

REVIEW

Targeting angiogenesis for cancer (gene) therapy

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Abstract: Suppression of development of new blood vessels in solid tumors provides a clear therapeutic benefit both in experimental animals and human patients. Molecules targeting multiple pathways with VEGF pathway being one of the best described are currently under consideration to reach use in clinical settings. Even though some success has been observed using traditional protein-based inhibitors, alternative strategies and new approaches to inhibit excessive tumor angiogenesis are being developed and tested. Gene therapy represents a powerful tool for therapeutic intervention to angiogenesis. Delivery of genes encoding endogenous angiogenesis inhibitors and decoy receptors for proangiogenic factors may bear an advantage over classic non-gene therapy in terms of specific targeting, cost-effectiveness and safety. Modern approaches focused on gene targeting such as RNA interference and microRNA will show the future direction in the field of angiogenesis inhibition for cancer treatment (Ref. 68). Full Text in free PDF www.bmj.sk.

Key words: gene therapy, cancer treatment, angiogenesis, biomedicine, experimental animals, proangiogenic factors.

First indications that the growth of human tumors is often associated with increased vascularization were observed more than 100 years ago (1). The presence of tumor – produced factors was postulated as early as 70 years ago. However, the actual rationale for studying the role of angiogenesis in tumorigenesis has become the Folkman's hypothesis in 1970s saying that solid tumors larger than 1–2 mm are not able to ensure cell nutrition only by diffusion and, thus, neovascularization takes place here (2). Hence, tumors are dependent on active angiogenesis. This hypothesis triggered a new wave of research focused on explaining the molecular basis of tumor angiogenesis and identification of angiogenic inducers/inhibitors as molecules of targeted therapy (3). Angiogenesis research has emerged as one of the most comprehensive research areas in biomedicine, and development of novel drugs by targeting angiogenesis has become one of the main focuses among pharmaceutical giants (4).

The pathway of VEGF-A and its receptors has become the best described signal pathway in developmental as well as reproductive and bone angiogenesis. Its importance is further confirmed by the fact that loss of one allele leads to embryonic lethality. Moreover, extensive experimental and clinical data has verified the role of VEGF in pathological angiogenesis and, thus, the rationale for the therapeutic use of VEGF inhibitors (5). VEGF-A binds both receptors, VEGFR-1 and VEGFR-2. It is generally accepted that mitogenic, angiogenic and permeability

increasing effects of VEGF-A are mediated by VEGFR-2, whereas the role of VEGFR-1 in angiogenesis is more complex. Under certain conditions, VEGFR-1 can serve as a decoy receptor sequestering VEGF and blocking its interaction with VEGFR-2. It also has a role in hematopoiesis and activation of monocytes and other cells originating from bone marrow, which can settle down in tumor vasculature and support tumor angiogenesis. At last, VEGFR-1 may be eventually expressed by tumor cells, what further broadens the actions of this receptor in tumor growth (6).

Expression of VEGF-A in tumors is stimulated by hypoxia. The central role in this regulation is played by transcription of hypoxia inducible factor (HIF), which, under normoxic conditions, is ubiquitinated and degraded. It has been proven that the expression of VEGF-A is markedly increased in many human tumors. This expression cannot be contributed solely to hypoxia and mutation/inactivation of tumor suppressor genes (such as von Hippel-Lindau factor, VHL), but also to the effect of oncogenes like mutated ras, erbB-2/Her2, activated EGFR and bcr-abl. Mabweesh and Amir emphasize the role of increased HIF-1 expression in tumorigenesis and its correlation to clinical status and prognosis (7).

Several preclinical studies showed that monoclonal antibodies against VEGF-A inhibit tumor growth *in vivo* in mice and this effect was proven on various tumor cell lines regardless of tumor origin (8, 9). Tumor growth inhibition *in vivo* has also been observed using different anti-VEGF approaches including application of dominantly negative VEGFR-2 (10), antibodies against VEGFR-2 (11), low molecular weight inhibitors VEGF RTK (12), soluble VEGF receptors (13) and VEGF vaccination (14). Recently it was found that an IgG-like fusion protein molecule VEGFR31-Ig that simultaneously binds VEGF-A and VEGF-C potently blocked tumor angiogenesis and lymphangiogenesis, ef-

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fectively inhibiting primary tumor growth and metastasis in a highly metastatic human hepatocellular carcinoma mouse model (15).

Besides cancer treatment, anti-VEGF therapy also has applications in different disorders. Age-related macular degeneration is the most common cause of serious irreversible loss of vision in the elderly. One of the treatments approved by the American Food and Drug Administration (FDA) is ranibizumab – recombinant humanized Fab antibody binding and neutralizing all human VEGF-A isoforms (16). It is further known that besides its vascular activity, VEGF represents a trophic signal for neurons, epithelial lung cells and cardiomyocytes, explaining why a decreased VEGF level may contribute to neurodegeneration, respiration distress and heart failure (17).

The other signal molecules playing a clear role in development and differentiation of vascular wall are represented by a family of PDGF (PDGF-B/PDGFR- β) and angiopoietins (Ang) acting as ligands of Tie2 receptor. The role of PDGF-B consists of activation of pericytes and maturation of microvasculature. Inhibiting the PDGFR- β signalization in tumor leads to an enhanced dependency of tumor vascular net on VEGF-mediated signals. Within this context, it is interesting that newly formed vessels, regardless of their origin, are particularly sensitive to absence of VEGF-A, whereas mature vessels covered by extracellular matrix and pericytes may be resistant to VEGF inhibitors and other antiangiogenic substances. Therefore, the combination of VEGF and PDGF inhibitors represents an attractive antivasular and anticancer strategy (18). Angiopoietin-1 is necessary for further remodeling and maturation of initially immature vasculature. In addition, the function of angiopoietin-2 in tumor angiogenesis has been recently uncovered, making the inhibitors of this molecule another candidate for therapeutic application (19). Selective inhibition of angiopoietin-2 has been found to be efficient in slowing the tumor growth by limiting the tumor angiogenesis and, more importantly, this effect is complemented by concurrent inhibition of VEGF leading to reduced proliferation and increased apoptosis of tumor cells (20).

Angiogenesis is a finely regulated process, which is under control of positive as well as negative regulation factors. Even though several endogenous negative regulators of angiogenesis have been described so far, their function in physiological angiogenesis regulation is still not completely clear. Thrombospondin is a large multifunctional glycoprotein secreted by epithelial cells into extracellular matrix that inhibits angiogenesis related to tumor growth and metastasis. Apart from that, several fragments of large proteins have been described as endogenous angiogenesis inhibitors, including endostatin, angiostatin, tumstatin and vasostatin. Specific mechanism of action of these proteins is still yet to be fully explained, although a few hypotheses have been published (21, 22).

Inhibition of tumor angiogenesis

Inhibition of tumor angiogenesis can be achieved by several strategies and on various levels. The classic targeted “non-gene” antiangiogenic strategies comprise targeting of:

a) endothelial (progenitor) cells

The most famous antiangiogenic substances of this group are VEGF inhibitors. Clinically significant are anti-VEGF antibody bevacizumab, VEGF₁₆₅ aptamer and various RTK inhibitors targeting VEGFR and other receptors pathways. The others include antibodies against VEGFR-1, VEGFR-2 and PlGF.

b) blood vessel mural cells and stromal cells

Pericytes differentiate from perivascular progenitor cells mobilized as a response to PDGF-BB. They increase the stability of vessels through a local production of VEGF and angiopoietin 1. Combined administration of RTK inhibitors against VEGFR and PDGFR β can have a synergistic effect even in rigid and resistant advanced tumor stages

c) hematopoietic cells

Some chemokines such as IL-8 directly stimulate the growth of endothelial cells. Inhibition of these chemokines, thus, reduces tumor growth. Blocking the signals enabling infiltration of leukocytes into tumors can therefore inhibit tumor angiogenesis.

d) neoplastic cells

Targeted cytotoxic, radiation or biological therapy of tumors also includes some antiangiogenic approaches. Tumor cells secrete angiogenic molecules and induce the expression of angiogenic receptors in tumor vessels (EGFR and VEGFR). EGFR inhibitors inhibit the growth of neoplastic epithelial cells. Moreover, tumor cells themselves express the receptors for VEGF, PDGF, FGF, EGF and other angiogenic factors on their surface. Therefore, antiangiogenic factors can directly kill tumor cells by blocking the signal pathways necessary for cell survival and increasing sensitivity to a different kind of therapy. Broad-spectrum RTKs Sorafenib and Sunitinib inhibit proliferation of endothelial and tumor cells through various pathways at the same time, thus being effective in monotherapy of certain tumors (23). In a recent phase II trial safety and efficacy of pazopanib, a multi-targeted tyrosine kinase inhibitor against VEGFR-1, -2 and -3, PDGFR α and β and c-Kit was tested in recurrent glioblastoma patients (24). However, this multi-targeted drug failed to show significant response at the maximum tolerated dose. Results from several other phase I studies using multi-targeted angiogenesis inhibitors have been recently published with partially successful outcomes (25, 26).

Combination therapy using angiogenesis inhibitors is not limited only to parallel application of cytotoxic chemotherapy, however, several preclinical and clinical studies analyzed the combination of different angiogenesis inhibitors with other targeted therapies such as EGFR/Her2 inhibitors (cetuximab, erlotinib and trastuzumab), PDGFR/bcr-abl inhibitors (imatinib), proteasome inhibitors (bortezomib) or integrin inhibitors (27).

A number of clinical studies focused on proving the effect of antiangiogenic therapies are ongoing worldwide in parallel. The National Institutes of Health (NIH) website provides a basic summary of antiangiogenic drugs that were or are still currently under clinical investigation (<http://www.cancer.gov/clinicaltrials/>)

developments/antiangiogenic-table). The best known drug for therapy of tumors is bevacizumab – a monoclonal antibody against VEGF-A. Results from several clinical trials have been published showing promising data with using bevacizumab (28, 29). The Food and Drug Administration (FDA) has approved bevacizumab (Avastin®) for use with other drugs to treat colorectal cancer that has spread to other parts of the body, some non-small cell lung cancers, glioblastoma and some breast cancers that have spread to other parts of the body. Bevacizumab was the first angiogenesis inhibitor proven to delay tumor growth and, more importantly, extend the lives of patients. The FDA has also approved other drugs with antiangiogenic activity as cancer therapies for multiple myeloma, mantle cell lymphoma, gastrointestinal stromal tumors and kidney cancer.

Interestingly, angiogenesis inhibitor therapy may not necessarily kill tumors, but may instead keep tumors stable. Therefore, this type of therapy may need to be administered over a long period. Because angiogenesis is important in wound healing and in reproduction, long-term treatment with antiangiogenic agents may cause problems with bleeding, blood clotting, heart function, the immune system, and the reproductive system (30). A meta-analysis of randomized controlled trials has shown that treatment with bevacizumab may significantly increase the risk of cardiac ischemic events in cancer patients (31). Since angiogenesis inhibitor therapy is still under investigation, all of the possible complications and side effects are still unknown. Moreover, results from the first of clinical anti-VEGF adjuvant cancer therapy studies were disappointing, stimulating extensive debate as to the potential of this approach. It will require additional clinical studies before we realize whether the effects of angiogenic blockade are durable, and if they are able to cure a subset of patients with early stage cancer (32).

Although much success with antiangiogenic therapy has been reported in preclinical and clinical studies, resistance to this therapy is a problem in human medicine (33). There are several mechanisms of resistance development. Even though still many patients do benefit from VEGF inhibitors therapy, there are some indications that VEGF-A signal pathway could be substituted by a different proangiogenic pathway during progress of the disease. Other reasons include the selection of tumor cells resistant to hypoxia (nondependent on angiogenesis), the remodeling of tumor vessels leading to decreased sensitivity to antiangiogenic therapy, the overproduction of angiogenic chemokines from macrophages infiltrating tumors, but also the heterogeneity and genetic instability of endothelial cells in tumor blood vessels. Recent studies have confirmed that VEGF, besides playing a crucial role in angiogenesis, does indeed serve multiple additional functions (34). These findings have important implications for the use of VEGF antagonists and VEGF receptor antagonists in patients for whom inhibition of pathological angiogenesis is the therapeutic goal.

Angiogenesis and gene therapy

Considering the fact that the fundamental discoveries and new findings in medicine are being crystallized on genetic and

genomic levels, gene therapy is one of the potential mechanisms for therapeutic intervention to angiogenesis. Gene therapy in a broad sense, i.e. all the therapeutic strategies employing nucleic acids as carriers of genetic information, found its utilization in most areas of medicine, including angiogenesis research.

Similarly to classical “non-gene” therapy, the angiogenesis research in gene therapy is happening on the preclinical level using appropriate animal models, with cancer and cardiovascular diseases being the most abundant indications. There are several different strategies known. Besides the delivery of therapeutic gene (replacement of the mutated gene by a functional one or delivery of the gene because of lack of the gene product), novel strategies are also being widely used based on blocking the function of a specific gene by application of RNA interference inducing sequences, antisense inhibition etc. Vectors for transfer of therapeutic sequences into target cells can be divided into three basic groups: viral, non-viral (naked DNA) and bacterial. Vectors and delivery systems, their construction strategies, pros and cons as well as application in therapy of specific diseases are reviewed in our paper (35). Since then, several improvements and new findings have been published though (36, 37).

The first clinical studies focused on therapy of genetic diseases of the immune system were initiated twenty years ago and gene therapy has been proven to be useful in almost all groups of diseases ever since, including infectious (38), psychiatric (39), but also post-injury states (40). One of the key advantages of gene approaches is the endogenous production of the therapeutic molecule. Furthermore, targeted gene delivery specifically into the target tissue or only to a certain cell type can dramatically decrease the likelihood of adverse effects. Along with the development of new vectors and regulatory systems, the ability to control the expression of therapeutic gene in time and space is being improved. This is of great importance in affecting such complex and complicated processes as angiogenesis. Currently, almost three quarters of indications addressed by gene therapy clinical trials are represented by cancer and cardiovascular diseases.

Antiangiogenic gene therapy of cancer

Gene therapy-based angiogenesis inhibiting strategies have gained much attention thanks to their advantages over the conventional antiangiogenic treatments. Given that effective inhibition of pathological angiogenesis requires long term treatment, gene therapy may be of importance for selective gene transfer to the affected areas and prolonged expression of therapeutic genes. Apart from that, gene therapy provides a possibility to circumvent the issues associated with recombinant proteins production, stability and solubility. Gene transfer allows for appropriate folding and stability of encoded proteins *in vivo* in the natural environment. An interesting advantage is also the ability to selectively target the gene transfer into certain tissues enabling localized expression and high regional drug concentration without increasing systemic levels. One of the key justifications for using gene therapy is also an insufficient efficiency of “non-gene” therapies based on inhibition of VEGF and other growth factors

signal pathways in humans (41, 42). The most commonly used gene therapy approach in cancer is so called suicide cytotoxic therapy using thymidine kinase or other chemosensitizing genes that allow the conversion of inactive prodrug (ganciclovir) into a cytotoxic product (43, 44).

Preclinical studies

Antiangiogenic gene therapy of cancer has been tested on a preclinical level in various carcinogenesis models. Most of the studies performed so far have used viral vectors (adenoviruses, retroviruses, lentiviruses, adeno-associated viruses, herpes simplex viruses) encoding endogenous angiogenesis inhibitor genes such as cytokines/chemokines (IFN- α , IFN- β , IFN- γ , CXCL10, IL-12, IL-18, TNF- α), VEGF blockers (sFlt-1, Flk-1), proteolytic fragments (angiostatin, endostatin, vasostatin, tumstatin) and others (45). For example, in a colorectal cancer model an adenovirus-based therapy using genes encoding IFN- β (46) and endostatin (19) as well as plasmids encoding Flk-1 (47) and tumstatin (48) have been successfully applied. In a model of malignant melanoma, retrovirus vectors carrying genes encoding CXCL10 (49), lentiviruses encoding PEX gene (50) and plasmids encoding vasostatin (51) and MCP-1 (52) genes have been successfully used, all exerting a clear antiangiogenic effect. Recently, a systemically available antiangiogenic gene therapy using adenovirus bearing soluble VEGF receptor gene has been proven to be effective in suppressing tumor growth in various oral cancer cell line xenografts in mice (53). Similarly, a tumor-selective replicating adenovirus expressing IL-18 could exert potential antitumor activity via inhibition of angiogenesis in melanoma-bearing mice (54).

Several studies have been performed using gene delivery of endogenous angiogenesis inhibitor endostatin. A liposome-encapsulated adenovirus encoding endostatin was applied in therapy of ovarian cancer (55). Systemic administration was well-tolerated and resulted in marked suppression of tumor growth, which was associated with a decreased number of micro-vessels and increased apoptosis of tumor cells. An interesting novel therapeutic approach for pancreatic cancer has been employed in a study using vaccinia virus encoding the endostatin-angiostatin fusion gene (56). Besides high selectivity of the used vector, inhibition of angiogenesis and a clear antitumor potency has been observed. A combined immunostimulatory and antiangiogenic gene therapy (IFN- γ -endostatin gene delivery) together with radiotherapy provided a potent antitumor effect in a murine metastatic breast tumor model (57). In another study, combined antiangiogenic and proapoptotic gene therapy involving endostatin and sTRIAL (soluble tumor necrosis factor-related apoptosis-inducing ligand) effectively suppressed hepatocellular carcinoma growth and angiogenesis in nude mice (58). At last, adenovirus-mediated endostatin gene delivery combined with cisplatin treatment was effective in a lung cancer murine model (59). These studies represent a future direction in cancer research in which instead of targeting a single molecule, a combinatorial approach targeting multiple factors and/or an additional thera-

peutic approach is applied to cover multiple pathways of cancer progression.

Clinical studies

Despite a relatively high number of clinical studies using cancer gene therapy, specifically antiangiogenic gene therapy has only been exploited in a few studies. Intratumoral injection of adenovirus encoding immunostimulatory cytokine IL-12 has been tested in patients with advanced gastrointestinal cancer (liver, colorectal, pancreatic tumors) in phase I study (60). The therapy was well tolerated, although only a moderate antitumor effect was observed. In another study, plasmid bearing IL-12 gene was applied to patients with malignant melanoma (61). In two out of nine patients, the disease was stabilized for period of over three years and a complete remission was achieved in one patient. In these patients, a localized reduction in angiogenesis has been proven by immunohistochemistry. However, the rest of the patients showed only temporal response to the therapy. A recent phase I clinical trial of IL-12 plasmid/lipopolymer complexes has also shown a clinical benefit in treatment of recurrent ovarian cancer without adverse events (62). In a different phase I study, adenovirus vector carrying IFN- α gene has been used in therapy of malignant pleural mesothelioma (63). In all the above-mentioned studies, however, inhibition of angiogenesis was not the primary goal, yet a part of the antitumor effect. The need for more clinical studies primarily targeting the angiogenic factors is of crucial importance for the whole field of cancer gene therapy in order to move forward. Both, employing the new molecular targets successfully tested in preclinical setting as well as targeting several angiogenesis pathways at the same time seem to be good perspectives for promoting antiangiogenic gene therapy from the bench to bedside.

Future directions

Angiogenesis is currently one of the target processes for cancer therapy. Several protein-based angiogenesis inhibitors have been successfully tested in preclinical as well as clinical studies. Some of them even reached the status of approved drugs for treatment of solid tumors. However, growing resistance to these molecules along with adverse effects and high cost all support the need for alternative strategies.

The explosion of new findings on angiogenesis in the last 15 years goes hand in hand with development and improvement of modern techniques and knowledge from molecular biology. Genetic studies on model organisms provided a new view on the key mechanisms and molecules that regulate the growth of blood and lymphatic vessels. Gene therapy has been proven as a promising and rapidly growing field of basic and clinical research with angiogenesis being one of the target processes to affect. Further studies on angiogenesis inhibition employing gene-targeting techniques such as RNA interference (RNAi) will show future directions in the field of cancer therapy. One of the most important papers in recent years was the study of Kleinman et

al., who have reported a sequence- and target-independent angiogenesis suppression by short interfering RNA (siRNA) via toll-like receptor 3 (TLR3) (64). Here, the non-specific siRNA suppressed dermal neovascularization in mice as effectively as VEGF-specific siRNA. The effect was mediated through cell surface TLR3, its adaptor TRIF and induction of IFN γ and IL-12. These results suggest that all siRNA-based RNAi strategies activating TLR3 have to face non-specificity, which, however, does not have to be considered a disadvantage. Even though a specific silencing is desired, a different approach/vector should be used to avoid activating of TLR3 pathway. Apart from RNAi, another big area of small RNA-related research that is gaining much more attention these days is the microRNA research. More importantly, microRNA has been found to play a key role in regulation of angiogenesis, both in cancer and ischemic diseases, indicating that the development of clinically relevant therapies can be expected in a short time period (65–68).

Considering the short time period taken from discovery to clinical testing of the above mentioned molecular pathways (RNAi and microRNA), it is likely that new yet undiscovered mechanisms will emerge from basic research that could possibly change the direction of current clinical research on cancer angiogenesis. We hypothesize that apart from the well-proved strategies of anticancer therapy completely new ones will take the lead in pursuit of better cancer treatment in 10 years. Angiogenesis-targeted gene therapy represents an excellent tool to reach this goal.

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