malignant skin tumors in the mouse [16]. Tissue inhibitors of matrix metalloproteinases (TIMPs) are natural inhibitors of MMP activity, creating a balance in MMP/TIMP function; therefore, an increase in MMP activity and/or a decrease in TIMP function may cause MMP-dependent remodeling of ECM and subsequent tumor invasion. Nevertheless, this issue seems to be far more complex, as many tumors, such as bladder cancer, show a high TIMP production [17], and TIMPs may exert some growth-promoting effects [18].

Manipulation of MMPs has provided the most direct data about MMP involvement in tumor occurrence. For instance, overexpression of MMP-9 in rat embryo fibroblasts enhanced their metastatic potential in immunocompromized mice [19], whereas ribozyme-mediated inhibition of its expression reduced lung colonization [20]. In addition, the expression of MMP-2 cDNA in a bladder carcinoma cell line augmented its ability to form lung metastases [21].

Carbonic anhydrase 9

Carbonic anhydrase 9 is an enzyme that catalyzes the conversion of carbon dioxide and water into carbonic acid, protons, and bicarbonate ions. It takes part in intracellular pH control and, therefore, protects the tumor cell from hypoxia-induced apoptosis [22].

Fibroblast growth factors

bFGF was the first molecule to be identified as a proangiogenic factor [23]. It binds to heparin sulfate proteoglycans on the cell surface and ECM, becoming stabilized from proteolysis; therefore, it interacts with FGF receptors, leading to endothelial cell proliferation, regulation of integrin and cadherin expression, and modulation of cell-to-cell interactions [24]. It also has a synergistic action with VEGF, generating a significant angiogenic response in target cells [25].

Platelet-derived endothelial growth factor

Platelet-derived endothelial growth factor is also known as thymidine phosphorylase. Its mechanism of action is not well elucidated, but it could favor tumor invasion and metastatic potential through higher expression of VEGF and MMPs [26]. Thymidine phosphorylase also augments levels of HIF-1α during hypoxia, and increases cellular oxidative stress.

Cyclooxygenases

Cyclooxygenase (COX) is involved in the prostaglandin synthesis pathway as two isoforms, COX1 and COX2. COX2 is proinflammatory, and recently it has been shown to have a proangiogenic role. COX2 increases the expression of VEGF and bFGF, but also seems to stimulate antiapoptotic pathways [27].

Integrins

Integrins are transmembrane proteins that mediate interaction between the cell and the ECM, and are functionally involved in determining tumor angiogenic response during cancer progression to metastatic disease [28]. Through the recognition of the major adhesive components of the ECM, laminin, and fibronectin, integrins regulate cell proliferation, cell survival, and migration. Increased expression of some integrins, such as $\alpha_v\beta_3$, is detected in growth factor-activated endothelial cells in tumoral blood vessels [29]. Integrins also contribute to signal transduction from the extracellular environment to the intracellular network mediated by integrin-activated molecules, such as focal adhesion kinase, phosphatydil-inositol-3-kinase, and members of the extracellular signal-regulated kinase 1 and 2-mitogen-activated protein (2 MAP) kinase family to regulate cell proliferation, migration, and apoptosis [6].

Angiopoietins

Angiopoietins are ligands for TIE-2 receptors, located in the endothelial cells. They act through these receptors increasing the recruitment of pericytes and smooth muscle cells, and enhancing vascular permeability [30].

Interleukin 8

Interleukin 8 (IL-8) is a cytokine that is involved in the angiogenic process. Koch *et al.* [31] found that human recombinant IL-8 was a potent proangiogenic agent when implanted in a rat cornea and induced proliferation and chemotaxis of human umbilical vein endothelial cells. The question of how IL-8 exerts its angiogenic activity is not completely understood. It seems to be involved in the upregulation of MMP-2 expression and activity [32], and also acts directly on vascular endothelial cells as a survival factor [33].

Neutralization of IL-8 through a fully humanized antibody (ABX-IL8) proved to be active in human melanoma cell lines, as it caused a decrease in angiogenesis and tumor growth, and inhibition of MMP-2 activity and increase of tumor cell apoptosis [34]. The same strategy was followed in bladder cancer cell lines and xenografts, although ABX-IL8 had no clear effect on urothelial carcinoma cells *in vitro*, it achieved a significant decrease in tumor growth in the ortothopic nude mouse model. It also inhibited the activity of MMP-2 and MMP-9, resulting in a decrease in invasion through reconstituted basement membrane *in vitro* [35]. In a recent, large-scale, real-time reverse transcription PCR study in bladder carcinoma, IL-8 was found to be one of the major drivers of angiogenesis, together with VEGF-A [36].

Epidermal growth factor receptor

The oncoprotein, epidermal growth factor receptor (EGFR), is overexpressed in 31–48% of bladder cancer, and is associated with poorer outcomes [37,38]. EGFR also plays a role in angiogenesis, as it regulates the activity of VEGF, IL-8, bFGF, and MMPs [39]. Studies in bladder carcinoma cell lines showed that EGFR inhibition produced a decrease in VEGF, IL-8, and bFGF levels, three of the most important proangiogenic factors [40].

Major antiangiogenic factors

Thrombospondin-1

TSP-1, which is upregulated by p53, exerts a potent antiangiogenic action [41]. It interacts with several receptors, such as CD36, which mediates reduced cell motility and induction of apoptosis. By inhibiting β1-integrins, TSP-1 reduces VEGF-mediated cell migration. It also upregulates Bax, downregulates Bcl-2, and activates caspase-3 through the intrinsic pathway, therefore resulting in a proapoptotic stimulus.

Angiostatin–endostatin

Angiostatin–endostatin were the first endogenous angiogenesis inhibitors to be identified. Angiostatin, a fragment of plasminogen, has been shown to bind to some extracellular cell surface proteins, inhibiting ATP synthesis and ultimately leading to caspase-mediated apoptosis [42]. It also induces cell death by anoikis, that is, the detachment of cells from the matrix, which results in apoptosis [43].

Endostatin is a fragment of the type XVIII collagen, which interacts with many cell surface proteins. It interferes with VEGFR-2, leading to reduced cell motility, proliferation, and survival [44]. It also inhibits the activity of MMP-2, blocks cell cycle progression at the G1/S transition, and impairs proliferation by reducing the mRNA levels of several proliferative genes, such as *MAPK-1*, *MAPK-2*, or *c-myc*.

Platelet factor-4

Platelet factor-4 is a chemokine naturally secreted by platelets, which binds and blocks heparin-like glycosaminoglycans in the cell surface, therefore inhibiting migration by blocking the upregulation of MMP-1 and MMP-3 [45]. It also interferes with the cell cycle, mainly by decreasing pRB phsophorylation and consequently reducing cyclin E-CDK2 activity.

Correlation with prognosis

Some of these factors have been associated with differences in the prognosis of bladder carcinoma. MVD is a method to assess and identify tumor vasculature through an antibody that targets endothelial cells, and it has been shown to be an independent predictor of survival [46]. In the study by Dickinson *et al.* [46] MVD was assessed in 45 resected bladder tumors through CD31 immunohistochemistry, and was clearly correlated with survival, being as informative as stage to predict overall survival. Similar results were shown in a study by Chaudhary *et al.* [47], using the same technique to measure MVD. In addition, increased MVD has consequently been linked with increased recurrence rates, lymph node metastases, and stage progression. Bochner *et al.* [48] analyzed 166 tumor

tissue samples from the patients with invasive urothelial tract carcinomas. MVD was measured using HPCA-1, a monoclonal antibody directed against CD34. High MVD was associated with disease progression in patients with organ-confined tumors, tumors extending through the bladder wall, and tumors that had spread to regional lymph nodes. Tumor angiogenesis was found to be an independent prognostic indicator when evaluated in the presence of histological grade, pathological stage, and regional lymph node status.

Analysis of the levels of VEGF and bFGF has also been carried out, and has shown that elevated serum levels of VEGF had high sensitivity and specificity for predicting metastatic disease [49]. In urine, high levels of VEGF also correlated with tumor recurrence [50]. An interesting study published by Chikazawa *et al.* [51] examined the expression of VEGF, bFGF, and IL-8 after orthotopic implantation of human urothelial cancer cell lines in athynic nude mice. This study showed that bFGF and IL-8 enhanced expression, especially in early stages, regulated tumor growth and subsequent spontaneous lymph node metastases, and therefore could be promising therapeutic targets.

With regard to MMPs, increased expression of MMP-1, MMP-2, and MMP-9 seemed to be an independent predictor for survival in bladder cancer [52,53]; they were also correlated with high-grade tumors and more invasive tumors. Osteonectin, also known as BM40, is a key protein in the regulation of MMPs, and it has a positive correlation with bladder cancer progression [54].

The close relationship between p53 and TSP-1 has also a proven role in the prognosis of urothelial carcinoma. Tumors with p53 alterations are associated with low TSP-1 expression, and these tumors are more likely to show a higher MVD. Decreased TSP-1 levels were associated with increased recurrence and reduced overall survival rates in a study published by Grossfeld *et al.* [55]. They measured TSP-1 expression in tumors from 163 patients, together with MVD and p53 expression. Patients with low TSP-1 expression exhibited increased recurrence rates and decreased overall survival. TSP-1 expression was an independent predictor of disease recurrence and overall survival after stratifying for tumor stage, lymph node status, and histological grade, but it was not independent of p53 status. TSP-1 expression was significantly associated with p53 expression status and MVD counts. Tumors with p53 alterations were significantly more likely to show low TSP-1 expression, and tumors with low TSP-1 expression were significantly more likely to show high MVD counts.

Antiangiogenic agents in urothelial carcinoma

Bevacizumab

Bevacizumab is a humanized monoclonal antibody that binds to all VEGF isoforms, thereby preventing ligand binding to both VEGFR-1 and VEGFR-2. Although it was the first VEGF inhibitor approved by the FDA and it is a part of standard therapies in a wide variety of neoplasms, such as breast, colorectal, or lung cancer, bevacizumab research in urothelial tumors is very scarce.

In the 2009 American Society of Clinical Oncology (ASCO) meeting, Hahn *et al.* [56] presented the results of a phase II trial in metastatic urothelial carcinoma treated with the combination of cisplatin, gemcitabine, and bevacizumab. A total of 45 patients were enrolled, and 36 patients were evaluated for response. Of the patients, 17% achieved a complete response, 50% a partial response, and 28% a stabilization lasting more than 12 weeks. More mature results were presented in the 2010 ASCO meeting [57]. The overall response rate was 72, with 21% of patients achieving a complete response and 51% of the patients a partial response. Stabilization lasting more than 12 weeks was seen in 16% of the patients. The median progression-free survival (PFS) was 8.2 months, and median overall survival was 20.4 months. These data suggested that this schedule can be useful in the treatment of these patients. An intergroup phase III trial is currently evaluating this combination.

In the neoadjuvant setting, bevacizumab is being tested in combination with cisplatin and gemcitabine in a phase II trial conducted in the US. Bevacizumab is administered concurrently with these two drugs as neoadjuvant treatment, followed by surgery, and adjuvant therapy with paclitaxel and bevacizumab in case there is residual disease. There is another phase II trial testing bevacizumab in the neoadjuvant setting, being conducted at the MD Anderson Cancer Center. In this case, bevacizumab is combined with cisplatin, adriamycin, methotrexate, and vinblastine. Preliminary results from these two trials are pending.

An interesting approach that is currently being evaluated is the combination of hypericin-mediated photodynamic therapy (PDT) with bevacizumab. In a study published by Bhuvaneswari *et al.* [58], mice bearing xenograft bladder carcinoma tumors were treated with PDT, bevacizumab, or PDT and bevacizumab combination therapy. Combination therapy-treated tumors showed the most posttreatment damage, and VEGF expression was significantly downregulated in these tumors.

Aflibercept

Aflibercept is a fully humanized, soluble decoy VEGF receptor that binds and inactivates VEGF. This molecule also binds other VEGF-family members, such as VEGF-B and placental growth factor. This binding is potentially 100-fold tighter than is achieved with bevacizumab. In the ASCO 2009 Annual Meeting, Twardowski *et al.* [59] presented the results of a phase II trial with this agent in patients with recurrent or metastatic transitional carcinoma. Twenty-two patients were accrued, and all of them had received a platinum-containing regimen earlier. One

patient achieved a partial response, with a median overall survival for the whole group of 3.5 months, showing a limited activity in platinum-pretreated patients.

Sunitinib

Sunitinib is an oral tyrosine kinase inhibitor (TKI), with activity against c-kit, VEGFR1-3, platelet derived growth factor receptor (PDGFR), and Flt-3. In the 2008 ASCO Genitourinary Cancers Symposium, first results of a phase II trial with sunitinib in cisplatin-ineligible patients were presented. Of the 17 enrolled patients, clinical benefit was shown in 64% of them, with two partial responses and a median PFS of 5.9 months. This study has presented more recent data at the ASCO 2010 Annual Meeting, confirming the activity of the drug in a total of 37 patients, and correlating high levels of IL-8 with a lack of response [60].

Final results of a phase II trial have been recently published [61]. This trial evaluated the use of sunitinib in patients with advanced, earlier treated urothelial carcinoma, testing two schedules: the traditional one 4 weeks on–2 weeks off with 50 mg per day, or continuous therapy with 37.5 mg per day. Clinical regression or stable disease was achieved in 43% of the patients in the whole group, with median PFS of 2.4 months and median overall survival of 6 months. Sunitinib did not reach the preplanned threshold of greater than 20% activity following RECIST (Response Evaluation Criteria In Solid Tumors) criteria but seeing the objective responses achieved, the investigators proposed that further research with sunitinib in urothelial tumors should be warranted.

Sunitinib is also being evaluated in combination with chemotherapy. In the ASCO 2010 Annual Meeting, Galsky *et al.* [62] presented a study combining sunitinib with cisplatin and gemcitabine in advanced urothelial carcinoma. Despite intriguing preliminary activity, this combination at this dose and schedule was not tolerable, with very considerable toxicity, mainly hematological, with an 80% of grade 3–4 neutropenia and 60% of grade 3–4 thrombocytopenia.

At present, there are several phase II clinical trials evaluating the role of sunitinib, not only in metastatic disease, but also in the neoadjuvant setting in combination with cisplatin and gemcitabine, as adjuvant therapy after neoadjuvant chemotherapy and radical cistectomy in high-risk patients, or as maintenance therapy after chemotherapy in advanced disease.

Sorafenib

It is a small, oral molecule that inhibits various targets along the EGFR/MAPK signal transduction pathway, and also through VEGFR and PDGFR families. Final results of a phase II trial with sorafenib in advanced urothelial cancer were published in 2009 [63]. Patients should have received one and no more than one earlier line of chemotherapy for advanced disease. Among the 27 patients treated with the drug, no objective responses were observed, with a 4-month PFS of 9.8% and an overall survival of 6.8 months.

In the 2010 ASCO Annual Meeting, Krege *et al.* [64] presented a study testing cisplatin and gemcitabine with or without sorafenib in advanced urothelial carcinoma. Safety and toxicity analyses were shown, with no differences between the two arms of the trial. Efficacy and survival data are awaited.

Pazopanib

Pazopanib is a novel, multitargeted, TKI against VEGFR1-3, c-kit, and PDGFR. Preclinical evaluation has shown a high antiangiogenic and antitumor activity, and its clinical development is more advanced in other tumor types, such as renal cell carcinoma or ovarian cancer. In urothelial carcinoma, there are phase II trials recruiting patients, in combination with paclitaxel or in monotherapy, for platinum-pretreated advanced disease.

Vandetanib

Vandetanib is a dual inhibitor that targets VEGFR, EGFR, and rearranged during transfection. The first studies in the cell lines showed their potential to sensitize cancer cells to cisplatin [65]. There is currently a phase II trial in combination with docetaxel for stage IV pretreated patients with advanced urothelial carcinoma.

Cetuximab

Cetuximab is a chimeric murine antibody that binds to the extracellular domain of EGFR, leading to receptor downregulation and inhibition of downstream signaling. Cetuximab can inhibit bladder tumor cell growth *in vitro* and *in vivo* [40]. In combination with paclitaxel, cetuximab shows synergistic growth inhibition in mice with metastatic human urothelial carcinoma [66]. At present, there is an ongoing phase II trial testing the combination of cisplatin and gemcitabine with or without cetuximab, in advanced urothelial carcinoma [67]. As with bevacizumab, PDT is being studied in combination with cetuximab, with promising results showing that combination of cetuximab with PDT strongly inhibits tumor growth in bladder tumor xenograft models [68].

Gefitinib

Gefitinib is an orally bioavailable, small molecule, reversible EGFR TKI that selectively inhibits EGFR by competitively blocking the intracellular ATP-binding domain. Its antiproliferative effect on urothelial carcinoma has been showed *in vivo* and *in vitro* [69]. In combination with chemotherapy, Philips *et al.*[70] published a phase II trial with cisplatin, gemcitabine, and gefitinib in advanced urothelial carcinoma. The overall response rate was 42%, with a median PFS of 7.4 months and a median overall survival of 15.1 months, appearing to be an active and

well-tolerated regimen. Gefitinib has also been tested as monotherapy after the failure of an earlier chemotherapy regimen [71]. Results were a little discouraging, with just one confirmed partial response (3%) and an estimated median survival of 3 months.

Conclusion

Urothelial carcinoma has experienced very few therapeutic successes in the last few years, whereas targeted therapy has caused a dramatic change in the treatment of other tumor types, such as breast or colorectal cancer. Research efforts in the field of angiogenesis should warrant not only the development of new and effective agents, but also the discovery of predictive factors for response to antiangiogenic therapy, to better select the subgroup of patients with more probabilities to benefit from this therapeutic approach.

The initial results are promising, and clinical trials exploring this strategy, not only in advanced disease, but also in the neoadjuvant and adjuvant setting, are currently accruing patients; future data regarding these trials are eagerly awaited and, hopefully, will help us to improve the survival and quality of life of our patients.

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