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Antiangiogenic Gene Therapy in Cancer

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Abstract

One of the most recent and exciting approaches in cancer gene therapy is the ability to target the developing blood supply of the tumor. An appealing feature of antiangiogenic gene therapy is that the tumor vasculature is a readily accessible target, particularly when the carrier and its gene are administered systemically. This is in contrast to several other gene therapy approaches in which the tumor vasculature represents a major obstacle to achieving high levels of transfection of the tumor cells. Several gene-based viral or non-viral therapies that target tumor angiogenesis have shown efficacy in pre-clinical models. Genes that encode antiangiogenic polypeptides such as angiostatin and endostatin have significantly inhibited tumor growth, inducing a microscopic dormant state. The products of these genes are thought to act extracellularly to inhibit angiogenesis. An alternative approach that investigators have used successfully in tumor-bearing mice is to target angiogenic growth factors or their receptors that are essential for tumor growth. Levels of angiogenic factors such as vascular endothelial growth factor (VEGF) have been reduced by either antisense methods or the use of genes encoding truncated angiogenic decoy receptors. Despite these promising findings of tumor reduction with antiangiogenic gene therapy, advances in the viral and/or non-viral delivery systems are essential for this therapy to have clinical utility. In this review, we will discuss the mechanisms of angiogenesis/antiangiogenesis, and the current status and future directions of antiangiogenic gene therapy.

Introduction

One of the key advances in our understanding of tumor biology is the realization that tumor growth is dependent on angiogenesis, the formation of new blood vessels (1). This fundamental principle states that tumor progression beyond 2 mm in diameter requires angiogenesis (2). Activation of growth factors that stimulate angiogenesis together with suppression of angiogenic inhibitors are frequent hallmarks of tumor growth (Table I). Therefore, antiangiogenic gene therapy attempts to reduce tumor growth either by reducing angiogenic growth factors or by increasing angiogenic inhibitors within a tumor (3).

In contrast to the situation with many gene therapy methods, the vasculature is not a significant obstacle to the antiangiogenic gene therapy approach. Since the tumor vasculature is readily accessible to systemic therapy, targeting mitogenic endothelial cells of the tumor increases the likelihood that gene therapy with antiangiogenic genes will be successful. Antiangiogenic gene therapy may also circumvent the problem of acquired

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anticancer drug resistance. If endothelial cells are targeted by this therapy, the relative genomic stability of the endothelial cells within tumors may reduce the emergence of drug resistance (4, 5). Another potential advantage of antiangiogenic gene therapy is the significant homology of the angiogenic/antiangiogenic proteins and their receptors found in animal models and man. As a result, animal models may have more utility in predicting tumor responses of antiangiogenic genes in man than other gene therapy approaches.

There are now several reports demonstrating the utility of antiangiogenic gene therapy in tumor-bearing mouse models. Investigators, first using *ex vivo* and later *in vivo* models, have successfully inhibited tumor growth by gene therapy approaches that inhibit angiogenic glycoproteins (VEGF, angiopoietin 2) and/or their receptors (6–9). More recently, gene therapy experiments with angiostatin and endostatin have also shown marked antitumor efficacy (10–12). With these successful experiments, there has been increased interest in antiangiogenic gene therapy. Whereas gene therapy with antiangiogenic genes was unrealistic a few years ago, there are now several gene therapy studies demonstrating its antitumor efficacy in pre-clinical models.

I. Molecular pathways controlling angiogenesis/ antiangiogenesis

Angiogenesis is a normal part of physiologic processes that occur in wound healing and in the formation of the corpus luteum. Many of the factors that play a role in physiologic angiogenesis are also important components in tumor angiogenesis. Increases in proangiogenic factors with concomitant reduction of angiogenic inhibitors are essential for tumors to grow (Fig. 1). In this section, we will first discuss the physiological role of the angiogenic or antiangiogenic factor and then consider its role in neoplastic processes. We have limited the scope of this review primarily to factors that have been targeted with gene therapy.

A. VEGF and Receptors

VEGF, a disulfide-linked 46-kDa dimeric glycoprotein, is a specific mitogen for endothelial cells (13, 14). VEGF, formerly known as vascular permeability factor (VPF), can also produce vascular hyperpermeability which is believed to be important in angiogenesis (15, 16). Hypoxia has been shown to increase the expression of VEGF in many cell types (15). One mediator of hypoxia-induced VEGF expression is adenosine, which binds to the adenosine subtype 2B receptor (17, 18). Furthermore, a number of cytokines such as transforming growth factor β (TGF β) and tumor necrosis factor α (TNF- α) can stimulate the production of VEGF. By promoting angiogenesis, deregulated VEGF expression contributes to the development of diseases such as psoriasis, diabetic retinopathy, and cancer. Five alternatively spliced VEGF mRNAs, encoding the isoforms of 121, 145, 165, 189, and 206 amino acids, have now been characterized. The predominant isoforms of VEGF, 121 and 165, are expressed in many different tissues.

Adding to the complexity of the alternatively spliced VEGF glycoproteins are the two VEGF receptors, VEGFR1 (also named *flt-1*) and VEGFR2 (also named *flk-1* or KDR). Both VEGFR1 and VEGFR2 are tyrosine kinase receptors with seven extracellular immunoglobulin-like domains. These receptors are specifically localized to mitogenic

endothelial cells. Whereas the VEGFR1 receptor appears to be important for migration of endothelial cells, the VEGFR2 receptor is essential for mitogenesis. Upon binding to VEGF, these receptors dimerize and subsequently may induce divergent signal transduction pathways (14). An extracellular soluble form of VEGFR1 that may modulate the activity of VEGF has also been identified (19, 20).

In addition to these high-affinity VEGF receptors, there are co-receptors that can alter the affinities of certain VEGF isoforms for their receptors. These membrane-bound receptors include heparin-sulfate proteoglycans, and neuropilins 1 and 2 (14, 21). Although the presence of these co-receptors on endothelial cells enhances the binding and bioactivity of VEGF to high-affinity VEGF receptors, the co-receptors by themselves do not activate signal transduction pathways (21).

In tumors, VEGF appears to be the most important and specific angiogenic factor in stimulating blood vessel development (22). Indeed, elevated VEGF levels are found in the vast majority of tumors. Increased serum levels of VEGF are found in cancer patients and correlate with a poor prognosis in several different types of cancers (breast, ovarian, lung, gastric, and colon). Furthermore, blocking antibodies to VEGF have been shown to inhibit tumor development in mice (23).

A number of early studies established that when a tumor reaches 2 mm or greater in diameter, angiogenesis is required for further tumor growth (2, 24, 25). These experiments suggest that VEGF plays a significant role in early tumor growth (Fig. 2). However, VEGF and its interaction with other angiogenic components in the tumor such as angiopoietin may be more complex than previously understood (see section on angiopoietin).

Although VEGF levels and angiogenesis are markedly increased at early stages of tumor growth when tumor cells are transplanted into tissues with low vascularity (e.g., subcutaneous), one recent study demonstrated that VEGF levels remained low even when metastases to vascular organs (e.g. brain or lung) were greater than or equal to 2 mm in diameter (26). At these early stages, the growing tumor, instead of promoting angiogenesis, co-opted the vessels of the normal tissue to obtain essential nutrients (Fig. 2). When the tumor grew to several millimeters in size, however, VEGF levels were up-regulated and became essential for angiogenesis and further tumor growth.

B. Angiopoietins 1,2 and the Tie Receptors

Angiopoietin 1 and 2 are highly homologous cytokines that bind with similar affinities to the endothelium-specific receptor, Tie2. Nevertheless, the physiological consequences of binding by these ligands are different. Angiopoietin 1, which appears to have a significant role in stabilizing vessels, activates the tyrosine kinase Tie2 receptor and the subsequent signal transduction pathway (Fig. 3). Despite the high homology with angiopoietin 1, angiopoietin 2 binds to but does not activate the Tie 2 receptor. During development, angiopoietin 2 causes apoptosis of the vessel in the absence in VEGF. In the presence of VEGF, however, angiopoietin 2 stimulates angiogenesis perhaps by disrupting the integrity of the vessels (Fig. 3). This interplay between VEGF and the angiopoietins has an important

role in the corpus luteum. Although Tie 1 and 2 are highly homologous tyrosine kinase receptors, a ligand has not been identified for the Tie 1 receptor (27).

Similar to the interplay that occurs in the corpus luteum, it appears that angiopoietins cooperate with VEGF to remodel tumor vasculature. Investigators recently found that newly formed tumor metastases in vascular tissues do not obtain most of their nutrient and oxygen from angiogenic vessels but rely on existing vessels in the target organs (26). As stated previously, the developing tumors co-opt these existing host vessels. These co-opted vessels do not immediately undergo angiogenesis but instead regress, leading to massive tumor cell loss. The remaining tumor is then rescued by a classical angiogenesis response. The expression patterns of VEGF and angiopoietin 2 correspond to the changes seen in the tumor. Early in tumor development, the levels of VEGF are low, whereas angiopoietin 2 is up-regulated and diffusely scattered throughout the tumor. Thus, in the absence of VEGF, angiopoietin 2 may mark the endothelial cells for destruction. At a later stage when the tumor is large with central necrosis, both angiopoietin 2 and VEGF are up-regulated primarily in the peripheral zones of the tumor (Fig. 2B), and there is marked angiogenesis and tumor growth in these zones. Thus, the presence of VEGF determines whether angiopoietin 2 induces apoptosis of endothelial cells or stimulates angiogenesis and tumor growth.

C. Fibroblast Growth Factors

In contrast to VEGF and the angiopoietins which specifically affect the endothelium, fibroblast growth factor 1 (FGF1, acidic) and fibroblast growth factor 2 (FGF2, basic) exhibit a variety of biological functions in many tissues and organs (22). FGF1 and FGF2 stimulate the proliferation of several cells including fibroblasts, endothelial cells, astrocytes, oligodendrocytes, neuroblasts, keratinocytes, osteoblasts, and smooth muscle cells. In addition, FGF2 is chemotactic for endothelial cells. Whereas VEGF induces the $\alpha_v\beta_5$ integrin pathway, FGF2 stimulates angiogenesis through the $\alpha_v\beta_3$ integrin pathway (28). FGF1 and FGF2 bind to one of four high affinity tyrosine kinase receptors. In addition, cell surface low affinity glycosaminoglycan co-receptors have been found to enhance FGF binding to the high affinity receptors.

Whether FGF2 has a direct role in tumor angiogenesis is inconclusive. Many investigators have extrapolated from *in vitro* and *in vivo* angiogenic assays utilizing FGF2 to explain the promotion of tumor growth *in vivo*. However, there is little evidence to show that fibroblast growth factor receptors (FGFR) are present in the microvessels of a tumor (29). The lack of measurable FGFR on the tumor endothelium is in marked contrast to the expression of the VEGF receptors. It has been suggested that FGF2 *in vivo* may increase angiogenesis by inducing other angiogenic growth factors such as VEGF (30). Although its role in angiogenesis *in vivo* is uncertain, there is evidence to suggest that FGF2 has a role in promoting tumor growth. Neutralizing antibodies to FGF2 have been shown to prevent development of tumors in pre-clinical models (31). Furthermore, elevated FGF2 levels, which have been detected in several types of malignancies, are associated with a poor prognosis (32, 33).

D. P53 and thrombospondin

Although the vast majority of studies with p53 have emphasized its ability to inhibit the cell cycle, p53 is able to stimulate several antiangiogenic mechanisms (discussed in more detail below). It was reported initially that inactivation of p53 did not affect embryological development, as studies on knockout mice primarily focused on the rapid development of tumors in these mice. Nevertheless, there appear to be subtle effects on embryological development regulated by p53 (34). It has been suggested that p53 plays a role during development in neuronal apoptosis and in controlling mutagenesis (35). At present, there is no direct evidence that p53 has a role in vasculogenesis during embryogenesis or in normal physiologic processes related to angiogenesis

In vitro experiments have shown that p53 may affect angiogenesis either by down-regulating VEGF and/or by inducing expression of thrombospondin 1 (Fig. 4) (36, 37).

Thrombospondin 1 (TSP1), a large 480 kd trimeric protein, has been found to inhibit endothelial proliferation and migration in assays *in vitro* (38). Although mice lacking TSP1 do not appear to have defects in vasculogenesis (39), potent antiangiogenic peptides have been isolated from the monomeric polypeptide of TSP1 (38). Besides antiangiogenesis, TSP1 is involved in attachment, spreading, and motility of several cell types. Until recently, little was known about the mechanisms of TSP1 action except that its binding to the endothelial surface receptor, CD36, was essential for inhibition of endothelial cell proliferation (Fig. 4) (40). Investigators have recently shown that inhibition of angiogenesis and the induction of apoptosis by TSP1 requires the sequential activation of CD36, p59fyn, caspase-3 like proteases, and p38 mitogen-activated protein kinases (41). The roles of TSP1 and 2 in physiologic processes are at present unknown.

Thrombospondin 2 (TSP2) has high structural similarity to TSP1. In contrast to the normal vasculature observed in TSP1 null mice, mice lacking TSP2 have a significant increase in the number of blood vessels (42). Further evidence supporting a role in angiogenesis for TSP2 is its strong inhibition of endothelial proliferation (43). Although p53 induces the transcription of TSP1, it is unknown whether p53 induces TSP2.

Several studies have correlated the presence of wild-type p53 and increased levels of TSP1 with an antiangiogenic effect on tumors. Loss of functional p53 in small cell lung cancer specimens correlates with increases in VEGF (44). In addition, there is evidence to suggest that p53 could affect the growth of the tumor by inducing an antiangiogenic protein, TSP1. Interestingly, lower TSP1 levels in a variety of cell lines correlate with a more malignant phenotype (45). The MDA-MB-435 cell line, a very aggressive and metastatic breast cancer line when injected in nude mice, secretes the lowest levels of TSP1 when compared to less malignant breast cell lines. Similar to TSP1, an inverse relationship has been found between the levels of TSP2 and progression/vascularity of the tumor (46–48).

E. Angiostatin and endostatin.

Angiostatin and endostatin, both potent polypeptide antiangiogenic inhibitors of tumors, are proteolytic products of plasminogen and collagen XVIII, respectively (49, 50). Neither full-length plasminogen nor collagen XVIII has any antiangiogenic activity. In angiostatin, the

antiangiogenic activity is confined to the first three of the four kringle structures. Furthermore, the 2nd and 3rd kringle domains of angiostatin are synergistic in their anti-proliferative tumor activity (51). In contrast to angiostatin, the precise location of the antiangiogenic activity of endostatin is unclear. Indeed, there may be several domains important for the antiangiogenic activity of endostatin (52–54).

The roles that angiostatin or endostatin play in physiology is unknown. Interestingly, with the use of antibodies “endostatin” can be detected in the serum of individuals without any evidence of tumors. It is possible that endostatin has a prophylactic function in preventing tumorigenesis. Alternatively, since full-length collagen XVIII (which contains endostatin) has no antiangiogenic activity (50) and can be detected by the polyclonal antibody to endostatin, the “endostatin” that is detected in the serum may not represent functional endostatin. This was suggested by Hefler and colleagues who found no difference in endostatin levels in patients with uterine cancer of various stages and aggressiveness (55).

Recently, investigators have found that angiostatin binds tightly to an ATP synthase on the endothelial cell surface (56). These receptors are specifically expressed on mitogenic endothelial cells, which may explain why there are few side effects with angiostatin. The antiproliferative effect of angiostatin was decreased by as much as 90% in the presence of anti-alpha-subunit ATP synthase antibody. At least part of the mechanism of action of angiostatin may be through this cell surface receptor. No receptor has yet been identified for the endostatin polypeptide.

Interestingly, a few tumors have enzymatic activity that generates angiostatin or endostatin (49, 50, 57, 58). It has been speculated that these proteins are mobilized to regulate carefully the angiogenic responses present in tumors.

F. Antithrombin III

Antithrombin III (AT), a plasma serpin, is a 58-kD glycoprotein (59) that is the principal inhibitor of thrombin and the blood-clotting proteinase factor Xa (60, 61). Antithrombin is relatively inactive as an inhibitor of coagulation proteases until it binds to the heparin side chains that line the microvasculature. The binding of heparin-like molecules to AT results in the proteolytic cleavage of AT at its C-terminal end, which confers antiangiogenic activity to AT (62). It has been hypothesized that the cleaved AT with an altered conformation (“relaxed” AT) can assume a new antiangiogenic function by either binding to cell surface receptors or to the extracellular binding proteins. Although these data provide evidence that the clotting and fibrinolytic pathways are directly involved in the regulation of angiogenesis, further experiments are required to substantiate this interesting association.

The antitumor efficacy of antithrombin III was recently demonstrated in a pre-clinical study. By selective *in vivo* passage of a small cell lung cancer, O'Reilly and colleagues isolated a potent antiangiogenic form of antithrombin III with a “relaxed” conformation (62). It is unclear if the antiangiogenic form of antithrombin III has any role in most tumors. Nevertheless, the relaxed form of AT has been found to inhibit markedly tumor growth in mouse models. Compared to angiostatin and endostatin, antithrombin III is a significantly more potent tumor agent on a molar basis. Although AT is a promising antiangiogenic

polypeptide, there are no gene therapy studies with this agent that have demonstrated antitumor efficacy.

G. Cytokines

Several cytokines including IL-12, Platelet factor 4 (PF-4) and IP10 have been associated with an anti-angiogenic effect. IL-12, an inflammatory cytokine, has a number of diverse biological functions. It has been demonstrated that IL-12 can inhibit angiogenesis and reduce tumor growth (63), and that this angiostatic response can be inhibited by blocking antibodies to IFN- γ , suggesting that IFN- γ mediates the antiangiogenic component of IL-12. It has been further hypothesized that IP-10, an angiostatic protein induced by INF- γ , may mediate IL-12 effects on angiogenesis (64, 65).

Platelet factor 4 (PF-4) and IP10 belong to a family of C-X-C chemokines. These are heparin-binding basic proteins with well-characterized functions in tissue repair and inflammation (22, 66). These proteins inhibit proliferation and migration of endothelial cells (67, 68). Although they were initially thought to be angiostatic because of their ability to bind heparin, a mutated form of PF-4 lacking the heparin-binding domain showed angiostatic properties equivalent to those of the natural peptide. It now appears that the mechanism involves decreased receptor binding of VEGF and FGF2 (69).

Although there is no current role for IL-12 and PF-4 in tumor biology, administration of these cytokines does inhibit tumor growth in a mouse model (70, 71). While both IL-12 and PF-4 appear to inhibit tumor growth by an antiangiogenic mechanism, IL-12 also inhibits tumor growth by the recruitment of specific immune effector cells.

H. Pigment Endothelial Derived factor (PEDF)

PEDF appears to play an important role in preventing vessels from invading the cornea and the vitreous of the eye. PEDF is produced by retinal cells and its levels are directly correlated with oxygen concentrations (72). Loss of expression may play a role in ischemic conditions such as diabetic retinopathy and cancer. Fetal expression has not been studied, but PEDF is up-regulated 18 days postnatally in the pigment endothelial layer. In an endothelial migration assay, PEDF was a more potent inhibitor than angiostatin, thrombospondin 1, or endostatin. Nevertheless, the half-life of antiangiogenic inhibitors *in vivo* may be more important in determining their potency (63). No known antiangiogenic functions in tumors have yet been described for PEDF.

I. Tissue inhibitors of Metalloproteinases (TIMPS)

The matrix metalloproteinases (MMPs) are a family of secreted and membrane-bound zinc-endopeptidases; these enzymes can together degrade all the components of the extracellular matrix. MMPs are thought to be essential for the diverse invasive processes of angiogenesis and tumor metastasis. Counterbalancing the MMPs are the tissue inhibitors of metalloproteinases (TIMPS). TIMPS are naturally occurring proteins that specifically inhibit matrix metalloproteinases, thus maintaining balance between matrix destruction and formation. An imbalance between MMPs and the associated TIMPS can alter the physiologic angiogenic processes. This is demonstrated in Sorsby's fundus dystrophy, a

genetic disorder in which mutations occur in TIMP-3. In this disease, inappropriate neovascularization of the choroidal layer is a prominent phenotypic feature (73).

To date, several studies have demonstrated that there is a close association between expression of various members of the MMP family by tumors and their proliferative and invasive behavior. Furthermore, inhibitors of metalloproteinases (TIMP 1, 2, and 3) have been shown to reduce tumor-induced angiogenesis in experimental systems. Although TIMPS may not be suitable for pharmacological applications due to their short half-life *in vivo*, two synthetic, low molecular weight MMP inhibitors (Batimastat and Marimastat) have been found to have antitumor activity (74).

II. Applications and Mechanisms: Anti-Angiogenic Proteins in Gene Therapy

The previous section discussed many angiogenic and antiangiogenic factors that may be useful in developing effective antiangiogenic strategies toward cancer. In this section, we will discuss the various antiangiogenic genes that investigators have thus far used in pre-clinical and clinical trials (Table II).

A. The tumor suppressor, p53

Although p53 has long been considered to inhibit tumor cell growth by altering the regulation of the cell cycle or apoptosis pathways, it is likely that there may be other mechanisms to explain tumor inhibition with p53 gene therapy. A bystander effect has been suggested for the observed difference between the relatively low transfection efficiency of the tumors with p53 and the reduction of tumor growth. Tumor inhibition mediated by gene therapy carriers expressing p53 appears to be due in part to angiogenesis inhibition. We determined that liposomes in complex with a plasmid encoding p53 inhibited tumor growth in a mouse model (75). We subsequently discovered that blood vessel density was reduced with this therapy (76). Other studies with liposomes carrying p53 plasmids have also demonstrated reduction in tumor growth in mouse models (77, 78). Besides liposomes, adenoviral and retroviral carriers expressing the p53 gene significantly reduced tumor growth and blood vessel density (79, 80). Moreover, adenoviral vectors expressing p53 significantly reduced VEGF levels within tumors. p53 may also decrease angiogenesis by inhibiting the cell cycle and/or enhancing apoptosis of the endothelial cells (76), inhibiting differentiation of endothelial cells (79), or by inducing the antiangiogenic protein, thrombospondin 1 (81).

B. Inhibitory Polypeptides with an Extracellular Site of Action

Several antiangiogenic peptides have potential antitumor efficacy, including laminin peptide, thrombospondin 1 and its peptides, a polypeptide fragment of prolactin, PF-4, IL-12, angiostatin, endostatin, and antithrombin III. Of these, gene therapy with PF-4, IL-12, thrombospondin 1, angiostatin, and endostatin have shown antitumor activity in a mouse model (10–12, 39, 48, 64, 82–85). Although these proteins have different mechanisms of action as discussed above, we consider them in one group because they probably all act extracellularly.

i. Thrombospondin—*In vitro* experiments in which TSP1 was used to reduce endothelial cell growth show conflicting results. Nonetheless, peptides isolated from thrombospondin 1 clearly have antiangiogenic effects *in vitro* (38), but surprisingly, there have been no published reports demonstrating that these natural peptides have antitumor activity *in vivo*. It is unclear if the reduced functional activity of these peptides *in vivo* is due to a short half life. However, synthetic antiangiogenic thrombospondin peptides in the D-conformation do exhibit *in vivo* antitumor activity (18). In contrast to peptide therapy, three studies have suggested that antiangiogenic gene therapy with TSP1 or peptides derived from TSP1 exhibit antitumor activity (46, 83, 84). Initially, an *ex vivo* study demonstrated that a tumor clone expressing high levels of TSP1 peptide reduced tumor growth (84). Recently, an *ex vivo* study showed that tumor clones expressing TSP2 had significantly more antitumor efficacy than clones expressing TSP1. More impressively, tumor clones expressing TSP1 and 2 were synergistic in their antitumor efficacy as demonstrated by the complete lack of tumor growth (46). In addition, investigators have demonstrated that p53 and an antiangiogenic peptide of TSP1 in complex with liposomes were synergistic in their inhibition of tumors (83). These liposome:DNA complexes were delivered intravenously at a site distant from the tumor.

ii. Angiostatin and Endostatin—It has been suggested that the half-life of antiangiogenic proteins may be the main determinant of their antitumor efficacy. Gene therapy offers the advantage of producing these proteins over an extended period of time and prolonging the effective ‘half-life’ of these proteins. Thus, gene therapy by delivery of short-lived proteins such as TSP1 would be expected to be advantageous compared to peptide therapy alone. Gene therapy delivery of some polypeptides, however, has not been as effective as the direct administration of these polypeptides to the tumor-bearing mice. For example, both angiostatin and endostatin polypeptides have shown marked inhibition of a variety of tumors in animal models. In one particularly dramatic experiment, tumors with a mean size of 500 mm³ were completely eliminated after therapy with the endostatin polypeptide. There was also no evidence of resistance developing to endostatin in these tumor-bearing mice.

In contrast, several gene therapy studies with angiostatin and endostatin have demonstrated only modest antitumor efficacy in animal models (11, 12, 85). Therefore, the antitumor efficacy of the gene therapy approach has been far less effective than the polypeptide therapy. The most obvious reason for this is that systemic levels of endostatin and angiostatin after gene therapy are lower than are those after direct administration of these polypeptides. High extracellular levels of endostatin and angiostatin polypeptides are required to reduce tumor growth. With *in vitro* angiogenic assays, endostatin levels of 125ng/ml are required to inhibit endothelial cell growth. Although these *in vitro* and *in vivo* results may not be easily translated to the gene therapy studies, they do suggest that high levels of these proteins are necessary for significant inhibition of tumor growth. Thus, although the approach of gene therapy with antiangiogenic polypeptides is relatively straightforward (at least in animal models), achieving adequate levels of the gene product remains a significant obstacle.

iii. **PF-4**—An adenovirus expressing the antiangiogenic peptide PF-4 (82) was found to inhibit endothelial cell proliferation specifically *in vitro*. More importantly, the vector-mediated PF4 transduction inhibited tumor growth of established gliomas and prolonged animal survival. This was the first report showing the efficacy of antiangiogenic gene therapy on established tumors.

iv. **TIMPS**—Although TIMPS may have half-lives that are too short for them to be administered as proteins, gene therapy may prolong delivery of these proteins. Nevertheless, the genes encoding these proteins should probably be delivered in close proximity to the tumor to enhance their efficacies. Investigators have discovered that adenovirus-mediated overexpression of TIMP-1, 2, and 3 inhibited invasion of several different tumor cells through artificial basement membrane (86). Nevertheless, only TIMP-3 promoted apoptosis, suggesting that this factor may be more effective in reducing tumor growth than either TIMP-1 or 2. To our knowledge, *in vivo* gene therapy with Timps has not reported (86, 87).

C. Truncated receptors- VEGFR2, VEGFR1, sVEGFR1, Tie2

An early watershed study demonstrating the utility of antiangiogenic gene therapy targeted the VEGF receptor, VEGFR2. Co-administration of a truncated VEGF receptor (VEGFR2) with tumor cells was shown to inhibit the growth of the tumor (6). It is likely that this retrovirus-transduced receptor acts through a dominant-negative mechanism to inhibit the wild-type VEGF receptor. The study not only showed the potential of this approach but also demonstrated that VEGF has an essential role in tumor development. More recently, investigators have demonstrated that antiangiogenic therapy with the truncated VEGF receptor suppressed angiogenesis and intracerebral glioma tumor growth (88). Survival time of rats with intracerebral tumors increased with treatment. In these two studies, the truncated VEGFR2 targeted the wild-type receptor of the transfected cell. However, the clinical utility of this approach is somewhat limited owing to the low transfection efficiencies of the vector delivery systems. One method to circumvent the low transfection efficiency of vector delivery systems is to engineer a secreted therapeutic protein. This was demonstrated by Goldman and colleagues (9) where tumor cells transduced with a secreted soluble VEGF receptor (sVEGFR1) inhibited implantation of a primary tumor. Furthermore, when tumor cells expressing high levels of sVEGFR1 were injected intravenously, mice displayed fewer lung metastases.

In addition to the VEGF and VEGF receptors, other receptors have been targeted for antiangiogenic gene therapy. An adenovirus expressing a soluble Tie 2 receptor was recently shown to inhibit tumor growth when injected intravenously, by binding to angiopoietin 2 (8). With plasma concentrations of the soluble recombinant Tie2 receptor exceeding 1 mg/ml for an 8-day period, established tumors treated by the intravenous injectable route in this study were significantly reduced. In addition, co-administration of the adenovirus-Tie2 vector with tumor cells greatly reduced the number of lung metastases. A potential disadvantage of this therapy is that the adenovirus-Tie2 construct may also inactivate angiopoietin 1, a protein known primarily for its ability to stabilize blood vessels.

III. Antisense approaches

An alternative approach to reduce tumor growth is to target growth factors with antisense strategies. Antisense gene therapy has been used to reduce FGF2 levels within a tumor and to decrease the tumor size (89). In addition, stable transfection of tumor cells with plasmids expressing antisense VEGF did not affect the growth of tumor cells *in vitro* but reduced tumor growth *in vivo* (7). Targeting VEGF with a retrovirus producing cell line has also prolonged survival of a glioma-bearing rat model (90). VEGF levels may also be down-regulated with antisense approaches directed toward the adenosine 2B receptor. As discussed previously, adenosine induces VEGF mRNA and protein after binding to the adenosine 2B receptor. Although this approach has not shown antitumor efficacy *in vivo*, investigators have found that mitogenesis of endothelial cells is reduced with antisense oligonucleotides. The major difficulty of targeting VEGF *in vivo* with antisense approaches is that if most of the VEGF is expressed in tumor cells and not the tumor endothelial cells, it may be difficult to achieve adequate expression of the antisense construct.

IV. Routes of administration for antiangiogenic genes

As with other gene therapy approaches, the major limitation of antiangiogenic gene therapy lies in the choice of vector delivery systems. Although there has been an incremental improvement in these systems, precisely when an adequate vector system will be developed for antiangiogenic genes is difficult to forecast. Since proteins of antiangiogenic gene therapy may require secretion, several tissues other than tumor endothelial cells can express these proteins. Consequently, secretion of antiangiogenic proteins allows the route of gene delivery to be varied. In this section, the advantages (and disadvantages) and recent advances of major delivery systems will be examined in terms of the route of delivery (for reviews on non-viral and viral vectors, see (91, 92)). The routes of delivery for antiangiogenic genes include intravenous, intratumoral and intramuscular injection. In animal models, all three modes of delivery have demonstrated antitumor efficacy with antiangiogenic genes.

A. Intravenous

Intravenous injections of both viral and non-viral vectors expressing antiangiogenic genes have demonstrated antitumor efficacy in animal models. Three studies have used adenovirus to express antiangiogenic genes and their gene products (8, 9, 80). Besides being the most common viral vector in current use, adenovirus vectors were probably selected because intravenous injection of adenovirus results in impressively high transfection rates in the liver, compared to other viral or non-viral vectors. It is not known from these studies how much antiangiogenic protein is expressed in the tumor endothelial cells. In one report, an antiangiogenic protein, the truncated Tie2 receptor, was expressed at levels above 1mg/ml that were sustained for nearly a week, but there was only moderate reduction in the tumor size. Similarly, two other studies with adenoviral vectors expressing either angiostatin or sVEGFR1 also showed only moderate effects on tumor growth. The most significant problem with adenoviral vectors is that they provoke a strong immune response in humans. In pre-clinical studies, tumor-bearing animals were injected only once with the adenoviral carrier to minimize an immunological response.

Considerable effort has been expended to avoid immunological and other toxic responses. Second, third, and now fourth generation adenoviral systems have been developed and the expression of genes has been progressively prolonged. Since these newer generation viral vectors contain minimal amounts of viral DNA within them, the immunological reactions toward these viral vectors appear to be reduced; pegylation of adenoviruses has resulted in a further reduction in immunological reactions toward these vectors. Although initial results with these pegylated viral vectors appear promising, further studies are needed before determining the effect of these viruses on transfection (93). Despite these pre-clinical advances in the adenoviral delivery vector, concerns about the safety of adenovirus have only been heightened with the death of a patient due to the therapy. It seems likely that lessons learned from the use of adenoviral vectors can be applied to other viral vectors such as AAV and lentivirus.

Non-viral vectors have also demonstrated antitumor efficacy when injected intravenously with a plasmid encoding endostatin. In one pre-clinical study with cationic liposomes as a carrier, moderate reduction in the tumor size of mice bearing MDA-MB-435 cells was observed after the third injection (11). In addition to liposomes, which are the most commonly used non-viral delivery system, several cationic polymers such as polyethylenimine or poly-L-lysine have shown effective DNA transfer *in vivo* (94, 95). The most promising aspect of intravenous administration of non-viral vectors is their low toxicity. In addition, non-viral vectors such as cationic liposomes, when administered intravenously, can selectively target the mitogenic tumor endothelial cells rather than quiescent endothelial cells (96). Adding ligands that specifically target the tumor endothelial cells may further increase the specificity and efficacy of cationic liposomes (97, 98). Three significant limitations hamper the *in vivo* use of these non-viral vectors, however. One is the low *in vivo* transfection efficiency of organs or vessels within organs. Efforts have been made to increase the *in vivo* transfection of these vectors by modifying the cationic lipids or adding cationic polymers to these liposomes. Both of these modifications appear to increase the transfection of liposomes significantly (99–101). A second significant limitation of these non-viral vectors is that they have half-lives of only a few minutes in the blood stream. The potential long-term expression of the antiangiogenic protein mitigates to some extent against this limitation. Nevertheless, an increase in the half-life of these vectors may enhance antitumor efficacy when the primary targets of transfection are the mitogenic endothelial cells of the tumor. Since angiogenesis is a continuing process within the tumor, prolonging the half-life of the vectors might increase the peritumoral level of the antiangiogenic protein. The half-lives of cationic non-viral vectors have been increased at least in part by pegylating the surface of these vectors (101, 102). Although enhancement of *in vivo* transfection and the increase in the liposome half-life appear promising, it remains to be determined whether these modifications will increase the antitumor efficacy. The third problem with non-viral vectors is a non-specific antitumor effect that has been seen with liposome:DNA complexes (76). This antitumor effect, which occurs in the absence of a therapeutic gene product being expressed, may be mediated by an immunostimulatory response to the liposome:DNA complexes (103). Unfortunately, we have found that the antitumor immunostimulatory effect is inversely correlated to the antitumor effect of the therapeutic product (76). It is not known whether this non-specific effect occurs with other non-viral delivery systems such as

polymers (e.g. polyethyleneimine, Superfect) (104–107). Despite these problems, we expect that non-viral vectors will have a major role in targeting vessels of tumor.

B. Intramuscular Somatic

Blezinger and colleagues have recently shown the utility of somatic antiangiogenic gene therapy by injecting a cationic polymer (PVP)-endostatin plasmid complex into mouse muscle (12). The tumors in these mice were significantly reduced and measurable levels of endostatin were detected in the blood stream. This is the only carrier thus far that has demonstrated the effectiveness of antiangiogenic therapy with intramuscular injections. Because of the prolonged expression of plasmids within muscle, we anticipate that this will become a common approach for administering antiangiogenic genes.

Several viral and non-viral delivery systems when injected intramuscularly may result in high levels of expression of antiangiogenic genes. Although it has not been determined whether systemic levels of an antiangiogenic protein with adeno-associated virus as a carrier can reduce tumor growth on a short- or long-term basis, it has been established that long-term expression of erythropoietin can be achieved by multiple injections into mouse muscles (108). The erythropoietin produced by gene therapy stimulated the hematocrit in these mice for nearly 6 months. Lentiviruses are another potential viral vector that may decrease tumor growth with intramuscular injections. The heralded advantage of lentivirus is that it can transfect both mitogenic and quiescent cells (109–111), although one report has cast doubt on this potential advantage (112). Both AAV and lentiviral delivery systems are significantly less immunogenic than are the adenoviruses. Nevertheless, it is not clear whether the immunogenicity of AAV or lentiviruses will be low enough to achieve long term elevated levels of antiangiogenic proteins in humans.

Two additional delivery systems to the muscle have the advantage of high expression without the toxicity of lipids, polymer, or viral carriers. Injection of naked DNA into muscle has been shown to express the gene product for prolonged periods of time (113). In addition, electroporation can significantly enhance the expression of a gene product compared to naked DNA injections alone, without subjecting the animal to the toxic effects of the vehicle (114). Nevertheless, particular care must be taken to prevent or minimize immunogenic or immunostimulatory responses in delivery systems with long term expression of antiangiogenic genes.

C. Intratumoral

Most of the carriers (liposomes, polymers, adenovirus, lentivirus, electroporation) discussed in the intramuscular injection section will be applicable to intratumoral injections of antiangiogenic genes. Except for lentiviruses, most retroviruses can only transduce mitogenic cells effectively. On certain occasions this property is desirable because the vector carrying the antiangiogenic gene will transduce the mitogenic cells of the tumor without transducing the normally quiescent surrounding tissue. Local injections of retroviruses expressing VEGFR2 and PF4 proteins have resulted in tumor reduction (6, 82, 88). In addition, locally injected liposomes in complex with angiostatin- or endostatin-containing plasmids also reduced the growth of a human breast cancer line in a mouse model (11).

V. Future Directions.

The utility of antiangiogenic gene therapy in a clinical setting will depend in large part on developing vectors with minimal toxicity and with increased *in vivo* transfection efficiency. Until these vectors are developed in pre-clinical models, clinical studies directed toward tumor inhibition will continue to yield marginal results. For a decade, there have been tremendous, and perhaps unrealistic expectations for gene therapy approaches. With the lack of success in clinical trials followed by the recognition that gene therapy may have significant side effects, there is now the risk of undue cynicism toward the field. We hope that more realistic expectations will prevail and engender the development of new gene therapy vectors and approaches toward neoplastic and non-neoplastic diseases.

One of the new gene therapy approaches that has attracted interest in the last few years is targeting of the blood vessels of the tumor. This approach is appealing since tumor vessels are readily accessible to therapy. Several studies have now demonstrated the “proof of principle” of antiangiogenic gene therapy with local and/or systemic administration. With the discovery of new antiangiogenic genes, more potent therapies are anticipated. Furthermore, combinations of antiangiogenic genes are currently being explored and will undoubtedly yield more potent antitumor responses. We believe that progressive improvement of antiangiogenic therapy against cancer and other angiogenesis-based diseases will continue as more potent antiangiogenic genes and vector delivery systems are defined. It must be remembered, however, that even though pre-clinical and clinical trials may demonstrate significant efficacy, this does not ensure that antiangiogenic gene therapy will usurp its counterpart, antiangiogenic peptide therapy. The success of antiangiogenic gene therapy compared to peptide/protein therapy will be contingent on several factors including, for example, antitumor efficacy, toxicity, half-life of protein, and cost-effectiveness. Nevertheless, antiangiogenic gene therapy has a number of potential advantages compared to peptide therapy. These advantages, which are dependent on the delivery systems and the route of delivery, include prolonged expression of the gene product, the ability to target the tumor or its vessels specifically, and the ability to control localization of the product. We believe that at least some of the potential advantages that antiangiogenic gene therapy has over its peptide/protein counterpart will be realized.

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Figure 1. Promotion of tumor growth by angiogenesis.

In order for tumors to progress from *in situ* to large tumors, increases in the proangiogenic factors coupled with decreases in the antiangiogenic factors are required (see Table I for list of activators or inhibitors). The thickness of the arrow represents the respective gradients of activators/inhibitor molecules.

Figure 2. Models of VEGF Expression in Tumor Growth.

A) In tissues with low vascularity, marked increases in VEGF and angiogenesis occur when the tumor size reaches 2 mm in diameter. B) and C) demonstrate a second model of VEGF expression in tumors that have metastasized to vascular tissues. In early stages of tumor growth (B), increases in VEGF and angiogenesis do not initially occur when the tumor is 2 mm in size. Instead, the tumor co-opts the vessels of the organ. At a later stage (C) when the tumor reaches several centimeters in size in the vascular tissue, VEGF, angiopoietin 2, and angiogenesis at the periphery increase, accompanied by central necrosis of the tumor.

Figure 3. Functions of various angiogenic regulators:

Two VEGF receptors, VEGFR1 and VEGFR2, are localized to mitogenic endothelial cells. VEGF, by binding to VEGFR1, induces migration of endothelial cells, while activation of VEGFR2 is essential for mitogenesis. In addition to VEGF/VEGFR, angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) appear to have important physiologic roles in vessel formation and stabilization. Although angiopoietins 1 and 2 have similar binding affinity to the endothelium-specific receptor, Tie2, the consequences of their binding are different. By activating Tie 2, Ang1 stabilizes vessels. In contrast, Ang2 does not activate Tie2. Rather, up-regulation of Ang2 stimulates angiogenesis in the presence of VEGF, but induces apoptosis in the absence of VEGF.

Figure 4. Proposed mechanisms for inhibition of angiogenesis by p53.

Two important mechanisms by which P53 may inhibit angiogenesis in neoplasms are induction of the antiangiogenic protein, TSP1, and decreased expression of the pro-angiogenic protein, VEGF. After wild-type p53 transcriptionally up-regulates TSP1 in the tumor cell, the secreted TSP1 binds to and activates the endothelial surface receptor, CD36. Several proteins, including p59fyn, caspase-3 like proteases, and p38 mitogen-activated protein kinase (p38MAPK), are then sequentially activated leading to apoptosis of the endothelial cell. Many of the mediators of the apoptotic signal transduction pathway downstream of p38MAPK have not yet been identified. A second mechanism by which p53 may inhibit angiogenesis is by decreasing the pro-angiogenic factor, VEGF. It is not clear whether down-regulation of the VEGF by p53 occurs at the transcriptional or at the post-translational stage. Pharmacological delivery of the p53 gene may result in the inhibition of angiogenesis by additional mechanisms (see section IIA).

Table I**Endogenous Regulators of Tumor Angiogenesis**

<u>Activators</u>	<u>Inhibitors</u>
Vascular Endothelial Growth Factor (VEGF)	p53
Fibroblast Growth Factor 1 (FGF1)	Thrombospondin-1 ¹
Fibroblast Growth Factor 2 (FGF2)	Thrombospondin-2
Platelet Derived Growth Factor (PDGF)	Tissue Inhibitors of Metalloproteinases (TIMPS)
Angiopoietin 2 + VEGF	Angiostatin
Angiopoietin 1 ²	Endostatin
Transforming Growth Factor- β ¹ (TGF- β)	Antiangiogenic Antithrombin III
Tumor Necrosis Factor- α ¹ (TNF- α)	Angiostatic C-x-C chemokines (PF4, IP10, MIG)
Interleukin-8 (IL-8)	Pigment Endothelial Derived Factor (PEDF)
Platelet Derived Endothelial Cell Growth Factor (PD-ECGF)	Interleukin-12 (IL-12)
Hepatocyte Growth Factor (HGF)	Interferon (INF)- α , β , γ

¹-Dose-dependent²-Weak Angiogenic Activator

Table II**Gene Therapy of Antiangiogenic Inhibitors**

Angiogenic Inhibitor	Tested <i>in vivo</i>	Mechanism	Method of Delivery
VEGF antisense	Yes	↓VEGF	<i>ex vivo</i> ; Co-injection
Adenosine Type II receptor antisense	No	↓Type II Receptor and VEGF	NA
VEGFR2-DN	Yes	Inhibits VEGFR	Co-injection
sVEGFR1	Yes	↓VEGF and inhibits VEGFR	<i>ex vivo</i> ; IV
Tie2	Yes	↓Angiopoietin 2	IV
FGF2 Antisense	Yes	↓FGF2	<i>ex vivo</i>
p53 ¹	Yes	Inhibits cell cycle ² , ↑ thrombospondin 1; ↓VEGF	<i>ex vivo</i> ; IT; IV
Thrombospondin 1	Yes ³	Activates caspases	<i>ex vivo</i> ; IV
Thrombospondin 2	Yes	?	<i>ex vivo</i>
Angiostatin	Yes	? Inhibits ATP Synthase	<i>ex vivo</i> ; IT; IV
Endostatin	Yes	?	IT; IV; IM
Antithrombin III	No	?	NA
IL-12 ¹	Yes	INF- γ	IT; IV; IM.
PEDF	No	?	NA

Symbols: VEGFR-DN, truncated VEGF receptor that dimerizes and inhibits the wild-type receptor; IT, intratumoral injection; IM, intramuscular injection; IV, intravenous injection; NA, not applicable

¹Ongoing Clinical Trials

²Inhibits cell cycle of endothelial cells

³Genes encoding antiangiogenic peptides of thrombospondin 1 have been delivered IV.