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Current Gene Therapy Strategy for Renal Cell Carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) is the third most common urologic neoplasm and its age-adjusted incidence has been increasing annually. Despite advances in cancer research and current therapy strategies, the effective treatment of advanced RCC remains elusive. Over the past two decades, clinical and genetic studies have shown that kidney cancer is not a single disease entity, rather a spectrum of different forms. This understanding has translated into therapies targeted against specific genetic targets. In this review, we highlight the scientific principles, current applications, and future direction of renal cell cancer gene therapy.

Keywords: antiangiogenesis, cancer, cytoreduction, kidney, metastases

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INTRODUCTION

Renal cell carcinoma (RCC) accounts for 2-3% of worldwide cancer incidence and 100,000 deaths annually. Interestingly, the age-adjusted incidence has been increasing for the past thirty years by 3% annually (Jemal *et al.* 2007). Conventional treatment for metastatic disease has been relatively poor with approximately 50% and 10% 1 and 5-year survival rates respectively (Motzer *et al*. 2004). To date the only effective therapy has been immunotherapy with IFN-α or IL-2. Unfortunately, response rates have only been approximately 10-15% (Rosenberg *et al*. 1987). In addition, RCC has been refractory to chemotherapy and hormonal agents (Yagoda *et al*. 1995; Motzer *et al*. 1996). Early stage disease can be treated surgically with radical nephrectomy but 30% of patients will develop future metastases (Elson *et al*. 1988). These limitations have prompted new investigation into effective novel therapy for advanced RCC (**Fig. 1**).

The past decade has witnessed dramatic advances in the understanding of molecular and genetic alterations that promote RCC carcinogenesis. Research has highlighted a multitude of these genetic abnormalities which direct initiation, promotion and progression of renal cell carcinoma. Renal cells acquire the ability to resist growth inhibitory signals, evade apoptosis, proliferate in a low-oxygen environment, avoid immunosurveillance, promote angiogenesis, and metastasize to distant sites (Hahn 2002). During this process, renal cells must acquire a vast array of genetic mutations and this is demonstrated in the genetic variance of RCC cell carcinomas (Pavlovich 2004). Also, this genetic heterogeneity helps explain the varied clinical behavior of renal cancer and its different histological subtypes arising from separate regions of the kidney. Each is caused by distinct genetic mutations (Linehan *et al*. 2004).

This insight has led to therapies that target specific genetic aberrations that alter the neoplastic process. Gene therapy for advanced RCC can be classified as immune-based, cytoreductive, corrective and antiangiogenic (Zisman *et al*. 2000). The immunomodulatory approach aims to produce a tumor-specific immune response from the host by inducing cytokines, transfection of cytotoxic lymphocytes, or autologous tumor vaccines. Cytoreductive gene therapy creates tumor toxicity by transfecting oncolytic, replication-competent viruses, or inducing suicide and apoptotic genes. Corrective gene therapy seeks to repair the acquired genetic mutations by inactivating oncogenes or replacing tumor suppressor genes. Finally, antiangiogenic gene therapy attempts to target endothelial cells resulting in loss of tumor vasculature and subsequent inhibition of growth. This paper serves as a review of these gene therapy approaches and their current status as therapeutic options for advanced renal cell carcinoma (**Table 1**).

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Invited Review

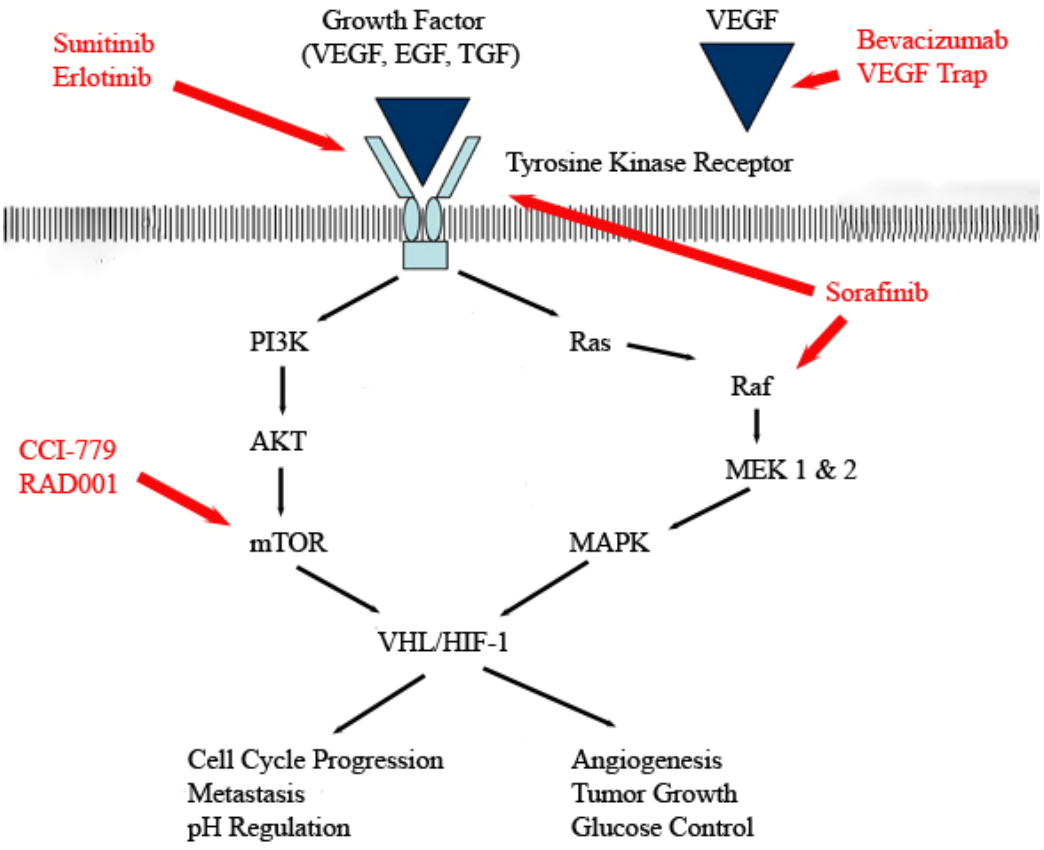

Fig. 1 Hypoxia-inducible pathway depiction. Growth factor binding to cell membrane bound receptor results in tyrosine kinase activation. Signaling leads to modulation of several pathways responsible for expression of genes controlling apoptosis, cell proliferation, metastasis and angiogenesis. A number of small molecule inhibitors are known to interfere with these pathways. Arrows represent key entry points for signal transduction inhibition and activity restriction.

Table 1 Classification of RCC gene therapy.

Immunomodulatory
Tumor Cell Vaccines: GM-CSF, B7-1(CD80), IL-2, *tag7*/PGRP-S
Dendritic Cell Vaccines: exogenous, endogenous RCC RNA, hTERT
Direct Cytokine Gene Transduction: IL-2,4,12, INF-α,β
Cytoreductive
Suicide/Apoptosis Genes: Thymidine kinase, Cytosine deaminase, TRAIL, apoptin, E4orf4
Replication-competent Oncolytic Viruses: Onyx-O15, MN
Corrective
Oncogene Suppression: *c-myc*, *pax-2*, PK-A, *bcl-2*, Ki-67, *TMSNB*, *PAR2*
Tumor Suppressor Gene Induction: VHL, RASSF1, Birt Hogg Dube, p7, Fumarate hydratase, Connexin 32, Int6
Antiangiogenic
Inhibitory Protein Expression: Angiostatin, Endostatin, Endostatin-Angiostatin fusion, soluble VEGF receptor

IMMUNOMODULATORY GENE THERAPY

Over the past 20 years there has been an impressive advancement in the understanding of the interaction between host immune systems and RCC tumors. Renal cell tumors actively evade immunosurveillance through a number of mechanisms, including down-regulation of major histocompatibility complex antigens and secretion of immuno-inhibitory cytokines (Radoja 2000). In contrast to direct administration of antitumor cytokines or adoptive immunotherapy, the goal of immune-based gene therapy is to selectively stimulate the host immune system to target tumor cells by the induction of genes encoding antigens, cytokines or growth factors. The tumor cells themselves will produce the cytokine, avoiding the toxicity related to systemic cytokine injection.

Transfection can be accomplished through multiple mechanisms. Viral vectors can deliver the immuno-modulatory gene to the tumor (Palmer 2005). Also, the desired gene or naked DNA can be directly injected into the neoplasm (Saffran *et al*. 1998). Alternatively, vectors encoding genes can transfect vehicle cells which subsequently target tumors. Genetically altered tumor cells or tumor-related dendritic cells can be used for this purpose. Transfection of vehicle cells can occur *in situ* or *ex vivo*. Cells altered *ex vivo* can be returned to the host for immune system stimulation, creating a tumor vaccine. This approach, a form of active immunotherapy, attempts to induce neoplasm-specific memory T cells as well as the total circulating amount of cytotoxic T cells to control tumor growth and potential recurrence (Kubler 2006).

Tumor cell vaccines

There are a number of clinical trials using tumor-cell based vaccines transfected with a variety of gene products that have demonstrated early potential benefit in treating metastatic RCC. Initial studies have found that granulocyte-macrophage colony-stimulating factor (GM-CSF) is an extremely potent inducer of tumor-specific immunity (Asher *et al*. 1991). A recent Phase I trial evaluating GM-CSF gene-transduced renal tumor vaccine elicited an objective partial response in one subject and 9 of 13 evaluated patients with Stage III-IV disease had delayed-type hypersensitivity and tumor specific CD8+ T cell proliferation (Simons *et al*. 1997). A second Phase I study from Japan demonstrated 1 of 4 patients with stable disease and 1 of 4 with mixed response when treated with GM-CSF-transduced autologous irradiated tumor cells. Similarly this trial displayed a marked immunologic response as 4 of 4 patients had positive delayed-type hypersensitivity and increased tumor-specific T cells (Tani *et al*. 2004). These early study results provide insight into the role of GM-CSF in promoting eosinophil infiltration, immune recruitment and antitumor activity.

Another study used genetically modified RCC cells to express the co-stimulatory molecule B7-1 (CD80) which is found on antigen presenting cells (APCs). The expression of B7-1, normally absent on tumor cells, strongly stimulates T cell activation. Of the nine patients with resultant metastases, 2 had partial responses and 2 developed stable disease (Antonia *et al*. 2002). Unfortunately these patients were also given adjuvant IL-2 and these results could be ascribed to the IL-2 administration in such a small trial. Other small Stage I trials have produced similar antigenic responses using tumor cells transduced with IL-2 genes and *tag7/*

PGRP-S, a novel gene involved in the innate immune response (Pizza *et al*. 2004; Moiseyenko *et al*. 2005). Inherent to all mentioned studies was the transfected tumor cells' ability to elicit delayed-type hypersensitivity reactions at the local tumor site thus recruiting immune activators.

Dendritic cell vaccines

Previous work interestingly suggests that the antigen-presenting cells that are recruited to the tumor locale, not the vaccinated tumor cells, are responsible for creating antitumor immunity (Huang *et al*. 1996). These APCs prime the CD4+ and CD8+ T cells that lead to systemic antitumor response (Huang *et al*. 1994). This knowledge led to the use of dendritic cells (DCs) as gene therapy vehicles. DCs serve as potent activators of CD4 T cells, CD8 T cells and NK cells, in addition to regulating response to tumor antigens through antibody production and inducing cytokine release (MacPherson *et al*. 1999). Further, DCs have been found to have defective maturation and function in cancer patients (Serafini *et al*. 2004). Therefore, immature dendritic cell can be genetically altered *ex vivo* and returned to the host in hopes of boosting antigen-specific responses (Frankenberger *et al*. 2005). The DCs are generated by either purification of peripheral blood precursors or culturing CD34 or CD14 cells and differentiating them into DCs with IL-4 or GM-CSF (Sallusto 1994; Fong 2000).

Gene therapy plays an integral role in "loading" the immature dendritic cells for use against RCC. In contrast to exposure to tumor cell lysates, peptides or cytokines directly, DCs can be transfected with a number of different immune stimulators. One study demonstrated tumor-specific T cell response *in vitro* to primary and metastatic RCC tumors by transfecting DCs with autologous renal cancer tissue RNA (Heiser *et al*. 2001). This same group subsequently conducted a Phase I trial on 10 study patients (Su *et al*. 2003). Using autologous dendritic cells transfected with tumor RNA, an unusually low number of patients progressed to lethal disease. Only 3 of 10 subjects died after a mean follow-up of 19.8 months. Unfortunately most patients concurrently received IL-2, surgery, or palliative irradiation, confounding the results.

The use of RNA allows presentation of a variety of tumor antigens which stimulate an aggressive immune response. Further, there may be shared antigens between renal cell carcinomas which are not patient-specific. Geiger *et al.* elicited T cell stimulation using DC vaccines transfected with RNA from generic RCC-26 cells (Geiger *et al*. 2005). This group illustrated that tumor-specific antigens from external sources can serve as stimulatory factors. This new approach could overcome tissue amount limitations in autologous vaccines by providing a limitless source of antigens. To take this approach a step further, Bontkes *et al*. combined dendritic cells transfected with mRNA encoding tumor-associated antigens producing biologically active IL-12 (Bontkes *et al*. 2007). Their technique allowed IL-12 production for up to five days post-transfection and effectively generated CTLs with high functional avidity.

Several studies have shown that the human telomerase reverse transcriptase gene (hTERT) can produce tumor-specific CTLs (Vonderheide *et al*. 1999; Minev *et al*. 2000). Transduction of dendritic cells with hTERT via an adenoviral vector was recently demonstrated to produce CTLs specific to gastric, osteosarcoma and hepatocarcinoma *in vitro* (Chen *et al*. 2006). Additionally, these hTERT-transduced DCs could increase secretion of IFN-γ. Since it has been observed that cancerous renal tissues express hTERT and telomerase activation plays an integral role in the oncogenic process of kidneys, this CTL-stimulating gene epitope could be used in the near future to induce anti-tumor immunity (Fan *et al*. 2005).

Direct cytokine gene transduction

Currently systemic cytokine administration, most notably IL-2, can result in metastatic tumor toxicity and produce complete and partial responses in patients with advanced metastatic RCC (Rosenberg *et al*. 1994). Though complete responses to IL-2 tend to be long lasting, significant patient side-effects and low response rates have limited the utility of systemic cytokine administration. Despite encouraging *in vitro* results, some have suggested that systemic administration fails to achieve adequate intratumoral concentrations before side effects occur (Fearon *et al*. 1990). Direct gene transfer of cytokine-encoding regions into malignant cells has the advantage of providing high local concentration of the desired cytokine without the toxicity. In addition, gene transfer has the potential to overcome deficiencies in T-cell receptor-directed signaling and the lack of co-stimulatory signals (Zier 1996).

Recent pre-clinical cytokine gene therapy trials with demonstrated efficacy include IL-2, IL-4, IL-12, IFN-α, and IFN-β (Hathorn *et al*. 1994; Blezinger *et al*. 1999; Figlin *et al*. 1999; Hoffman 2000; Nakanishi *et al*. 2003; Yu *et al*. 2004). As with vaccine therapy, the common mechanism of tumor inhibition is cytokine activation of CTL response. IL-2 has received the primary focus of cytokine gene therapy in recent years. Leuvectin (Vical Inc, San Diego) is a plasmid DNA expression vector containing the human IL-2 gene complexed with a cationic lipid mixure. Preclinical studies by Saffran *et al.* demonstrated successful IL-2 expression in an RCC cell line (Saffran *et al*. 1998). Subsequent Phase I/II studies have illustrated good toleration of therapy and adequate safety (Galanis *et al*. 1999). Of the 14 RCC patients available for follow-up, two achieved partial responses lasting from 16 to 19 months. An additional two patients had stable disease lasting from 3 to 18 months. Further, serial biopsy specimens showed increased IL-2 expression and improved CTL in the treated tumors.

A recent report by Galanis *et al*. has reviewed their cumulative experience in the treatment of metastatic RCC with intratumoral Leuvectin (Galanis *et al*. 2004). This current study included 31 patients with more extensive follow-up periods. 94% of these individuals had previously underwent nephrectomy and presented with metastatic lesions in multiple anatomic locations. Two patients had partial responses and one other patient had a pathological complete response, yielding an overall rate of 10%. Though this rate is close to the low end of response for systemic IL-2, an additional 23% of patients derived clinical benefit from disease stabilization (Goey *et al*. 1996). Interestingly, the median survival in the Leuvectin treated group, 11 months, was similar to those reported with systemic IFN-α (8.5-13 months) and IL-2 (12 months) (Negrier *et al*. 1998). The major advantage to using Leuvectin appears to be decreased side effects. None of the 31 patients displayed evidence of Grade 3 or 4 toxicities, in contrast to systemic IL-2 administration rates. Though only 10% of patients displayed durable responses, intratumoral injection of the IL-2 gene complexed with liposomes could provide an additional option for patients with metastatic RCC. Further studies are needed to identify patients most likely to benefit from this approach.

An alternative approach to the direct induction of cytokines is the gene transfer of co-stimulatory molecules which amplify the systemic effects of cytokine therapy. A triad of these molecules (TRICOM) has previously been described (Hodge *et al.* 1999). B7-1, ICAM-1 and LFA-3, designated TRICOM, have been shown to co-stimulate T-cell receptors and subsequent proliferation/signaling. Kudo-Saito and associates have recently reported the use of a replication-defective fowlpox vector encoding TRICOM to enhance tumor immunogenicity in an RCC model (2007). The intratumoral injection of this vector in combination with systemic IL-2, IL-15 or GM-CSF significantly reduces tumor burden, decreases metastasis incidence and extends survival in tumor-bearing mice than either therapy alone. These pre-clinical results confirm the potential of combining existing immunotherapy with novel gene therapy to provide enhances therapeutic effects. Though not reported by Kudo-Saito *et al.*, the simultaneous administration of co-stimulatory molecules

may allow a reduction in IL-2 dose and subsequently toxicity which has been a limiting factor of this therapy.

CYTOREDUCTIVE GENE THERAPY

Cytotoxic gene therapy strategies selectively kill tumor cells by employing two approaches: transduction of suicide or apoptosis-inducing genes, and replication-competent oncolytic virus transfection. In most cases, suicide genes encode for enzymes which convert benign pro-drugs into cytotoxic substances or encode protein products with direct tumor cytotoxicity. By administering the previously benign agent, high concentrations of this cytotoxic agent will accumulate in the tumor producing significant anti-tumor effects without systemic toxicity. A promising technique combines the delivery of the suicidal herpes-simplex thymidine kinase gene (HSV-TK) along with systemic ganciclovir (GCV), a purine analog. Only transfected tumor cells expressing HSV-TK are able to phosphorylate GCV into a monophosphate form. Subsequent conversion by cellular kinases yields GCV triphosphate, a false base. This product leads to tumor cell death by competitive inhibition of DNA polymerase and DNA synthesis. Success with other malignancies has led to attempts with renal cancer. An early animal model treated RCC tumors with either retrovirus-mediated *ex vivo* HSV-TK gene transfer or direct intratumoral adenovirus-mediated HSV-TK transfer (Pulkkanen *et al*. 2001). Though transduction efficiency was low at 22%, significant tumor regression was achieved with direct intratumoral adenovirus-mediated transduction, followed by intraperitoneal GCV. Additionally, increased apoptosis, macrophage tumor infiltration, reduced proliferation and significant survival prolongation was achieved with Adeno-HSV-TK and GCV treated mice.

Alternatively, cytosine deaminase (CD) plus 5-fluorocytosine (5-FC) has been applied to pre-clinical models of human RCC as a form of toxic gene therapy. In a similar approach as HSV-*tk* and GCV, adenovirus-mediated transfer of the CD gene into RCC tumors and the systemic delivery of 5-FC disrupts both cellular DNA and RNA synthesis, leading to tumor cell death. Both of these approaches have been compared in a recent study (Shirakawa *et al*. 1999). This group constructed adenoviral vectors containing the Rous sarcoma virus promoter driving CD (Ad-RSV-CD) or TK (Ad-RSV-TK). As compared to RSV-TK plus acyclovir, RSV-CD plus 5-FC demonstrated superior cell killing in both cell culture and animal models. Observed in both these forms of toxic gene therapy has been the "bystander effect." Although gene delivery does not have high efficiency, most early trials have demonstrated highly successful tumor toxicity. A suggested mechanism is that a portion of target cells are therapeutically transduced and their subsequent gene products diffuse to neighboring cells (Dilber *et al*. 1997). This spread depends largely on gap junctions, cell-cell interactions, soluble factors and apoptotic vesicles (Elshami *et al*. 1996; Hoganson *et al*. 1996; Mesnil *et al*. 1996).

Finally, a more recent adaptation for cytotoxic gene therapy with HSV-TK is the use of a hypoxia-inducible factor as a promoter. Much has been written concerning the loss of the von Hippel Lindau (VHL) gene in RCC and the resultant accumulation of hypoxia factors leading to tumor angiogenesis. Several published studies have shown that vector systems targeting hypoxic regions with solid tumors can regulate the expression of therapeutic genes (Dachs *et al*. 1997; Ruan *et al*. 2001). Based on these early results, the dysregulation of VHL and the upregulation of hypoxia factors in RCC appeared to be a potential target of this strategy. Its therapeutic potential was recently tested by combining the hypoxia-responsive promoter with a vector expressing HSV-TK (Ogura *et al*. 2005). *In vitro* and *in vivo* results indicate this approach has therapeutic efficacy. Xenografts treated with the cytotoxic gene therapy displayed marked regression without evidence of systemic toxicity indicating the desired specificity. In conclusion, this hypoxia-inducible vector system may have therapeutic potential for RCC with VHL mutations.

Induction of apoptosis is another approach towards producing tumor regression. While there are many agents which induce apoptosis, the best characterized to date has been the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL has been shown in its soluble form to induce apoptosis in a variety of tumor cell types, while having minimal toxicity again normal cells (Degli-Esposti *et al*. 1997; Griffith *et al*. 1998; Griffith 1998). A recent study has demonstrated that there is in fact TRAIL receptor expression in human RCC cell lines in variable amounts (Griffith *et al*. 2002). Interestingly, incubation with actinomycin D increased the expression of TRAIL receptors and decreased the inhibitory actions of survivin, thus improving TRAIL's usefulness as a therapeutic agent. Gene therapy provides significant potential in eliciting TRAIL's antitumor activity against renal cell carcinoma while eliminating the need for continuous administration of soluble TRAIL. Matsubara *et al*. have been able to introduce TRAIL plasmid into RCC cells via electroporation to produce potent *in vivo* effects (Matsubara *et al*. 2006). Further, concurrent systemic injection of 5-fluorouracil enhanced TRAIL-induced apoptosis. Another approach has been to use adenovirus-mediated transfection of TRAIL genes. Although a recombinant adenovirus encoding TRAIL(Ad-TRAIL) alone was unable to induce apoptosis in two RCC cell lines in a recent study, tumor cell apoptosis occurred when Ad-TRAIL was combined with histone deacetylase inhibitors (HDAC) (van Oosten *et al*. 2006). Because of their anti-proliferative effects, HDACs have themselves been proposed as antitumor agents. Significantly, they enhance recombinant adenovirus transgene expression and subsequently alter RCC sensitivity to Ad-TRAIL mediated apoptosis (Yamano *et al*. 2000). More recently, a lentivirus vector encoding TRAIL has been used to induce apoptosis (Wenger *et al*. 2007). This group observed direct toxicity problems with their vector but suggest TRAIL may be suited for *ex vivo* applications such as tumor cell vaccines mentioned above. An important conclusion from these studies is that combination therapy with chemotherapeutics plays a significant role in apoptosis-induction gene therapy. In the future, ionizing radiation may be used to augment TRAIL's apoptosis.

Other candidates for pro-apoptotic gene therapy are Apoptin and E4orf4. These genes, of viral origin, are capable of inducing apoptosis selectively in tumor cells. Their action is accomplished independently of caspase, p53 or Bcl-2 (Danen-van Oorschot *et al*. 2003). Recently, both genes were transduced via electroporation into RCC cell lines *in vitro* and *in vivo* to confirm their anti-tumor activity (Mitrus *et al*. 2005). In animals with established tumors, gene transduction of Apoptin and E4orf4 produced growth inhibition, though no tumors completely regressed. These early results suggest promise as potential cytoreductive therapy for RCC and further investigation is warranted.

The second approach to cytoreductive gene therapy utilizes replication-competent oncolytic viruses. These tumor-targeting viruses are genetically engineered and their intracellular replication is directly toxic to tumor cells. One possible candidate for this approach is ONYX-015 adenovirus. Replicating preferentially in p53-mutated cells, consistent efficacy and safety in phase I and II trials has been demonstrated with hepatocellular carcinoma and sarcoma metastatic sites (Makower *et al*. 2003; Galanis *et al*. 2005). Although RCC tumors manifest p53 mutations and may be a candidate for this form of gene therapy, ONYX-015 has not been applied to renal cell carcinoma. Other potential oncolytic viruses used in bladder and prostate cancers have included the herpes simplex viruses G207 and NV1020, and the adenovirus Ad-BSP-E1A. These viruses are promoter driven and as of yet, there has not been a RCC promoter discovered to produce the tissue specificity needed for cytotoxic gene therapy. Recent work with MN/carbonic anhydrase antigen has illustrated its potential as an RCC promoter. MN is a tumor-associated antigen found on many tumor cells in-

cluding human cervical, ovarian and renal cell carcinomas (Zavada *et al*. 1993; McKiernan *et al*. 1997). A 554 base pair MN promoter has been cloned into reporter vectors and activity successfully confirmed in multiple RCC cell lines (Ou *et al*. 2005). These early results suggest that the MN promoter can drive an adenoviral or retroviral vector carrying a drug-sensitivity gene, cytotoxic gene such as HSV-TK or other mentioned toxic gene therapies. Further profiling is warranted.

CORRECTIVE GENE THERAPY

Much has been learned over the past decade regarding the tumorigenic pathways of RCC. Most of these genetic alterations are characterized as the overexpression of an oncogene or the inactivation of a tumor suppressor gene by mutation. The goal of corrective gene therapy is to inhibit this aberrant oncogene expression or reinstitute normal tumor suppressor genes into malignant cells through vector transfection. Thus, normal cellular control mechanisms of cell cycle progression, apoptosis, DNA repair and transcriptional activity are re-established.

Oncogene suppression

Oncogenes represent mutated genes encoding for normal cell cycle regulation. Suppressive gene therapy attempts to arrest oncogene expression at the DNA, RNA or protein level. Four strategies have been described: anti-sense oligonucleosides (ASONs), ribozymes, dominant negative mutants and RNA interference. ASONs are short segments of DNA or RNA which bind to and prevent transcription or translation of particular oncogenes (Kausch 2002). Ribozymes are small segments of RNA which catalyze specific sequences of oncogene mRNA. Dominant negative mutants are altered oncogenes which produce large amounts of defective proteins. These non-functional products sequester or competitively inhibit critical targets in the normal oncogene function. RNA interference is a gene silencing approach where short segments of RNA inhibit homologous genes by degrading target mRNA. This final technique has advantages is that these inhibitory RNA sequences can target multiple genes involving numerous pathways.

Anti-sense oligonucleosides represent the most common strategy employed in oncogene suppression of RCC. Over the past decade, a range of oncogenes and gene products have been targeted in pre-clinical studies. *C-myc*, an oncogene initially discovered in a retrovirus, has been shown to participate in most aspects of cellular function. Many human tumors including RCC have been demonstrated to have amplification and over-expression of this gene (Nesbit *et al*. 1999). Subsequent *c-myc* ASON treatment of renal carcinoma cells *in vitro* has demonstrated significant susceptibility to lysis by lymphocytes and natural killer cells (Mizutani *et al*. 1995). Further, *c-myc* anti-sense oligonucleoside-treated RCC cells were also more susceptible to TNF-α mediated lysis.

Another oncogene target for ASON therapy is *pax-2*. Functional experiments have established that *pax-2* is an early activated gene and plays a role in a number morphogenetic processes of the developing kidney (Gruss 1992). Failure to down-regulate *pax-2* leads to severe kidney abnormlities including oncogenesis (Dressler *et al*. 1993). ASONs directed against the *pax-2* gene induced significant growth inhibition in a number of RCC-derived cell lines (Gnarra 1995). This data indicates that *pax-2* is required for cellular proliferation and differentiation and could be used as a novel therapy for RCC. More recent investigation has demonstrated that *pax-2* inactivation with ASONs sensitizes RCC cells to cisplatin-induced apoptosis (Hueber *et al*. 2006). Approximately 50-60% of previously cisplatin-resistant ACHN and Caki-1 cells were killed, suggesting that *pax-2* overexpression confers cisplatin resistence. Considering these findings, *in vivo* inactivation of *pax-2* may improve the efficacy of conventional chemotherapy against RCC.

Protein Kinase A (PK-A) has long been suggested as a target of anticancer treatment. PK-A plays an important role in cellular growth, differentiation and maintenance of malignant tumors (Gordge *et al*. 1996). It has further been shown to have higher activity in malignant versus normal tissue. Dose limiting toxicities restricted the utility of ASON treatment against PK-A in a phase I study including patients with RCC (Chen *et al*. 2000). Interestingly, another study investigated the effect of an anti-epidermal growth factor monoclonal antibody along with PK-A ASONs on RCC growth (Ciardiello *et al*. 1998). This group observed marked growth inhibition and increased apoptosis induction in RCC cells *in vitro* as well as athymic mice xenografts subjected to these agents in combination.

Bcl-2 is another oncogene which acts to promote tumorigenesis through suppression of apoptosis and programmed cell death. Increased expression of *bcl-2* is directly involved in the progression of many tumor types (Gautschi *et al*. 2001). Recent work has demonstrated that downregulation of *bcl-2* using ASONs has been shown to have effects on various cancers in phase I-III trials (Manion 2003). Initial application of this therapy was reported by Uchida *et al*. (2001). ASODs targeting the *bcl-2* oncogene were demonstrated to have significant inhibitory effects on *in vitro* proliferation of five RCC cell lines and growth of human RCC xenografts in athymic nude mice. Their results were dose dependent and associated with the induction of apoptosis. *Bcl-2* has also been shown to participate in the development of chemotherapy resistence (Bettaieb *et al*. 2003). RCC has proven highly resistant to chemotherapy in the past. A more recent study investigated whether down-regulation of *bcl-2* using ASONs may increase the chemosensitivity of human RCC tumors (Kausch *et al*. 2005). Though in this study transfection of RCC cells with *bcl-2* ASONs did not affect cell viability, combination therapy with cisplatin produced an 8-fold increase in apoptosis. These promising results indicate that for those patients with high tumor *bcl-2* expression, there may be advantage to combining ASON treatment with standard chemotherapy.

A further target of anti-sense therapy is Ki-67. This protein is only present in proliferating cell nuclei. This antigen is known to be an effective tumor marker and indicator of tumor grade in RCC (Visapaa *et al*. 2003). Only actively proliferating cell nuclei express Ki-67, thus inhibition of this protein could represent a promising anti-cancer therapy. Incubation of RCC cell lines with anti-Ki-67 ASONs resulted in Ki-67 protein reduction, inhibition of cell growth and increased apoptotic cell death in monolayer and spheroid cell culture (Kausch *et al*. 2005). Additionally, systemic application of these ASONs significantly decreased tumor burden in SCID mice xenografts. Though this study did not demonstrate an anti-angiogenic effect, the results suggest Ki-67 represents a potential target of therapy and ASONs could be used as anti-proliferative agents. These effects have been shown in other genito-urinary tumors and recently a phase I clinical trial has been started using intravesical Ki-67 ASON treatment for transitional cell carcinoma. This antigen could prove to be a potent RCC treatment in the future.

Two additional oncogenes have been identified which play an integral role in RCC growth and proliferation, *TMSNB* and *PAR2*. *TMSNB* directly inhibits actin polymerization and subsequent cytoskeleton formation. In addition, it has been implicated in apoptosis inhibition and angiogenesis (Iguchi *et al.* 1999). *PAR2* is a G-protein coupled receptor shown to promote angiogenesis and cancer metastasis (Richard *et al.* 2001). A recent microarray gene profiling of RCC cell lines implicated these genes in the tumorigenesis of RCC (Abdulrahman *et al.* 2007). This group found that overexpression of *TMSNB* and *PAR2* by direct gene transfer led to increased growth of RCC lines, cell cycle progression and hypoxia-independent growth. To further confirm their potential role as oncogenes, silencing of *TMSNB* and *PAR2* was achieved using siRNA. SKRC-18

cell transfected with siRNA demonstrated a statistically significant decrease in colony formation, growth rate and cellular motility. These results suggest that *TMSNB* and *PAR2* play a role in RCC tumorigenesis and could serve as potential therapeutic targets in the future.

While corrective gene therapy has not been studied in phase I clinical trials, numerous *in vitro* and *in vivo* animal studies have shown significant potential. The specific targeting of oncogene expression by these techniques provides significant anti-tumor effects and warrant further investigation. A theme from these pre-clinical studies is the synergism exhibited by combining suppressive gene therapy with other approaches such as chemotherapy. The potential role of multimodal treatment highlights the utility of oncogene suppression in renal cell carcinoma.

Tumor suppressor gene induction

Tumor suppressor genes regulate a cell's ability to proliferate by controlling cell-cycle progression, DNA repair, transcription and apoptosis. Alteration of these important genes can lead to malignant transformation. The goal of corrective gene therapy in this regard is to transfect wild-type tumor suppressor genes into cancerous cells, thus re-creating normal cellular control mechanism and producing tumor regression. The most recognized tumor suppressor gene in RCC literature is the VHL gene. It has been shown that the VHL gene, located on chromosome 3, is mutated in 60% of both inherited and sporadic forms of clear cell renal carcinoma (Whaley *et al*. 1994). Additionally, those patients with von Hippel-Lindau disease harbor an in-activating mutation in one VHL allele predisposing them to other tumors besides RCC, including pheochromocytomas, hemangioblastomas, pancreatic tumors, among others (Lonser *et al*. 2003). The VHL protein suppresses tumor formation by binding subunits of hypoxia-inducible factors (HIFs) responsible for downstream signaling of angiogenesis and promoting their ubiquitination and degradation. The upregulation of HIFs by the loss of VHL's regulatory control results in transcriptional activation of vascular endothelial growth factor (VEGF) gene and platelet-derived growth factor (PDGF) gene, further leading to vascular proliferation (Giaccia *et al*. 2004). Therefore the VHL gene serves as a potential target for corrective therapy.

Initial studies have demonstrated that correction of this gene with vectors encoding wild-type VHL leads to growth suppression *in vitro* (Chen *et al*. 1995; Lieubeau-Teillet *et al*. 1998). With preliminary work establishing feasibility, subsequent efforts have shown in animal models sufficient growth suppression in mouse xenografts with the re-introduction of the VHL gene (Iliopoulos *et al*. 1995; Gnarra *et al*. 1996). VHL protein was also found to regulate VEGF mRNA expression at a post-transciptional level, further leading to the conclusion that VHL is a first-line tumor suppressor gene. More recent approaches have utilized synergistic therapies combining anti-sense oligonucleotides against HIF-1α along with VHL plasmid (Sun *et al*. 2003). Intratumoral injection of subcutaneous tumors with an expression plasmid encoding VHL resulted in downregulation of HIF-1α, VEGF, increased tumor cell apoptosis and complete regression of small but not large tumors. However, combination of HIF-1α ASONs and VHL plasmids eliminated large tumors. Although, these results were obtained in thymic lymphoma xenografts, this approach may prove effective in RCC xenograft models.

Int6 is another recently-discovered tumor suppressor gene which plays an integral role in the angiogenesis cascade mediated by VHL. Recent results from Chen *et al.* indicate that Int6 induces proteosome HIF-2α degradation in RCC cells (Chen *et al.* 2007). Further, Int6 protein knockdown induced overproduction of VEGF, angiopoietin and basic fibroblast growth factor. Importantly this upregulation of angiogenic factors occurred under normoxic conditions and appeared VHL-independent. These early results suggest that Int6 is a critical factor in RCC angiogenesis and siRNA transfer of Int6 could become an effective therapeutic strategy in the future.

Another commonly mutated gene on chromosome 3 which appears to have tumor suppressor function for renal cell carcinoma is RASSF1. The hyper-methylation of promoter regions has been established as a method of inactivating tumor suppressor genes. The RASSF1 gene is hypermethylated in up to 56% of primary RCC tumors (Morrissey *et al*. 2001). Reversal of this inactivation can be accomplished by demethylating the tumor suppressor gene. Early work with RCC 786-O cell lines determined that RASSF1 transcription can be reactivated after incubation with a DNA methylation inhibitor 5-Aza-dC (Dreijerink *et al*. 2001). This agent acts by depleting methyltransferase activity, resulting in generalized demethylation. More recent investigation has noted a synergistic reactivation of RASSF1 when a histone deacetylase inhibitor, Trichostatin A, was added to 5-Aza-dC (Ibanez de Caceres *et al*. 2006). This additional agent can help re-express silenced genes by reversing the formation of transcription-inhibiting structures. Other synergistic approaches have been attempted with 5-Aza-dC to reactivate RASSF1. To reverse silencing, selective inhibition of DNA methyltransferase by antisense oligonucleotides was employed *in vitro* on RCC cells (Reu *et al*. 2006). RASSF1 was reactivated in multiple cell lines, leading to improved interferon-mediated apoptosis. A second major approach to reversing RASSF1 inactivation is transfection of RCC cells with functional RASSF1 genes using lentiviral, adenoviral, or plasmid vectors (Li *et al*. 2004; Ibanez de Caceres *et al*. 2006; Reu *et al*. 2006). *In vivo* animal studies indicated that re-expression of RASSF1 in tumors led to significant growth suppression and tumor regression. To date no clinical trials have been performed but these promising results suggest that the RASSF1 tumor suppressor gene plays a similar role as VHL in controlling cell-cycle progression and serves as an ideal target of corrective therapy.

In addition to VHL and RASSF1, many other tumor suppressor and cancer genes central to renal tumorigenesis are currently being identified. The Birt Hogg Dube gene appears to have the characteristics of a tumor suppressor gene in chromophobe renal carcinoma (Khoo *et al*. 2003). Also, the gene encoding the Krebs cycle enzyme fumarate hydratase appears to function as a suppressor in papillary renal carcinoma (Toro *et al*. 2003). An adenovirus expressing p7 was shown to induce cell cycle arrest and apoptosis induction in RCC cells (Katner *et al*. 2002). Further, the connexin 32 gene appears to be a promising tumor suppressor gene relating to the early stage of renal carcinogenesis (Yano *et al*. 2004). Furthermore, connexin 32 appears to potentiate the effects of chemotherapy on RCC cells (Sato *et al*. 2007). Connexin significantly enhanced the sensitivity of RCC to vinblastine-induced cytotoxicity and apoptosis induction in pre-clinical *in vivo* and *in vitro* trials. This suggests that siRNA treatment reintroducing connexin 32 into deficient-RCC cells could sensitize tumors to existing chemotherapeutic options and provide an exciting new approach for cancer therapy.

Genetic analysis of familial cohorts and ongoing clinical studies of recent years have demonstrated that renal cell carcinoma is in fact a spectrum of different cancer types occurring in a kidney, not a single disease entity (Linehan *et al*. 2003). Many genes participate in RCC development and regulation. Each one in turn could serve as a potential target of corrective gene therapy. Though limitations of this approach such as poor transfection efficiency and inadequate biological activity need to be overcome, the success of preclinical work suggests strong potential efficacy in the future.

ANTI-ANGIOGENIC GENE THERAPY

Many patients with unresectable RCC or metastatic disease at time of diagnosis remain refractory to standard systemic therapy. Anti-angiogenic therapy aims to inhibit the development of tumor blood vessels and represents a potential

treatment for this group of patients. This strategy targets endothelial cells rather than cancer cells themselves, resulting in loss of tumor vasculature and nutrient supply (Bergers *et al*. 1999). Tumor suppression is obtained by the resulting growth inhibition and apoptosis induction. There have been numerous endogenous angiogenesis inhibitors identified and ongoing clinical trials with systemic administration of these are demonstrating their efficacy against a range of malignant tumor types. Though these agents have not demonstrated limiting side effects, the majority of angiogenesis inhibitors are not cytotoxic and long-term potentiation of their effects would require chronic administration. Gene therapy allows potential long-term delivery of therapy without multiple administrations. Also, the cost of gene therapy vector production is less than purification of recombinant antiangiogenic proteins. Further, the peak/trough pharmacokinetics of chronic bolus administration of recombinant proteins may not be optimal for an antiangiogenic effect as compared to continuously elevated levels of antiangiogenic agents with gene therapy (Hahnfeldt *et al*. 1999). The major approach of antiangiogenic gene therapy suggested by Folkman is to administer agents systemically rather than selecting vectors which selectively target tumor cells (Folkman 1998). Because antiangiogenic agents lack direct cytotoxicity, systemic application would allow normal tissue production of antiangiogenic proteins for use against tumors.

Angiostatin is an internal fragment of plasminogen with demonstrated antiangiogenic and antitumor properties (O'Reilly 1997). Recent gene therapy attempts to produce this endogenous inhibitor have yielded promising results for RCC. *In vitro* studies have demonstrated that supernatant fluid from tumor cells transduced with the angiostatin gene inhibit endothelial cell proliferation (Nguyen *et al*. 1998). Numerous transfection vehicles have proven effective including adeno-associated viruses, adenoviruses, cDNA, retroviruses and liposomes. Fukumori *et al*. transfected RCC cell lines with angiostatin cDNA and subsequently established subcutaneous tumors (Fukumori *et al*. 2002). Three weeks after implantation the mean volume of angiostatin-transduced tumors was significantly less than controls suggesting that gene therapy expression of angiostatin can suppress RCC tumor growth. Additional work by Weiss *et al.* observed in a RENCA model that cell transfection of the angiostatin gene with nonviral electroporation significantly delayed and reduced primary tumor formation in nude mice (Weiss *et al*. 2004). Further, no metastases were detected in the lungs of mice that received angiostatin-transfected cells.

Endostatin, a fragment of type XVIII collagen, is a second endogenous anti-angiogenic protein. Specifically, it inhibits proliferation and migration of endothelial cells and induces their apoptosis (O'Reilly *et al*. 1997; Dhanabal *et al*. 1999). This agent also appears to have utility in the treatment of renal cancer. Szary and colleagues were able to intra-tumorally administer plasmid DNA containing the endostatin gene into mice RCC tumors (Szary 2001). Though *in vitro* growth is unaffected, *in vivo* tumors demonstrated slower growth. Additionally, a concurrent animal trial administering systemic endostatin plasmid further confirmed endostatin's ability to inhibit systemic RCC tumor angiogenesis (Blezinger *et al*. 1999). The intramuscular delivery of this antiangiogenic gene resulted in the inhibition of not only primary RCC tumors but also the development of metastatic lung and liver lesions. These results indicate that indeed normal tissues can be used to produce antiangiogenic proteins, in effect creating a "bioreactor".

Another theme of RCC gene therapy has been multimodal approach by combining multiple therapies acting by different mechanisms. Recently, Pulkkanen and colleagues have combined previously-mentioned HSV-TK with endostatin gene transfer to eradicate orthotopic RCC tumors in nude mice (Pulkkanen *et al*. 2002). This combination of cytotoxic and antiangiogenic gene therapy both improved

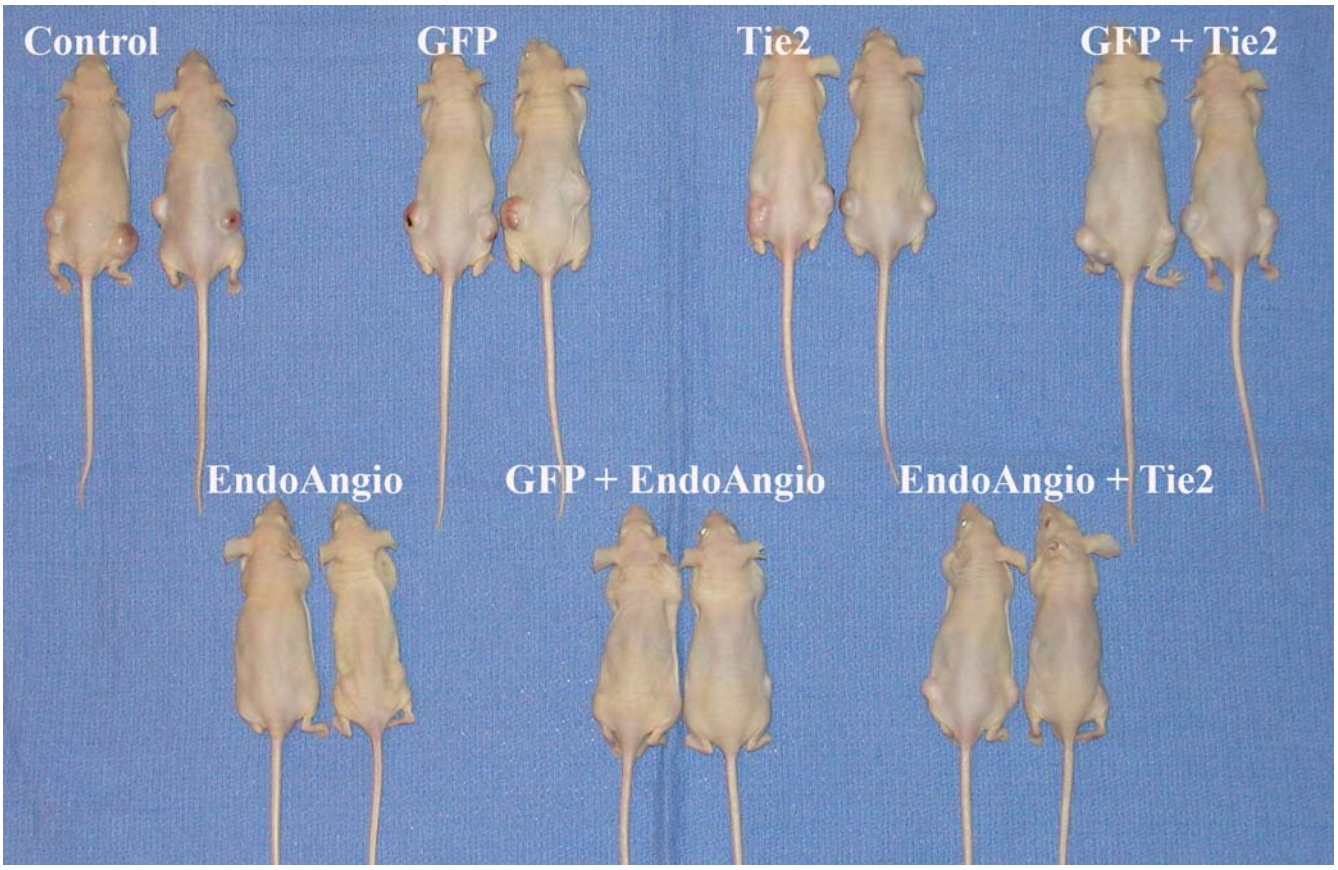

Fig. 2 Antiangiogenic gene therapy with Ad-hEndoAngio results in significant growth inhibition of injected and distant RCC subcutaneous tumors. Three replication-deficient adenoviral vectors were injected into flank-induced renal cell carcinoma tumors. Growth inhibition was compared to the saline and viral (Ad-GFP) control groups. Tumors treated with EndoAngio, GFP + EndoAngio, and EndoAngio + Tie2 demonstrated 82%, 83% and 87% growth reduction respectively ($p<0.001$). Neither Tie2 nor GFP + Tie2 resulted in significant inhibition of tumor growth.

the primary antitumor response as well prolonging survival time. Remarkably, complete tumor eradication occurred in 57% of the mice when combined therapy was used. This "choke and kill" strategy may prove effective long-term and could be used as an additional treatment option for RCC. Further, the inhibitors endostatin and angiostatin individually restrict angiogenesis effectively and could demonstrate synergism when applied together. Our laboratory has developed an adenoviral vector expressing a fusion protein, endostatin-angiostatin, to develop this principle (**Fig. 2**). Recently, this gene therapy approach was applied to RCC (Mellon *et al*. 2008). This adenovirus encoding endostatin-angiostatin produced 82% growth reduction compared to mock-treated groups. Additionally, *in vivo* imaging illustrated a reduction in blood vessel diameter and number.

Drugs targeting the vascular endothelial growth factor (VEGF) pathway have increasingly been used to treat metastatic renal cell carcinoma in recent years (**Fig. 1**). Strategies to inhibit VEGF include binding the VEGF protein and targeting its tyrosine kinase receptor. Both approaches are currently under investigation and the preliminary results from Phase I-III trials are encouraging. An alternative to using antibodies or small molecule inhibitors is gene therapy to express the human VEGF receptor as a means of inhibiting tumor development and metastases propagation. Lin *et al*. examined the effects of a soluble VEGF receptor encoded by an adeno-associated virus (Lin *et al*. 2005). The results of this study indicate that stable expression of this product successfully inhibits tumor-associated lymphangiogenesis, tumor development and regional lymph node metastases in a RCC model. As expected, the effective blockage of metastases is directly dependent on the amount of VEGF inhibitor produced. A second investigation of this technique was performed by Ichikura (Ichikura *et al*. 2006). An adenovirus expressing the extracellular domain of the VEGF receptor was intramuscularly injected into athymic mice distant to established RCC tumors. The application of this vector significantly suppressed the growth of the tumors, suggesting the gene-therapy expression of the soluble VEGF receptor can be effective in theory against RCC. Alternatively, plasmids containing the genes for IL-2 and soluble VEGF receptor respectively have been locally injected into subcutaneous tumors (Yockman *et al.* 2007). Local tumors as well as metastatic lesions were significantly reduced using a combination of these two agents. The IL-2/VEGF treatment reduced metastases by 56% over single agent therapy and prolonged survival by 50% in a murine model. Moreover, tumor-infiltrating lymphocytes were increased in the tumor microenvironment. This study further illustrates the potential of multi-modal gene therapy approach to RCC treatment.

To date, no clinical trials have been initiated but these early results are promising. Many of the pre-clinical antiangiogenic gene therapy studies indicate vector protein expression is most successful during early stages of vessel development, suggesting the best use might be as prevention therapy following surgery.

CONCLUSION

Renal cell carcinoma is associated with a poor prognosis and continues to remain refractory to traditional treatment options. New techniques and therapies are continually being discovered. One promising modality is gene therapy. The outlined approaches in this review have great potential as isolated therapy to transform RCC into a treatable condition or in combination with available conventional agents could provide curative options with decreased morbidity. Corrective, cytotoxic, immunomodulatory and antiangiogenic gene therapies directed against RCC have all demonstrated efficacy *in vitro* and *in vivo* in pre-clinical studies. While few clinical trials are available defining the role of gene therapy in renal cell carcinoma, it does appear that these approaches represent significant promise. As further understanding of the genetic mutations and immune dysregulations in RCC become available, it is apparent that gene therapy will continue to offer effective options in the management of this disease in the future.

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