REVIEW

ANGIOGENESIS IN RENAL CELL CARCINOMA: AN UPDATE

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ABSTRACT

The purpose of this article is to consolidate and update current information on the characterization and treatment of renal cell carcinoma, as well as the angiogenic phenomenon that underlies tumor development.

The article highlights the experimental methods that can be used in the study of angiogenesis, the growth factors that underlie the angiogenic process and their receptors, activation pathways, and the immune system as therapeutic targets.

Finally, the article points out the therapies currently carried out with the emphasis on therapies that involve the immune system but also therapeutic alternatives that require development in the future.

Keywords: renal cell carcinoma, angiogenesis, immunotherapy, tyrosine kinase inhibitors.

INTRODUCTION

Renal cell carcinoma (RCC) is one of the leading causes of death worldwide with gender ranking nine among men and fourteen among women. (1) The incidence of this pathology has increased significantly mainly due to imaging investigations often used in general patient evaluations. RCC is a category of tumors with molecular and histopathological heterogeneity, with multiple genetic factors that contribute to their occurrence, several familial syndromes of RCC and suppressor tumor genes have been identified that contributes to the development of this malignancy. (2)

Kidney cancer is characterized as one of the most vascularized encountered solid tumors because tumor cells can produce angiogenic factors and form their blood vessels that will connect to the systemic circulation. This process of formation of new blood vessels from pre-existing ones is called angiogenesis which is paramount for local tumor progression. The main activating mechanism of tumor neo-angiogenesis is hypoxia and mutations associated with the von Hippel Lindau gene. (3) The introduction of anti-vascular endothelial growth factor (VEGF) therapeutic agents and tyrosine kinase inhibitors (TKI) has been an important development in the treatment of RCC.

HOW TO INVESTIGATE ANGIOGENESIS IN RCC

The investigation of the angiogenic process is the evaluation of the pro or antiangiogenic activity of several endogenous and exogenous factors. These factors practically inhibit or stimulate the proliferation, migration, and organization of endothelial cells. There are currently several experimental models both in vitro and in vivo.

In Vitro experimental models:

Cell count

This technique evaluates the proliferation of endothelial cells by direct counting of them. The cells are grown and harvested and then stained with blue trypan and then exposed to angiogenic agents. (4)

Migration technic

This technique evaluates the movement of endothelial cells under the action of various pro- or antiangiogenic factors. Endothelial cells can proliferate, damage the basement membrane, and migrate in the form of sprouting under the action of proangiogenic factors.

Colorimetric assays

This technique evaluates the numerical change of the cell population by processing biomolecules by endothelial cells resulting in a metabolic product. The most commonly used colorimetric agent is 3-2,5-diphenyltetrazolium bromide (MTT). (5)

HDMEC sprouting

In this experimental model, progenitor endothelial cells (EC) suspended in fibrin gels and exposed to various angiogenic growth factors are used. The main purpose of the method is to evaluate the sprouting process of EC.

DNA evaluation method

It is a technique for assessing endothelial cell proliferation by introducing into the synthesis phase of DNA cellular replication measuring agents such as tritiated thymidine or bromodeoxyuridine. (6)

Matrix degradation

In the process of vascular sprouting, endothelial cells degrade the basement membrane and the extracellular matrix through the synthesis of proteolytic molecules. These molecules are aminopeptidases and matrix metalloproteinases and their detection is important knowing that many pro-angiogenic factors stimulate their production. (4)

Matrigel

In the process of angiogenesis, an important step is tubulogenesis which connects pre-existing vessels. In this experimental model, progenitor endothelial cells grown in cultures are introduced into the matrix for the evaluation of angiogenic properties. This medium (matrigel) is two-dimensional and has the characteristics of an extracellular matrix, containing cytokines and growth factors. (6)

Ex Vivo experimental models:

Ex Vivo Retina Angiogenesis

In this experimental model, the retina or fragments of mouse retina included in a three-dimensional fibrin gel containing VEGF are used. (7)

Thoracic Aorta Ring Model

In this model, fragments of the mouse thoracic aorta are taken from a gel containing collagen and fibrin and then cultured to form blood vessels. (8)

In Vivo experimental models:

Chick Chorioallantoic Membrane

It is an old method and often used in the study of angiogenesis due to the development of an important vascular network after only a few days of incubation. (4) The method is cheap, easy to reproduce and vascularization is easy to identify.

Corneal Angiogenesis

The cornea is a transparent and avascular organ which makes it a special model any new blood vessel being the result of the process of angiogenesis. (9)

Matrigel Plug

This method combines the experimental in vitro matrigel model with an in vivo subject. The matrix is injected subcutaneously using a mouse or rat and at 37 degrees Celsius, it becomes solid and can be inoculated with various proangiogenic substances. after an incubation period of several days, the matrigel is excised and evaluated from an immunohistological point of view.

Zebrafish

The method uses zebrafish embryos that are transparent and have a well-vascularized Yolk sac visible under a microscope. More recently, there have been genetically modified zebrafish embryos with fluorescent blood vessels with blood cells that emit different colors, making it easier to visualize new blood vessels. (10) (11)

PROGNOSTIC SIGNIFICANCE OF MICROVESSEL DENSITY

At the base of tumor growth and metastasis is the process of angiogenesis which, through the production of new blood vessels, supplies the tumor tissue with oxygen and nutrients. Microvascular density (MVD) is expected to be associated with tumor aggression as well as tumor extension and metastasis. MVD is defined as the ratio of the individual number of microscopic vessels detected in a given area. (12)

There are several studies that have demonstrated an inversely proportional association between MVD and survival in cancers such as melanoma, prostate cancer, breast cancer. (13,14,15) Other studies have shown a correlation between MVD and the expression of vascular endothelial growth factor, an important proangiogenic agent involved in the biology of several tumors and a therapeutic target. (16)

Currently, in the literature, there are several controversies related to the correlation between MVD and patient survival or disease aggression, some authors report associations of MVD with reduced survival in RCC (17,18) Also some authors suggest associations between MVD and VEGF expression as well. associations between MVD and mast cell tumor infiltration. (19,20,21) However, other studies did not report a significant link between MVD and VEGF secretion or between MVD and mast cell infiltration. (22,23) On the contrary, some authors have even suggested a correlation between increased MVD expression and prolonged survival. (24) We find the same phenomenon in the association of MVD with the tumor stage, some authors suggesting this correlation while other authors reported a negative correlation between MVD and the tumor stage. (25, 26, 27, 28)

In a recent study, Yanyuan et all. proposed a new concept of MVD assessment in the idea of a better correlation between it and the prognosis of the disease. This new concept is called total microvascular density (TMVD), which is basically the combination of MVD and vasculogenic or vascular mimicry (VM). If MVD is characterized by vessels of variable size, in different evolutionary stages, lined with endothelial cells, VM is represented by supply channel structures consisting of tumor cells, with the role of blood supply. In this study, MVD showed significant heterogeneity in patients with RCC and was not correlated with tumor grade or patient survival. MVD also did not correlate with VM, while VM together with the associated genes as well as other factors such as matrix metalloproteinase-9, galectin-3, caspase-3, and nodal differentiation factor had a significant impact on the progression-free survival rate (SPF) and global survival (OS). Also in the study, TMVD differentiated between patients with high and low MVD in terms of survival. Thus, it seems that TMVD has higher sensitivity compared to MVD or VM in predicting the evolution of the disease and the survival of patients with RCC. (29)

EXPRESSION OF ANGIOGENIC GROWTH FACTORS AND THEIR COGNATE RECEPTORS

Angiogenic growth factors in RCC have major implications in invasion and tumorigenesis and they result from the degradation of hypoxia inducing factors (HIF) that are a result of the alteration of the VHL complex. (30)

HIF complex comprises HIF-1α, HIF-2 α and HIF-β. HIF stimulates the following molecules: VEGF, PDGF, EGFR, TGF-α, GLUT1.(31) The increased expression of HIF leads both to the stimulations of endothelial cells in the extracellular space, to produce new blood vessels, and to the stimulation of tumor cells increasing their survival. (32)

Vascular endothelial growth factor (VEGF) is the main proangiogenic factor involved in renal cell carcinoma and comes from a ligand family composed of several subtypes whose activity is controlled by VHL and HIF. These subtypes bind to specific receptors- VEGFR1, VEGFR2 through which the two main pathways PI3K/AKT/mTOR and Raf-MEK-ERK are activated at the intracellular level, resulting in the stimulation of cell growth and proliferation of endothelial cells. (33,34)

Platelet-derived growth factor (PDGF) is another important proangiogenic factor also overexpressed by HIF action. PDGF activates the PDGFR (receptor) leading to the stabilization of new blood vessels created by the adhesion of pericytes.

Tumor growth factor-alpha (TGF a) is induced by HIF through activation of its receptor epidermal growth factor (EGFR). (35) VHL mutation leads to overexpression of EGFR by reducing its degradation in the cell membrane, thus increasing EGFR expression in renal cell carcinomas. (36)

Hepatocyte growth factor (HGF) and the corresponding proto-oncogene receptor c-Met are also involved in the proliferation and stimulation of tumor cells. The overexpression of HGF by c-Met is characteristic of hereditary papillary RCC. c-Met protein levels are increased by HIF and thus have a role in the angiogenesis of all RCC not only the papillary type.
HIF controls also the expression of *insulin-like growth factor 2 (IGF2*) and independent of HIF the alteration of VHL inhibits the receptor of insulin-like growth factor 1(IGF1R). (37) The presence of increased IGF in RCC correlates with a low survival rate.

Interleukin-6 (IL6) is another growth factor involved in renal cell carcinoma and has an autocrine effect, the increased expression of which is due to VHL mutation. (38) The binding of IL-6 to the appropriate receptor causes its dimerization and phosphorylation through the Janus protein family. Activation of the IL-6 receptor leads to the activation of STAT3 which ultimately leads to the proliferation of tumor cells in RCC. (39, 40)

Another important part of angiogenesis is played by *angiopoietin* and their receptor Tie 2 whose activation leads to stabilization and maturation of blood vessels, while inhibition will lead to their destabilization. In this case, destabilization leads to the proliferation of new vessels in the pre-existing vascular network, and decreased VEGF expression will lead to the death of endothelial cells. The VHL mutation is known to have an angiopoietin 4 expression inhibitory effect under normal conditions. There is also a relationship between the VHL mutation and angiopoietin 2, the decrease in Ang2 expression induced by hypoxia being achieved only in the condition of this mutation. These elements make Tie2 receptor antagonists in combination with VEGF inhibitors a possible treatment for clear cell RCC. (41, 42, 43)

MOLECULAR TARGETS FOR THERAPY

Vascular endothelial growth factor receptor

The most important molecular target of antiangiogenic agents in recent years, VEGFR belongs to a family of receptors (VEWGFR1-3), the most important being VEGFR2. (44)

This receptor can be stimulated by several factors including VEGF A, B, C, D, VEGFE, VEGFF, PIGF, NRP1-2. (45) On the other hand, it can be stimulated by non-canonical factors such as PECAM1, galectin-3 (45, 46) Once these factors are fixed on the corresponding receptor, the latter is activated and phosphorylation occurs in homodimers or heterodimers, the latter being more often involved in cancers. Activation leads to two-way intracellular signaling PI3K-AKT-mTOR and phospholipase C γ (PLC-γ).

PLC-γ path

With the activation of VEGFR2, phosphorylation of the PLC-γ pathway occurs. This will lead to the activation of protein kinase C (PKC), RAS-RAF-ERK, calmodulin, and calcineurin. Calcineurin causes the nuclear translocation of NFAT in T lymphocytes and the expression of CDK2 and 4. All these phenomena aim to increase cell proliferation and migration. (47,48)

PI3K-AKT-mTOR

This pathway is activated after VEGFR2 stimulation but also requires Src action. The synergistic action will lead to the activation of PI3K with the transformation of PIP2-PIP3-AKT1-mTOR and thus the stimulation of cell proliferation. (49,50)

Fibroblast grow factor receptor (FGFR)

FGFR is a family of 4 members of FGFR1-4 and can be stimulated by several ligands such as FGF1,4,7,8,9,11,15,19. (51) Binding of ligands to FGFR leads to phosphorylation of Srk-RAS / ERK-PI3K-AKT-PLC-γ-MAPK which ultimately leads to cell growth, proliferation and migration. (52)

PDGFR

This family includes PDGFR alpha and beta commonly expressed in mesenchymal, epithelial, endothelial and myeloid cells. Activation of the receptor has an autocrine effect with intracellular activation of known activation pathways. (53)

Other molecular targets are c-MET, AXL, CTLA-4, PD-1, PD-L1 and PDL2. The latter receptors are part of the new generation of therapeutic targets, many therapeutic agents being currently tested as first or secondary therapeutic line, alone or in combination with TK inhibitors.

ANTIANGIOGENIC DRUGS USED IN THE TREATMENT OF RCC

Anti-VEGF therapeutic agents

Tyrosine kinase inhibitors are currently the most important agents used in the treatment of RCC and target VEGF receptors. (54) They represent the first and the second line option in advanced and metastatic RCC demonstrating improved progression-free survival. Unfortunately, a majority of patients will develop resistance to treatment and thus consequent disease progression.

Sunitinib

Sunitinib is one of the first antiangiogenic drugs used in the treatment of patients with RCC. It targets several proangiogenic receptors and factors such as VEGFR1-3, PDGFR ALPHA and BETA, FLT3, KIT, RET and CSF-1R. This drug has been approved as the first line of therapy after several clinical trials compared to interferon-alpha. Sunitinib shows a higher rate of progression without recurrence compared to Interferon-alpha. However, the safety profile causes Sunitinib doses to be reduced due to important side effects, remaining a unique or combination therapy. (55, 56)

Axitinib

It is a specific inhibitor of VEGF1-3 receptors used as a secondary therapeutic line after previous treatment with first-line therapeutic agents. Therapeutic effects are less important due to low doses and secondary toxicity. However, this drug has also been used as a first-line treatment, and exposure to higher doses leads to an increase in progression-free survival, but even in this case the overall survival is not significantly improved.

Cabozantinib

Is an oral tirosin-kinase inhibitor (TKI) that is currently approved as a first-line treatment in advanced or metastatic RCC. Cabozantinib has multiple targets with inhibitory action on the VEGF receptor (VEGFR), Tie-2, MER, GAS6 receptor, and tropomyosin kinase receptor. (57) The most common side effects of the treatment with cabozantinib are similar to those of other VEGFR inhibitors and consists of hypertension, diarrhea, fatigue, anemia, palmar-plantar erythrodysesthesia. In various studies, it has been shown that cabozantinib is superior to everolimus or sunitinib in prolonging progression-free survival. (58)

Pazopanib

It is an anti-TK agent that targets VEGFR1-3, FGFR, PDGFR -α, - β and c-KIT. It has been used in several clinical trials and compared with Sunitinib. The comparative results between the two therapeutic agents are similar but with differences in toxicity profiles. This has led to Pazobanib being considered as the first alternative therapeutic line to Sunitinib. (59)

Tivozanib

This drug targets VEGFR1-3 and the main advantage effect and reduced side effects. Tivozanib has been used in several clinical trials both as first-line therapy in patients not previously treated with anti-TK agents, but also as a secondary line of treatment after first-line therapy. The therapeutic effect leads to an increase in the progression-free survival of the disease but so far we have no data on the increase in overall survival compared to Sorafenib. (60, 61)

Sorafenib

Sorafenib is one of the first antiangiogenic agents to target receptors such as VEGFR 1-3, c-KIT, RET, PDGFR BETHA, Flt-3. It has been used in patients with metastatic renal cancer to moderate and mild forms after other systemic treatments (with other non-antiangiogenic agents) and has shown increases in progression-free survival.

This has led to Sorafenib being used in many clinical trials as a control arm to date. (60, 62, 63)

Lenvatinib

It is an antiangiogenic agent that targets FGFR and is used as a secondary treatment line after the first line with anti-VEGFR agents. It has been used in several clinical trials and has shown an increase in progression-free survival compared to Everolimus (mTOR inhibitor), which has led to the inclusion of Lenvatinib as secondary therapy. (59)

Inhibitors of the PI3K-AKT-mTOR pathway

Agents such as temsirolimus and everolimus are the first generation of mTORC1 inhibitors. They inhibit mTORC1 kinase activity by binding to FKBP12 but do not block mTORC2 activity.

Sapanisertib is a pan-mTOR inhibitor that blocks both mTORC1 and mTORC2, this agent being included in phase I and phase II studies with antitumor effect in several types of solid tumors. (64)

Imunotherapeutic agents

It is now known that the immune system plays an important role in the behavior of kidney tumors, so new therapeutic agents have emerged that target the immune system. These therapeutic agents were initially used as secondary therapy after anti-VEGFR treatment.

The therapeutic target is the PD1 and CTLA-4 molecules. They have been used in therapeutic combinations (Nivolumab and Ipilimumab) and their effect has been compared with Sunitinib. The results are interesting, in the group of patients with intermediate and high risk the therapeutic combination showed increases in progression-free survival compared to Sunitinib but in the group of patients at low risk, the results were weaker compared to Sunitinib. These therapeutic agents of the immune system are currently used in clinical trials in combination with TK inhibitors as first-line therapy. Combinations such as Pembrolizumab plus Axitinib or Avelumab plus Axitinib showed increases in progression-free survival compared to Sunitinib. Studies have not been completed, and we currently have no data on the change in overall survival in these combinations. (65, 66, 67)

FUTURE DIRECTIONS AND PERSPECTIVES

The treatment of kidney cancer has changed in recent years, from tyrosine kinase inhibitors to anti-m-TOR agents and now to immune system agents.

There are currently several clinical trials in which immune agents are used in combination with anti-TK agents and also in combination with monoclonal antibodies. The results on progression free-survival are gratifying but we are still waiting for data on overall survival. Overall this therapeutic target is promising.

In addition to these therapies, other remedies are being prepared in the future, such as anti-tumor vaccines, agonists and, antagonists of T lymphocyte. For this, we need predictiveness in patient selection and this requires clearer future biomarkers.

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