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SYSTEMIC GENE THERAPY WITH P53 INHIBITS BREAST CANCER: RECENT ADVANCES AND THERAPEUTIC IMPLICATIONS

Qing-Rong Chen and A. James Mixson

University of Maryland Baltimore, Room 759, Bldg MSTF, 10 South Pine Street, Baltimore MD 21201

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TABLE OF CONTENTS

1. ABSTRACT

Development of gene therapy technologies is approaching clinical realization for the treatment of neoplastic diseases. The use of tumor suppressor genes has been one useful strategy in gene therapy. Modifications and development of vectors as well as increased knowledge of the anti-tumor mechanisms of the p53 will play a significant role in the further advancement of this therapy. Currently, several laboratories have demonstrated that intratumoral injection of a virus carrying the p53 gene decreases tumor size in pre-clinical and clinical studies. Our lab has focused on a tumor-bearing mouse model in which intravenous delivery of liposome: p53 complexes decreases tumor growth. Although a high transfection efficiency of the tumor was thought to be necessary for gene therapy to exhibit anti-tumor activity with tumor suppressor genes, marked inhibition of the tumor occurs even with a low transfection efficiency. p53 may exhibit its bystander anti-tumor effect, at least in part, through an antiangiogenic effect. We believe that understanding the mechanism by which the p53 tumor suppressor gene inhibits tumor growth will lead to improvement in cancer therapy.

2. INTRODUCTION

2.1. Vectors: limitations and advantages

Sufficient transfection of the target cell by vectors carrying their therapeutic genes has thus far been the rate-limiting step in gene therapy. Vectors carrying genes commonly are divided into viral and non-viral vector categories. Unfortunately, all vectors have significant limitations. Replication-deficient retroviral vectors can efficiently transfect dividing cells. However, retroviral vectors have the potential to cause insertional mutagenesis. As a result, their use has been limited to either direct injection of tumors or to *ex vivo* gene transfer trials. Unlike retroviral vectors, adenoviral carriers can transfect non-dividing cells and their ability to cause insertional mutagenesis is greatly reduced. However, their potential to activate the immune system on re-injection in humans is their major limitation (1). Attempts are underway to minimize the immunogenicity of the adenoviral vectors or to discover new viral vectors that circumvent these potential problems. In addition to adenoviruses and retroviruses, several other viral vectors including the lentivirus vector are now being tried (2). However, although viral vectors have a great deal of promise, safety issues and toxicity will likely limit their use for systemic delivery of genes in the near future.

Liposomes are the most commonly used non-viral carriers of DNA. The major advantage of liposomes over retroviruses is that DNA is not incorporated into the genome, and unlike adenoviral vectors, they are not immunogenic. The major limitation of liposomes is that they are not as efficient as viral vectors in transfecting many cell lines. Until recently, their medical utility was limited by their rapid uptake by phagocytic cells. Interest in liposomes as a drug carrier was rejuvenated by two

recent technological advances that in essence produced a renaissance in the field. Stealth liposomes represented a significant breakthrough in liposomes in that they are non reactive and are not readily taken up by the reticuloendothelial system (RES). These stealth liposomes are usually composed of lipids containing poly-ethylene glycol. These PEG containing lipids provide a stearic barrier outside the membrane. As a result, stealth liposomes remain in the blood for up to 100 times longer than conventional liposomes and can thus increase the pharmacological efficacy (3, 4). However, these liposomes are not particularly efficient in transfection of cells or as carriers of DNA.

The second significant advance was the use of cationic liposomes complexed to negatively charged DNA (5). Cationic liposomes can condense DNA and increase transfection yields several orders of magnitude. In the DNA-cationic liposome complex, the nucleic acids or oligonucleotides are not encapsulated but are simply complexed with small unilamellar vesicles by electrostatic interactions. The exact nature of the liposome: DNA complex is not clear but there appears to be a sandwich arrangement of the liposome and DNA complexes. With the liposome complexing with DNA complexes, liposome aggregation and fusion and DNA condensation also occur. This supramolecular complex is then added to cells *in vitro*, injected parenterally, or aerosolized for pulmonary therapies (6). Almost all gene therapy experiments with liposomes have been done with these cationic liposomes. The transfection efficiency of these cationic liposomes is dependent on the size, charge, and type of neutral and cationic lipids (7, 8). One potential disadvantage is that these liposomes: DNA complexes are not particularly effective in escaping the endothelial cells and the RES system when they are injected intravenously. The transfection efficiency of genes complexed with liposomes into a variety of tumors and tissues is less than 5 % (9, 10). A major challenge of gene therapy remains the systemic delivery of transgenes to the tumor or peritumoral area that will effectively decrease the size of the primary tumors and its metastases. Nevertheless, we have determined that a liposome: p53 gene complexes when given intravenously in the tail veins inhibit tumors that have been implanted into the mammary fat pad of mice. There are other non -viral carriers which hold a great deal of promise. For example, cationic polymers with buffering capacity complexed to therapeutic DNA have demonstrated promising transfection efficiency against several cell lines resistant to transfection with cationic liposomes (11-13).

2.2. Breast cancer and p53: frequently mutated in tumors including breast cancer

Mutations in the tumor suppressor gene p53 are known to occur in over 50% of human tumors. The lung, breast, colon, and breast cancer are among solid tumors which frequently have p53 mutations (14). Women with mutations in the p53 gene have a worse prognosis, a decreased disease free interval, and an increased resistance to chemotherapy. Breast cancer is second leading cause of death in women due to cancer in North America and Western Europe (lung cancer is the leading cause). Breast cancer affects nearly 10% of this population living to 80 years of age, and one million new cases are predicted by the end of this decade(15). Although the molecular basis of multistage carcinogenesis in breast cancer is not well understood, the metastatic potential of breast cancers has been correlated with the presence of point mutations in the p53 gene (16). Because of the number of women that die from breast cancer dying each year, novel therapies need to be developed.

2.3. P53 regulates a number of cellular functions

P53 mobilizes multiple responses to DNA damage. DNA damage results in an increase in the level of the p53 protein. Following DNA damage, an important function of wild-type p53 function is to control the progression of cells from G1 to S phase. Recently, several groups have found that p53 transcriptionally activated a p21 kD protein (also known as WAF1, Cip1, Sdi1, p20CAP, or Pic1), an inhibitor of cyclin-dependent kinases (CDKs) (17, 18). Inhibition of CDK activity is thought to block the release of the transcription factor E2F and related transcription factors from the retinoblastoma protein RB, with consequent failure to activate transcription of genes required for S phase entry (18, 19). Evidence consistent with the model that pRb is a downstream effector of p53-induced G1 arrest has recently been reported (20).

Since p53 is a central regulator in controlling the cell cycle, one might expect that restoration of wild-type p53 function could inactivate the proliferative effects of the mutated product. In fact, various groups have found that *in vitro* transfection of a tumor suppressor transgene into a variety of tumor cells decreased the tumor's growth rate *in vivo* or *ex vivo*. (21-25). From these experiments, one would anticipate that p53 would need to be transfected efficiently into a high percentage of the cells in order for it to be efficient. Nevertheless, various groups have now discovered that efficient transfection of the tumor with p53 is not necessary.

In addition to cell cycle arrest, p53 also induces apoptosis. The molecular details of this form of cell death are not fully understood, but conserved features include activation of proteases of the ICE/Ced-3 class, and regulation by members of the Bcl-2/Ced-9 family (26, 27). p53 has been shown to induce expression of BAX, a gene encoding a positive regulator of this pathway (28). A second way in which p53 may induce apoptosis is through the repression of transcription (29, 30). This function is mediated via TATA-box elements when sites for p53 binding are absent in the promoter of the target gene (31).

Besides BAX and p21, other genes that are transcriptionally activated by p53 include: MDM2, whose product is a negative regulator of p53 (32); thrombospondin I, which inhibits angiogenesis (33); IGF-BP3, which may be a autocrine/growth regulator through its interaction with IGF1 (34); cyclin G, the function of which has yet to be determined (35); and GADD45, which may play a role in DNA repair (35, 36). Besides inducing GADD45, there is also some evidence that p53 may play a role in DNA mismatch repair by directly binding to the DNA (36, 37).

Of the proteins that p53 is known to induce, only thrombospondin I and IGF-BP3 are secreted from the cell.

3. P53 INHIBITS BREAST CANCER

3.1. Intratumoral injections

Several groups have now determined that intratumoral injections of p53 reduce the growth of a variety of tumors expressing the mutant p53 (38-41). In 1994, Roth and co-workers made a seminal discovery in determining that at least part of p53's antitumor effect was due to a bystander effect (38). Even though there was a 90% reduction in tumor size, there was only a 30% transfection efficiency. The mechanism by which p53 exerted its bystander effect was unclear at that time. More recently, investigators have determined an increase antitumor efficacy with intratumoral injections of transferrin labeled liposomes complexed with the p53 gene (42). These pre-clinical models are being translated into human trials in which inhibition of lung tumors has been reported (43).

3.2. Regional Intravenous Administration

In addition to intratumoral injections, regional administration in the portal vein of an adenovirus expressing wild-type p53 has been found to decrease tumor size in the liver (44). The adenovirus is known to transfect the hepatocytes efficiently, even when given systemically. The immunological neutralization on re-administration of the adenovirus appears to be a significant hurdle if this therapy were to be administered to humans. Nevertheless, this finding demonstrates the efficacy of p53 when given by regional administration.

3.3. Systemic Intravenous Administration of liposome: p53 complexes

3.3.1. Inhibition but also regression with p53

We first demonstrated that systemic intravenous administration of the p53 gene significantly affects tumor growth and metastases of breast cancer cells injected into nude mice (45). In this study, we complexed a negatively charged plasmid encoding the p53 protein (BAP-p53) with a cationic liposome. The mechanism by which the intravenously delivered liposome: BAP-p53 complex inhibits breast cancer was initially unclear. However, nine of the 14 tumors in the p53 treated group showed not only inhibition, but these tumors also regressed in size in response to treatment. In contrast, only 1 of the 22 in the control group showed any evidence of regression. Many tumors which underwent regression had volumes greater than $375mm^3$. Although one might expect that transfection was extremely efficient based on these results and the adenoviral experiments discussed in 3.2., there was evidence that transfection efficiency was low (9). We found that less than 5% of the solid tumor was transfected by a systemically injected liposome: BAP-CAT complex (9). Thus, unlike *in vitro* and *ex vivo* experiments in which p53's transfection into each cell is efficient, systemic transfection of the tumor by the liposome: p53 complex is not efficient.

The growth patterns of individual tumors were examined to determine if these tumors not only were inhibited but regressed with therapy (45). In the p53-treated group, 8 of the 15 of the primary tumors showed a significant reduction in the size of the tumors ($p<0.001$) whereas only 1 of 22 of the control and empty vector groups had a reduction in size. One month after the discontinuation of therapy, all eight of the tumors that regressed in the p53-treated group showed no evidence of regrowth of their primary tumors.

In addition, tumors from the p53-treated mice revealed marked differences in histology as compared to tumors of the vector-treated and control mice (45). Histological examination of the primary tumors distinguish all 7 of the p53-treated animals from the vector-treated and control mice. Although apoptotic cells were scarce in the viable portion of tumors from control mice, apoptotic cells were widely scattered throughout the viable portion of tumors from p53-treated mice.

3.3.2. Effects of gene therapy with p53 are independent of p53 status of tumor

If mechanism was due to replacement of p53 in p53 mutated tumors, then one would expect that there would be little to no effect on tumors expressing wild-type p53. Nevertheless, we determined that the liposome: BAP-p53 complex inhibited both MDA-MB-435 tumors and MCF-7 tumors (9). The MCF7 tumors not only expressed high levels of thrombospondin I, but these tumors contained wild-type p53 as well. Thus, the antitumor efficacy of the liposome: BAP-p53 gene therapy was independent of the p53 status of the tumor.

3.3.3. Toxicity of the liposome: p53 complex

While some investigators have reported toxicity of liposome: DNA complexes at elevated concentrations, others found that systemically delivered liposome: DNA complexes are non-toxic (46, 47). Perhaps, part of these discrepancies about toxicity may be due to lipids used as carriers. As a result, we felt that it was important to determine whether the liposome: p53 complexes were toxic at dosages sufficient to inhibit tumor growth. Although the liposome: p53 complex appeared to be effective in reducing the tumor volume, we evaluated this therapy's toxicity on the organs of the mice. After gene therapy with p53, mice organs (heart, lungs, liver, pancreas, spleen, kidney, intestine, and skin) were processed for histopathological evaluation. No evidence of toxicity from the liposome: p53 complex was present when these organs were examined (9). Although cationic liposomes and DNA may form large complexes, embolic events were not evident in the lungs or other organs. Despite the fact that the spleen, liver, reticuloendothelial system, and bone marrow are known to efficiently take up liposomes (48), these organs were not adversely affected by this treatment. Since p53 has a significant effect on dividing cells, we also examined the skin and the small intestines for toxicity. Sections stained with hematoxylin and eosin revealed normal histological morphology without any evident apoptosis. Furthermore, the electrolytes and the blood counts between the various

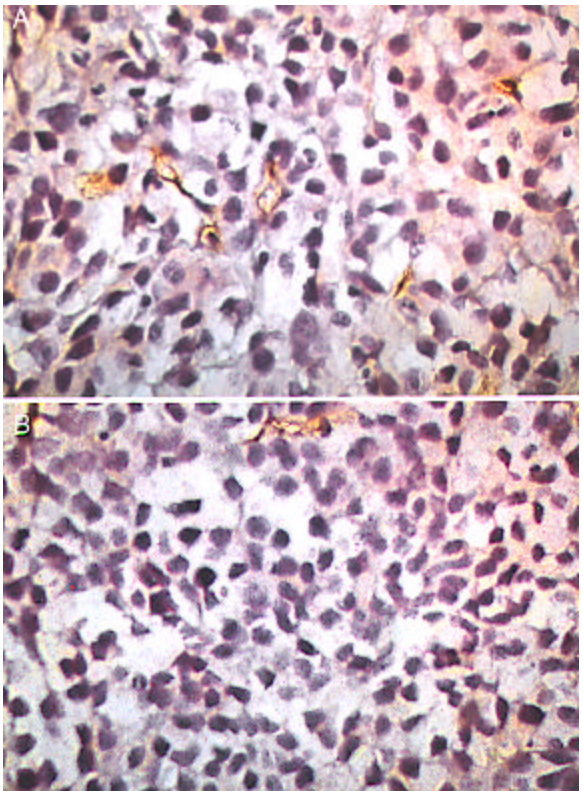

Figure 1. Photomicrographs of tumor sections stained with an antimouse CD31 monoclonal antibody by indirect immunoperoxidase. There was a significant reduction in the number and size of the blood vessels in the MDA-MB-435 tumors treated with BAP-p53/TSPf (X600) (B) compared to BAP (A).

treatment groups were similar with no significant differences among the treatment groups.

4. MECHANISM BY WHICH THE P53 GENE INHIBITS TUMOR GROWTH

Any mechanism that explains the antitumor effect of systemically delivered p53 must account for its transfer into a low percentage of tumor/peritumoral cells (9). The mechanism of p53 gene therapy must also account for the finding that the liposome:p53 complex is effective in reducing tumors with mutated p53 as well as tumors expressing wild-type p53 (9). We initially discovered that the liposome: p53 complex inhibited the blood vessel density of tumors suggesting that the p53 gene was acting through an anti-angiogenic effect. One potential mechanism explaining this antiangiogenic effect is that the p53-transfected portion of the tumor induces an inhibitor which acts on the nontransfected areas of the tumor. It is probably necessary for this inhibitor to be secreted in order to repress (directly or indirectly) the majority of untransfected tumor cells. Recently, evidence has been obtained from other laboratories to suggest that p53 affects the growth of the tumor by inducing an antiangiogenic protein (49). In p53 deficient fibroblasts, restoration of p53 induces thrombospondin I, a secreted protein that inhibits angiogenesis (33). Interestingly, decreased secreted thrombospondin I levels by a variety of cell lines correlates with a more malignant phenotype (50). For example, the MDA-MB-435 cell, a very aggressive and metastatic breast cancer line in nude mice, secretes the lowest levels of thrombospondin I when compared to less malignant breast cell lines such as MCF7 which secretes high levels of thrombospondin I. Thus, one would anticipate that if the mechanism of p53 was primarily due to the induction of thrombospondin I, the liposome: p53 therapy would be effective against MDA-MB-435 but not against MCF7 tumors.

An alternative antiangiogenic mechanism by which p53 inhibits tumor growth is that the endothelial cell is the direct target of the liposome: p53 complex. As a result, tumor growth inhibition can be explained by the occurrence of G1/S arrest or apoptosis of endothelial cells by p53. Endothelial cells, both *in vitro* (51) and *in vivo* (6, 7, 46, 52) are known to be targets of the cationic liposomes: DNA complexes. We initially found that the endothelial cells of the tumor were transfected by a liposome: DNA complex (9). More recently, other investigators have confirmed this finding as well as shown that endothelial cells are of the tumor are transfected preferentially with liposome: DNA complexes compared to other endothelial cells (53). Transfection of wild-type p53 into the mitogenic endothelial cells of the tumor could result in G1 arrest and/ or apoptosis of these endothelial cells. Although the effects of transfecting p53 into endothelial cells were not known before we began our studies, it had been established that apoptosis of endothelial cell occurs when they are exposed to nitric oxide (54). Furthermore, increased levels of p53 in endothelial cells are present following exposure to nitric oxide (54), and as a result, apoptosis may be mediated through increased p53 levels in these cells. We have discovered that the liposome: p53 complex significantly reduces the endothelial cell number *in vitro* as well as reduces the blood vessel density of the tumor (figure 1) (9, 55). Utilizing an adenovirus as a carrier of p53, Riccioni and co-workers have also suggested that the endothelial cell is the direct target of therapy with p53 (56). At the present time, there is evidence to support that gene therapy with p53 acts through both directly and indirectly to inhibit angiogenesis.

Since our cationic liposomes have a low uptake into the tumor endothelial cells (<5%) (9), we are particularly interested in increasing the transfection efficiency. It has been estimated that inhibition of a single endothelial cell can inhibit as many as 100 tumor cells (57). As a result, a modest increase in transfection efficiency may result in a significant reduction of tumor growth.

5. GENE THERAPY WITH GENES ENCODING ANTIANGIOGENIC PEPTIDES/ POLYPEPTIDES INHIBITS TUMOR GROWTH

The realization that effective systemic gene therapy might inhibit angiogenesis led us to examine whether genes derived from antiangiogenic peptides act synergistically with p53. Despite the evidence that antiangiogenic peptides are effective antitumor agents (58-64) as well as the great interest in targeting genes toward

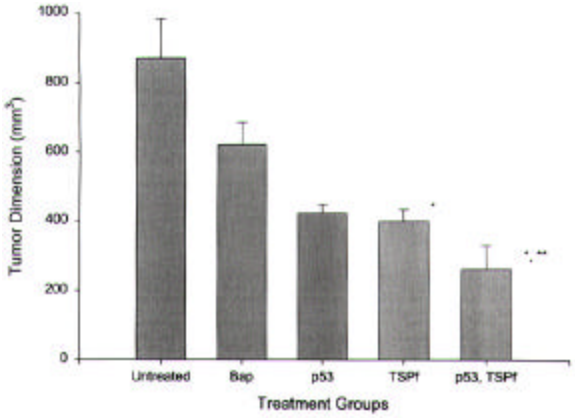

Figure 2. Systemic injection of liposome-DNA complexes into nude mice bearing MDA-435 tumors. Each group of mice received 5 injections of the different therapeutic genes and 14 µgs of DNA was delivered per dose. *, Untreated vs BAP-TSPf or BAP-p53/BAP-TSPf, $p < 0.05$; **, BAP vs BAP-p53/BAP-TSPf, $p < 0.05$.

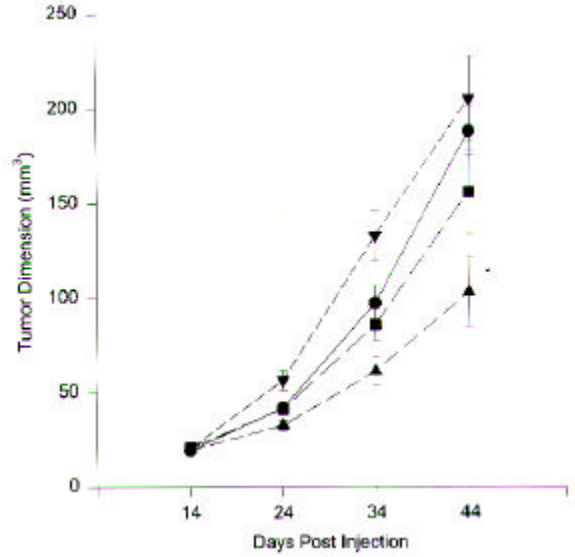

Figure 3. Systemic injection of liposome-DNA complexes into nude mice bearing MCF7 tumors. The dose of DNA given with each injection was 16 µgs as the initial dose followed by 12 µgs for the subsequent dosages. ▼ -- Untreated; ●-- BAP; ■-- BAP-p53; ▲ -- BAPp53:BAP-TSPf. *, BAP-p53 vs BAP-p53/TSPf, $p < 0.05$.

the vasculature, there have been few published reports on effective *in vivo* gene therapy regimens with established antiangiogenic DNA sequences (65-68). Thus far, antiangiogenic gene therapy studies are of limited usefulness in that reveal only modest reduction of tumor size with *ex vivo* design (65, 66) or intratumoral reduction (67). There have been no published studies utilizing anti-angiogenic gene therapy given systemically to eradicate tumors at a distant site. Furthermore, the development of effective carriers has been agonizingly slow in the field of gene therapy. Our approach of targeting the blood vessels of the tumor through intravenous administration of antiangiogenic genes has the potential of treating metastatic tumors.

Why consider developing gene therapy targeting blood vessels when antiangiogenic peptides therapy (i.e., a combination of angiostatin and endostatin) are being acclaimed as a significant breakthrough in cancer (at least in mice)? It is certainly true that gene therapy encoding antiangiogenic peptides is not as effective as anti-angiogenic peptides, but we believe that the efficacy differences between these therapies will diminish with usage of better carriers and therapeutic genes. Furthermore, gene therapy encoding antiangiogenic peptides has several potential advantages when compared to antiangiogenic polypeptide/ peptide therapy. These include a greater specificity toward the tumor, enhanced ability to localize the peptide intra- or extracellularly (via a signal peptide), a greater repertoire of therapeutic genes to utilize, and the ability to produce pharmaceutical amounts of the carrier and gene readily. As a result, we believe that antiangiogenic gene therapy will develop rapidly within the next several years.

One of these genes that encode an antiangiogenic product is a fragment of thrombospondin I. Thrombospondin I is a large trimeric glycoprotein composed of three identical 180 kD subunits linked by disulfide bonds (61). The majority of antiangiogenic activity is found in the central stalk region of this protein (61). There are at least two different structural domains within this central stalk region that inhibit neovascularization. It is this central stalk region of the thrombospondin I protein that is encoded by the BAP plasmid in our proposal. Since a paradoxical angiogenic effect at elevated dosages of thrombospondin I protein was reported with an *in vitro* angiogenesis assay (61), we chose to study the antitumor effect of the cDNA that encodes for the antiangiogenic fragment of TSP I to avoid any potential paradoxical results. We were also interested in determining whether p53 was synergistic with the antiangiogenic fragment of thrombospondin I.

After the mice were implanted with MDA-MB-435 cells and the tumor size was approximately 20mm3, the mice received two injections ten day apart of liposomes complexed to various therapeutic plasmids (55). We determined that the tumor size in the p53 treated group was significantly smaller than observed in the untreated group. In addition, liposomes complexed to BAP-TSPf (a plasmid encoding an antiangiogenic fragment of thrombospondin I) reduced tumor size similar to the liposome: p53 treated group. Thus, this delivery system appears to be a useful model upon which to develop more potent antiangiogenic gene therapies toward cancer. Of special interest, p53 in combination with the thrombospondin I fragment treatment group reduced tumor size more effectively than p53 alone.

We have confirmed that the combination of BAP-p53 and the thrombospondin fragment (BAP-TSPf) reduced the tumor size considerably more than BAP-p53 alone (figure 2) (55). Histological examination by the tunnel assay revealed increased apoptosis in the combination treatment group when compared to the p53 or untreated groups. We also examined this therapy in a second tumor model (MCF7) in which p53 is normal and thrombospondin I levels are increased. Interestingly, the combination therapy of p53 and TSPf showed marked antitumor activity in the MCF7 tumor model (figure 3).

The additional reduction of the tumor by p53 and the thrombospondin I fragment group compared to the p53 only treatment group suggest that these two agents have different mechanisms of action. This does not preclude that these gene products are both antiangiogenic since there are several pathways that can inhibit antiangiogenesis (68). Further support that the combination therapy has a marked antiangiogenic effect is demonstrated by a 45% reduction of blood vessel density in the p53 and TSPf treatment group compared to the untreated group. In addition to TSPf, we now have evidence that the angiostatin gene when complexed to liposomes inhibit breast cancer.

6. NON-SPECIFIC EFFECT

One of the more challenging aspects of the present project is to increase the antitumor therapeutic window. Lack of knowledge of this small therapeutic window has probably prevented others from being successful with systemic gene delivery of liposome: DNA complexes. As the dosage of the plasmid increases, the antitumor difference between the liposome: therapeutic gene and the empty vector groups decreases (15). Thus, one could potentially miss identifying a therapeutic gene due to the non-specific antitumor effect of the liposome: DNA complex. The synergistic antitumor action of p53 and TSPf has allowed us to increase this therapeutic window. However, the clinical utility of this approach depends on further increases in the efficacy of the therapeutic genes while limiting the non-specific antitumor effect of the DNA complexes. To express higher levels of the therapeutic gene in the tumor without significantly increasing the amount of plasmid, one might consider one or more of the following: 1) identify a promoter that allows higher expression than the β-actin promoter, 2) insertion of multiple promoters per plasmid, and/or 3) insertion of an IRES (internal ribosomal entry site) sequence between the two therapeutic genes allowing expression of the genes with one promoter. These suggested modifications of the plasmid might increase the efficacy of this therapy. Alternatively, carriers with minimal non-specific effects may exist. Future studies in our laboratory will focus on defining more effective carriers and plasmids expressing antiangiogenic genes to further reduce tumor growth.

7. PERSPECTIVE

We believe that systemic therapy with genes encoding anti-angiogenic peptides/polypeptides will rapidly expand. In addition to the p53 and the TSPf genes, we have determined that several genes that encode antiangiogenic peptides have significant antitumor activity. These include the laminin peptide, a fragment of angiostatin, and the FLK-DN receptor. The further development of this therapy will be dependent on increasing the specificity and efficiency of the carriers as well as identifying more potent combinations of therapeutic genes.

8. ACKNOWLEDGMENT

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Send correspondence to: Dr. A. James Mixson, University of Maryland Baltimore, Room 7-59, Bldg MSTF, 10 South Pine Street, Baltimore MD 21201, Tel:(410)-706-3223, Fax:(410)-706-8414, E-mail: amixson@umaryland.edu