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Lung cancer gene therapy

Review Article

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Abbreviations: small cell lung cancer (SCLC); non-small cell lung cancer (NSCLC); intratumoral injection of Ad-p53 (INGN 201); murine double minute-2 (MDM2); cisplatin (CDDP); active metabolite of irinotecan (CPT-11); O(6)-methylguanine-DNA methyltransferase (MGMT); retinoblastoma (RB); fragile histidine triad (FHIT); tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); triplex forming oligonucleotides (TFO); melanoma differentiation associated gene-7 (mda-7); carcinoembryonic antigen (CEA); replication-deficient adenovirus vector, Ad-mda7 (INGN 241); MAPK-activated kinases (Rsks);). Insulin-like growth factor binding proteins (IGFBPs); cyclooxygenase (COX)-2; ganglioside G(D2); Herpes simplex virus 1 (HSV); thymidine kinase (tk); ganciclovir (GCV); gastrin-releasing peptide (GRP); neuron specific enolase (NSE); Cre recombinase(Cre)/loxP; hypoxanthine-guanine phosphoribosyl transferase (HGPRT); Trypanosoma brucei (Tb); sodium iodide symporter (NIS); thyroperoxidase (TPO); matrix metalloproteinase (MMP); secret form of human platelet facter 4 (Spf4); vascular endothelial growth factor (VEGF); soluble flt-1 (sFLT-1); Tie2-expressing mononuclear (TEM); dendritic cells (DCs); Lewis lung carcinoma (LLC); natural killer (NK) cells; tumor necrosis factor receptor (TNF-R); 1,3) Galactosyl epitopes (·Gal); proliferin-related protein (PRP); interferon-inducible protein 10 (IP10); interferon (IFN)

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Summary

Lung cancer is the most lethal cancer worldwide. Although progress has been made in prevention, early detection, and treatment, mortality from this disease is still increasing. Current treatments in clinical trials have yielded only very limited results, and it is therefore necessary to develop new therapeutic strategies. Gene therapy is a novel field of medicine that may signal a more promising future for patients with lung cancer. Several studies on lung cancer therapy have held out the promise of treatment methods, including the alteration of intracellular molecular defects, the introduction of suicide genes, the inhibition of angiogenesis, and the augmentation of specific antitumor immunity. Various methods have been used to achieve specific gene transduction and effective gene expression. Clinical trials indicate that a combination of different treatment modalities is needed to obtain better results in lung cancer therapy. This review will summarize and discuss some recent advances and the potential future applications of gene therapy approaches in lung cancer.

I. Introduction

Lung cancer is the most common cause of death by cancer in both men and women, accounting for 18% of all cancer cases around the world. The average worldwide incidence of lung cancer is 37.5 per 100,000 persons, though this number varies greatly by country. The incidence is highest in Eastern Europe and lowest in Africa. The 5-year survival rate for lung cancer is 11% worldwide. In most countries, mortality from this disease is still increasing, especially in Southern and Eastern Europe (Parkin et al, 1999). The American Cancer Society estimates that 171,900 new cases of lung cancer will be diagnosed in the United States in 2003 alone. About 157,200 people will die of this disease: 88,400 men and 68,800 women, accounting for 28% of all cancer deaths. More people die of lung cancer than of colon, breast, and prostate cancers combined (Jemal et al, 2003).

Lung cancer is divided into two main histologic groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Approximately 80% of lung cancer cases are NSCLC, with small cell lung cancer (SCLC) accounting for the remaining 20%. Lung cancer arises from a series of morphological and molecular changes in which a normal epithelium transforms into an invasive cancer. To date, no efficient and safe therapy has yet been introduced for lung therapy.

Gene therapy, although still a comparatively young discipline, has made rapid strides in the past decade (Xu et al., 2003). Considerable efforts have been made to improve protocols for human gene therapy. Four main strategies for the treatment of cancer have been reported: alteration of mutated genes; introduction of suicide genes;

antiangiogenic gene therapy; and immunotherapy. Clinical trials have already been initiated. The number of approved protocols in clinical trials has increased, and at least 50% are designed for cancer (Folkman, 1998). Nervethless, a central challenge is perfecting methods for delivering therapeutic genes to the appropriate cells. The ideal gene transfer systems should be tailored to the specific tissue or cells requiring modification, to the needed duration of gene action and to the desired physiological effect of the gene product. Practical and theoretical limitations currently exist for the application of gene therapy in cancer patients, Most of these approaches have yet to pass even the most preliminary clinical tests demonstrating their overall safety and efficacy, but these ideas may lead to better cancer treatments in the future.

The molecular changes and underlying mechanisms of lung cancer have been continuously identified. The accumulation of the progresses may actually offer a thorough understanding of the disease and clinical context, and offer many targets for gene therapy. The improvements in gene transfer systems offer promise for the development of an efficient, specific-targeted and nontoxic gene delivery system, and thus there is very good reason to believe that greater success will be achieved in the near future.

II. Alteration of mutated genes

Gene alteration therapy is potentially a very powerful tool, targeting intracellular mutant genes of lung tumors. These gene products are specific molecular mediators of cancer development and progression.

A. Tumor suppressor genes

1. p53

p53 mutations, with frequencies up to 50% in NSCLC and 80% in SCLC, are the most common genetic lesions observed in lung cancers (Salgia et al, 1998). Mitsudomi et al, (2000) have shown by meta-analysis that p53 mutation or overexpression was an indicator of poor prognosis, especially in patients with adenocarcinoma. Roth et al, (1996) first reported the use of the strategy of replacing p53 in the treatment of nine lung cancer patients by local injection of retroviral vectors encoding wild type p53. Tumor regression was noted in three patients, and tumor growth stabilized in three other patients. In the second Phase I trial performed by this group, an adenoviral vector was used. Repeated intratumoral injections of Ad-p53 appeared to be well tolerated, resulted in transgene expression of wild-type p53, and mediated antitumor activity in a subset of patients with advanced NSCLC (Swisher et al, 1999). Because these completed studies have demonstrated only modest response rates, several protocols have been developed that combine the p53 gene transfer approach with other treatment modalities. No enhanced radiosensitivity of normal cells was noted when the ability of Ad-p53 (INGN 201) in NSCLC cell lines and human fibroblast cells was compared (Kawabe et al, 2001).

Recently, the same group reported the results of their Phase II study on 19 patients with NSCLC. The group found that intratumoral injection of Ad-p53 (INGN 201) in combination with radiation therapy was well tolerated, and also demonstrated evidence of tumor regression in primary injected tumors. Additionally, they found BAK expression was significantly increased 24h after injection of Ad-p53 (INGN 201), providing the first demonstration of induction of an apoptotic pathway by tumor suppressor gene expression in actual human cancers (Swisher et al, 2003). Schuler et al, (1998) reported the results of another Phase II trial, in which Ad-p53 gene therapy appeared to provide no additional benefit in patients receiving first-line chemotherapy for advanced NSCLC. To elucidate the combined effects of p53 gene transfer, chemotherapy, and radiation therapy on lung cancer growth in vitro and in vivo, Nishizaki et al, (2001) evaluated the synergistic, additive, or antagonistic efficacy of these therapeutic agents in four human NSCLC cell lines at the ID50 and ID80 levels. Synergistic inhibitory effects on tumor cell growth were noted both in an in vitro and a murine model with H1299 and A549 xenografts. Using two human cancer cell lines, H157 and H1299, Osaki et al, (2000) evaluated several anticancer agents, and suggested that cisplatin (CDDP) and an active metabolite of irinotecan (CPT-11) would be suitable candidates for a combination of chemotherapy and gene therapy for NSCLC. Srivenugopal et al, (2001) demonstrated that enforced expression of wild-type p53 curtailed the transcription of O(6)-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein that confers tumor resistance on many anticancer alkylating agents. This finding suggests that a combination of MGMT-directed alkylators with the p53 gene should achieve improved antitumor efficacy. Several studies (see below) have indicated the benefits of combination therapy on lung cancer, as one of the functions of p53 is to keep the cell from progressing through the cell cycle if there is damage to DNA present (Lowe et al, 1993).

Since wild type p53 reconstitution was not completely effective in all cases, mutants of p53 were explored for their ability to prevent p53 inactivation. A p53 derivative vector, in which the p53 domains bound by its inhibitor (murine double minute-2, MDM2) were replaced, was significantly more efficient than the p53 vector in tumor models overexpressing MDM2. Both in vitro and in vivo, a higher inhibition of tumor growth with the mutant p53 vector correlated with a higher induction of apoptosis (Bougeret et al, 2000).

2. RB

Abnormalities of retinoblastoma (RB), consisting of the tumor suppressor pRb/p105 and related protein p107 and pRB2/p130, are detected in more than 90% of SCLCs and in 15% to 30% of NSCLCs (Forgacs et al, 2001). Immunohistochemical studies of the expression patterns of the Rb family members in 235 specimens of lung cancer suggest an independent role for pRB2/p130 in the development and/or progression of human lung carcinoma (Baldi et al, 1996; 1997). Loss of pRb2/p130 expression is

also associated with an unfavorable clinical outcome in lung cancer (Caputi et al, 2002). The effects of expressing pRB2/p130 in a human lung adenocarcinoma cell line H23 have been analyzed, and it has been reported that retrovirus-mediated delivery of wild-type RB2/p130 to H23 potently inhibits tumorigenesis in vitro and in vivo. When tested in established tumors in nude mice, this approach reduced tumor mass twelve times more effectively than the control viruses (Claudio et al, 2000). These results offer promise for the potential future use of RB2/p130 in lung cancer gene therapy.

3. p16

In many instances, p53 and Rb are activated to promote senescence by the two products of p16 gene, protein p16(INK4a) and protein p14(ARF) (Lowe et al, 2003). p16(Ink4a) engages the Rb pathway by inhibiting cyclin D-dependent kinases that would otherwise phosphorylate and inactivate Rb. p14(ARF), on the other hand, increases the growth suppressive function of p53 by interfering with its negative regulator, MDM2. Clinical studies suggest that p16(INK4a) is a positive prognostic marker for NSCLC (Gessner et al, 2002). Several studies have suggested that polygene therapy with the p16 and p53/Rb gene may contribute to a greater antitumor effect (Kawabe et al, 2000; Tango et al, 2002). In vitro studies using adenoviral vector have demonstrated that p-16(INK4a)-mediated cytotoxicity is closely associated with the presence of functional pRb. Kawabe et al, (2000) also used adenoviral delivery systems to show that p16(INK4a) mediated radiosensitization of tumor cells depended on intracellular p53 status. Coinfection of Ad-p14(ARF) and Ad-p53 in human lung cancer cells resulted in a significantly higher *in vitro* cytotoxicity than Ad-p53 infection alone, coupled with an increase in expression of p53-inducible genes. Intratumoral injection of these two vectors significantly inhibited tumor growth in vivo (Tango et al, 2002). These results suggest that the p16 gene should be considered for possible applications in human lung cancer therapy.

4. FHIT gene

Alteration of the FHIT (fragile histidine triad) gene occurs as an early and frequent event in lung carcinogenesis (Sozzi et al, 1998). Small cell lung tumors (80%) and non-small cell lung cancers (40%) have shown abnormalities in RNA transcripts of FHIT, and 76% of the tumors exhibited loss of FHIT alleles (Sozzi et al, 1996). FHIT-negative patients tend to correlate with a worse prognosis (Pavelic et al, 2001). Seven lung cancer cell lines and three cervical cancer cell lines showed induction of apoptosis in all Fhit-negative cell lines, together with activation of caspase-8 by adenovirus vector-mediated FHIT gene expression (Roz et al, 2002). Consistently, increased level of BAK in FHIT-reexpressing cells linked the tumor-suppressor activity of FHIT to its proapoptotic function (Sard et al, 1999). *In vivo* reintroduction of wild type FHIT not only suppressed the tumorigenicity of lung cancer cells in nude mice (Ji et al, 1999), but also inhibited tumor development in heterozygous Fhit(+/-) knockout mice, which were prone to tumor development after carcinogen exposure (Dumon et al, 2001). With an improved liposome vector, successful treatment of primary and disseminated murine tumors and human lung tumor xenografts was achieved. This treatment suppressed tumor growth and prolonged animal survival with minimal toxicity (Ramesh et al, 2001). Further studies on this interesting gene are required, but FHIT gene therapy may eventually offer a promising clinical approach for the prevention and treatment of lung cancer.

5. p27

p27(Kip1), a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, may also function as a potential tumor suppressor gene. Significantly reduced p27(Kip1) expression is frequent in NSCLC, and is associated with shortened patient survival (Esposito et al, 1997; Yatabe et al, 1998). p27(Kip1) might play a distinct biological role in SCLC as a CDK inhibitor, conferring on SCLC cells the ability to escape from apoptosis under conditions unfavorable for cell growth (Masuda et al, 2001). The transfer of full-length human p27 cDNA by an adenoviral vector into lung cancer cell lines showed that induction of growth arrest and apoptosis by over-expression of p27 required expression of pRB (Naruse et al, 2000). With two adenoviruses expressing wild-type p27 (Ad-p27wt) and mutant p27(Ad-p27mt), Park et al, (2001) demonstrated the anti-tumor effects of p27 *in vitro* and *in vivo* in nude mice, and demonstrated that Ad-p27mt, which was believed to bind cyclin E/CDK2 more stably, had more potent anti-tumor effects than Ad-p27wt.

B. Apoptotic signaling checkpoints in response to DNA damage

Defects in apoptosis underpin both tumorigenesis and drug resistance, and because of these defects chemotherapy often fails (Johnstone et al, 2002). Tumor response to radiotherapy is regulated by endothelial cell apoptosis (Garcia-Barros et al, 2003). SCLC and NSCLC represent the two major categories of lung cancer, and they differ in their sensitivity to apoptosis (Joseph et al, 1999). It is therefore important to understand the molecular events that contribute to drug- and radiation-induced apoptosis, and how tumors evade apoptotic death, as it may be possible to harness this knowledge for novel therapeutic approaches.

1. BCL-2 family

The BCL-2 family of proteins, consisting of both antagonists (e.g. BCL-2, BCL-XL) and agonists (e.g. Bax, Bak) that regulate apoptosis and compete through dimerization (Reed 1994), are among the most closely studied apoptotic molecules in lung cancer. p53 is a regulator of bcl-2 and Bax gene expression *in vitro* and *in vivo* (Miyashita et al, 1994), and Bax acts as a tumor suppressor and as a component of the p53-mediated apoptotic response (Yin et al, 1997). Tumors harboring a

Bcl2-mediated apoptotic block undergo a drug-induced cytostasis involving the accumulation of p53, p16 (INK4a), and typically acquire p53 or INK4a mutations upon progression to a terminal stage (Schmitt et al, 2002). Bax (Kagawa et al, 2000) and Bak (Pataer et al, 2000) retained an impressive antitumor ability in the absence of chemotherapeutic drugs, and were able to effectively kill both p53-sensitive and p53-resistant tumors *in vitro* and *in vivo*. To avoid their toxicity to the packaging cell line, a binary adenoviral vector system was used. Usui et al, (2003) used the Cre-loxP system to propagate adenoviruses expressing the N-terminally truncated Bax (ΔN Bax), which was not blocked by Bcl-2 or Bcl-xl, and intratumoral injection into nude mice showed a significantly stronger suppression of tumor growth (74%) than full-length Bax (25%). The synergic effects of Bax and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were evaluated by human telomerase reverse transcriptase promoter-driven and adenovirus-mediated gene expression *in vitro* and *in vivo*, and it was found that combined Bax and TRAIL therapy produced more profound cell killing in human lung cancer line H1299 and prolonged survival in mice with ovarian cancer xenograft (Huang et al, 2002). As these are strong proapoptotic genes, targeted expression of the genes is highly desirable when they are used as a therapeutic agent. When Bax was expressed under the control of human vascular endothelial growth factor (VEGF) promoter, adenovirus-mediated overexpression of Bax resulted in apoptosis in human lung cancer cells and also in normal human bronchial epithelial cells (Kaliberov et al, 2002). Like Bax, BID also counters the protective effect of BCL2. Sax et al, (2002) suggested that BID was a p53-responsive 'chemosensitivity gene' that may enhance cell death response to chemotherapy. Fukazawa et al, (2003) noted adenoviral Bid overexpression could induce apoptosis in NSCLC cell lines and enhance chemosensitivity in the absence of p53. The function of BCL2 could also be blocked by silencing this gene with triplex forming oligonucleotides (TFO) (Shen et al, 2003a), or by down-regulation of its transcripts using antisense oligonucleotides (Buck et al, 2002).

2. p21 and Myc

Activation of the tumor suppressor p53 by DNA damage induces either cell cycle arrest or apoptotic cell death. The cytostatic effect of p53 is mediated by transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor p21(Cip1) (Bunz et al, 1998). *In vitro* experiments have suggested that p21 could serve as a marker for biological response to p53 gene therapy (Tango et al, 2002; Choi et al, 2000; Dubrez et al, 2001). A similar result was later obtained from biopsy examinations: p21 expression was up-regulated in NSCLC patients after treatment, especially when injections of higher doses of p53-expressing adenovirus were combined with simultaneous chemotherapy (Boulay et al, 2000). Joshi et al, (1998) have provided preliminary evidence for growth inhibition of NSCLC by p21WAF1 adenoviral gene transfer *in vitro* and *in vivo*. Myc was involved in this apoptotic signaling in response to DNA damage by selectively inhibiting bound p53 from activating p21(Cip1) transcription (Seoane et al, 2002). Downregulating *c-myc* expression by the combination treatment of *c-myc* antisense DNA with all-trans-retinoic acid resulted in inhibition of cell proliferation of small cell lung cancer *in vitro* (Akie et al, 2000). In a Lewis lung syngeneic drug-resistant murine tumor model, chemotherapeutic drugs in combination with c-Myc inhibition (which was specifically achieved by using non-toxic antisense DNA chemistry) suppressed tumor growth dramatically, but only with a regimen in which cisplatin or taxol treatment preceded the antisense compound (Knapp et al, 2003).

3. mda-7

It has been reported that adenoviral-mediated overexpression of the mda-7 gene exhibited cancer cell-specific growth inhibition irrespective of the status of other tumor suppressor genes, such as p53, RB, and p16 (Mhashilkar et al, 2001). When this attractive gene was used in lung cancer, similar results were noted in NSCLC cells in which the product of the transgene induced G2/M cell cycle arrest and an increase of Bax and Bak (Saeki et al, 2000). The induction of apoptosis was associated with activation of specific caspase cascades (Saeki et al, 2000; Pataer et al, 2002). *In vivo* studies correlated well with *in vitro* inhibition of lung tumor cell proliferation and endothelial cell differentiation mediated by Ad-mda7. Besides its proapoptotic properties, Ad-mda7 also demonstrated antiangiogenic abilities (Saeki et al, 2002). As a potent radiosensitizer, Ad-mda7 has been shown to enhance the radiation sensitivity of NSCLC cells, but not of normal human lung fibroblast lines (Kawabe et al, 2002). A Phase I/II dose-escalation trial of intratumoral injection with a replication-deficient adenovirus vector, Ad-mda7 (INGN 241), will be performed in combination with radiation therapy in patients with locally recurrent breast cancer (http: //www4.od.nih.gov /oba/rac/PROTOCOL.pdf).

4. Fas/Fas ligand

The interaction between Fas and Fas ligand (FasL) is involved in the apoptotic death of a number of cells, including lymphocytes. Hahne et al, (1996) proposed that FasL-expressing melanoma cells might induce apoptosis of Fas-sensitive tumor infiltrating cells. Human lung cancer cells have been shown to express FasL, enabling them to destroy T lymphocytes expressing Fas (Niehans et al, 1997). Moreover, apoptotic FasL-expressing tumor cells suppressed antitumor immunity, in contrast to the potent tumor-specific protective immunity generated by viable FasL-expressing tumors (Tada, 2003). Direct *in vivo* transfection of antisense FasL produced a systemic decrease in soluble FasL, and reduced tumor growth and invasion (Nyhus et al, 2001). However, membrane-bound FasL had opposite effects. Tada et al, (2002) demonstrated that forced expression of membrane-bound FasL in murine lung carcinoma cells produced anti-tumor effects through

an apoptotic mechanism by Fas/FasL interaction. Adenoviral infection with the Fas-associated death domain protein gene in lung cancer cell lines resulted in activation of caspase-8 and dose-dependent apoptosis (Kim et al, 2003). Shin et al, (2002) noted that the inactivating mutations of the genes in the pathway of Fas-mediated apoptosis were associated with nodal metastasis in NSCLC. Using adenoviral vectors to restore wild-type p53 function in a human lung cancer cell line, Thiery et al, (2003) reported that this restored not only Fas expression but also the Fas-mediated apoptotic pathway, and suggested that the wt p53-induced optimization of tumor cell killing by specific CTL may involve at least in part a Fas-mediated pathway via induction of Fas expression by tumor cells. Wt p53-dependent Fas-mediated apoptosis has been reconfirmed in human cancer cells expressing a temperature-sensitive p53 mutant (Li et al, 2003).

C. Growth factor pathway targets

Continuous growth of tumors depends on the altered regulation of the cell cycle, which is in turn modulated by signals from growth factors and their receptors, which provide the therapeutic targets.

Growth factors directly inactivating a critical component of the cell-intrinsic death machinery may result in continuous tumor growth. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death (Yang et al, 1995). It links p53 pathways with AKT and MAPK pathways, as phosphorylation of Bad by AKT or MAPK-activated kinases (Rsks) blocks pro-apoptotic activity to promote cell survival (Datta et al, 1997; Bonni et al, 1999). An *in vitro* model of variant differentiation in SCLC, which was chemo- and radio-resistant, elevated activation of AKT and MAP kinase associated with increased levels of phosphorylated BAD and activated NF-κB (Kraus et al, 2002). Therapeutic modalities that overcome the antiapoptotic function of AKT and Rsks are expected to be a novel strategy for lung cancer treatment. A combination of Bad with Bax resulted in a successful treatment in experimental tumor models (Zhang et al, 2002). IκBα, a specific inhibitor of NF-κB, has also been shown to be able to increase cytotoxicity in lung cancer cells (Batra et al, 1999). In addition, reduction of NF-κB activation in lung cancer cells was induced by TNF-α (Batra et al, 1999; Jiang et al, 2001). Evidence has been accumulated that IκBα is responsible for strong negative feedback that allows for a fast turn-off of the NF-κB response, whereas IκBβ and -ε function to reduce the system's oscillatory potential and stabilize NF-κB responses during longer stimulations (Hoffmann et al, 2002). IκBβ appeared to block the IGF-1 signaling pathway in IκBβ-expressing lung adenocarcinoma cells, and metastatic growth of such cells in the lungs of nude mice was significantly inhibited (Jiang et al, 2001).

Besides activating the AKT pathway to block apoptosis, IGF-IR (the type 1 receptor for insulin-like growth factor) activates other two signaling pathways to phosphorylate BAD protein and suppress apoptosis, one of which involves ras-mediated activation of the map kinase pathway. IGF-IR mediates cell survival and growth in response to its ligands IGF-I and IGF- II. Blockade of IGF-I and IGF-IR demonstrated antitumor effects on lung cancer (Hochscheid et al, 2000; Sueoka et al, 2000; Pavelic et al, 2002; Lee et al, 2003). Antisense oligodeoxynucleotides to IGF-IR and IGF- II were recruited to suppress the proliferation of lung cancer cell lines *in vitro*, and concomitant treatment inhibited growth up to 80% (Pavelic et al, 2002). Dominant negative IGF-IR has also shown potential for gene-based cancer therapy. Two kinds of defective IGF-IR expressed by adenoviruses effectively blocked IGF-I-induced Akt kinase activation and significantly suppressed growth in lung cancer xenografts (Lee et al, 2003). Insulin-like growth factor binding proteins (IGFBPs) are another promising candidate (Hochscheid et al, 2000; Sueoka et al, 2000). Ad-IGFBP6 reduced NSCLC cells growth *in vitro* and *in vivo* in xenografts through activation apoptosis (Sueoka et al, 2000). Damage of downstream target IGF-IR-regulated gene, such as ras, may be an alternative solution to inducing apoptosis. The antitumor effect has been demonstrated in human lung tumor xenografts using an anti-K-ras ribozyme adenoviral vector (Zhang et al, 2000).

D. New targets and approaches

The list of potential therapeutic genes promises to expand considerably with the identification of additional genes related to human lung cancer.

1. Survivin

A high level expression of survivin, a novel apoptosis inhibitor, has been noted in lung and breast cancers (Shen et al, 2003b). RT-PCR assay on tumor samples from a group of 83 NSCLC patients demonstrated that the survivin gene was expressed in samples from 71 patients who showed poorer overall survival than the other 12 patients (Monzo et al, 1999). Down-regulation of survivin by a targeted antisense oligonucleotide (Olie et al, 2000) or a TFO (Monzo et al, 1999) induced apoptosis in human lung cancer cells. Although further studies are required, this gene might provide promising clinical benefit in patients overexpressing survivin.

2. Cyclooxygenase-2

An increase in cyclooxygenase (COX)-2 expression, which is an important biomarker for biologically aggressive disease in NSCLC (Khuri et al, 2001; Brabender et al, 2002), may be associated with the development of human lung cancers and enhanced tumor invasiveness (Hida et al, 1998). Tumor COX-2-dependent invasion seems to be mediated by a number of factors (Dohadwala et al, 2001; 2002). Recently, Heuze-Vourc'h revealed a novel mechanism that, due to the deficiency of IL-10Rα on the surface of NSCLC cells and the unresponsiveness of COX-2 to IL-10 (known to potently suppress COX-2 in normal cells), contributes to the maintenance of elevated COX-2 and its product in the lung

tumor environment (Heuze-Vourc'h et al, 2003). These findings suggest the potential efficacy of COX-2 targeted gene therapy, and offer new targets for the further development of prevention and therapy.

3. Galectin-3

Galectin-3, a member of the β-galactoside-binding animal lectins, was recently identified as a key factor in tumor metastasis in NSCLC cancer (Yoshimura et al, 2003). Galectin-3 has been implicated in tumor invasion and metastasis (Inohara et al, 1998). Compared with healthy individuals, Galectin-3 serum levels in patients with lung cancer and some other cancers were significantly elevated, especially in patients with metastatic disease (Iurisci et al, 2000). In vitro experiments have suggested that Galectin-3 expression may play a role in NSCLC cell motility, invasion, and metastasis (O'Driscoll et al, 2002). A population (10/30) of the NSCLC samples from cell lines and biopsy tissue were found to overexpress the Galectin-3 protein at levels three times higher than those of normal epithelial cells (Yoshimura et al, 2003). Accordingly, Galectin-3 may represent a novel target molecule in NSCLC therapy.

Multiple genes are implicated in lung cancer development and progression to malignancy. Preliminary studies have proven the tumor suppressor activity of these new candidates, such as ganglioside G(D2) (Yoshida et al, 2001; Chen et al, 2003), uteroglobin (Lee et al, 2003) and several genes in the human chromosome 3p21.3 (Ji et al, 2002). However, further investigation is necessary to resolve a number of uncertainties before human trials can begin.

III. Suicide gene therapy

A. HSV-tk

Although the Herpes simplex virus 1 (HSV) thymidine kinase (tk) suicide gene together with ganciclovir (GCV) have been successfully used for the in vivo treatment of various solid tumors in recent clinical trials, a careful assessment and improvement of the efficacy and safety of such a strategy in different tissues in animal models of human lung cancer is essential before they can be used clinically. With the aim of establishing an effective therapy for pleural metastasis of lung cancer, liposome-mediated transfer of HSK-tk was performed in a nude mice model. Direct eradication together with a bystander effect contributed to a therapeutic outcome (Nagamachi et al, 1999). Using an orthotopic lung cancer model employing immunocompetent mice, Fukunaga et al, (2002) have assessed the therapeutic potential of adenovirus-mediated HSV-tk. Prolonged survival rates were obtained in mice treated with adenovirally HSV-tk-transfected tumor cells, and were related to gene transduction efficiencies.

In order to obtain the specific transduction of HSV-tk into human lung cancer cells, several tumor-specific promoters have been evaluated. In vitro and ex vivo experiments have demonstrated the specific expression of using gastrin-releasing peptide (GRP) promoter in SBC5 human SCLC cell line, in which GRP mRNA expression was detected (Inase et al, 2000). However, another experiment on the same cell line showed that neuron specific enolase (NSE) was not optimal for use in suicide gene transfer to SCLC cells, although NSE mRNA was expressed more abundantly in the SBC3 human SCLC cell line than in other cancer cell lines (Tanaka et al, 2001). Myc-Max response element demonstrated potential for specific expression of HSV-tk in any myc- overexpressing SCLC cells (Kumagai et al, 1996; Nishino et al, 2001). In vivo injections with Ad-MycTK followed by GCV administration selectively and markedly suppressed the growth of myc-overexpressing tumors established in the subcuties or in the peritoneal cavity of athymic mice; and in contrast to treatment with Ad-CATK, which conferred strong but nonspecific expression of HSV-tk, no apparent side effects were observed (Nishino et al, 2001). These results emphasis the importance of cell type-specific promoter selection to target different subpopulations.

Carcinoembryonic antigen (CEA) promoter is another practical choice to reduce toxicity to normal cells, because CEA is found in lung and other cancers (Konishi et al, 1999; Goto et al, 2001). Goto et al, (2001) exploited a Cre recombinase(Cre)/loxP system consisting of two adenoviral vectors (one expressing the Cre gene under the control of the CEA promoter (Ad.CEA-Cre), and the other the herpes simplex virus thymidine kinase (HSV-TK) gene) to provide a sutilized Cre recombinase(Cre)/loxP system to enhance antitumor effects together with minimal adverse reactions in HSV-tk gene therapy against disseminated CEA-producing cancer cells in the peritoneal cavity of mice. This provided an effective tool against disseminated cancer cells without significant side effects.

Modification of the HSV-tk gene itself or the prodrug should offer a practical way of improving this therapeutic system. Delivery of the HSV-TK mutant TK30 in a VSV-G pseudotyped retroviral vector, which was found to enhance the efficacy of prodrug therapy, provided a therapeutic efficacy after subsequent GCV application in human NSCLC cell lines in a preclinical murine xenotransplant model (Kurdow et al, 2002). Recently, two HSV-tk mutants transferred by adenoviral vector showed more tumor growth inhibition than the wild-type when tested in several cell lines, including human lung cancer and in their flank tumor models (Wiewrodt et al, 2003). On the other hand, a novel guanosin analog A-5021, which can be used more safely than GCV, demonstrated cytotoxic activity as potent as that of GCV in response to retroviral mediated HSV-tk-transduced human lung cancer cell lines, but did not exhibit a inhibitory effect on bone marrow progenitor cells and colony formation (Hasegawa et al, 2000).

B. New targets and approaches

1. Hypoxanthine-guanine phosphoribosyl-transferase

Like HSV-tk, the newly-discovered enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), expressed by the parasite Trypanosoma brucei

(Tb), can serve as a suicide gene, as it converts allopurinol, a purine analogue, to a cytotoxic metabolite. Retrovirus-mediated TbHGPRT expression can sensitize five NSCLC cell lines to allopurinol to levels 2.1 to 7.6 higher than control values, and represents a practical approach in lung cancer therapy (Trudeau et al, 2001).

2. Thyroperoxidase-mediated retention of radioiodide

In much the same way as such gene-prodrug treatment strategy, the sodium iodide symporter (NIS) gene, that allows rapid internalization of iodide into cells, can be used to obtain radionuclide accumulation by radioactive iodide administration for tumor cell killing. A combination of the NIS gene and the thyroperoxidase (TPO) gene, which can catalyze iodination of protein, resulted in an augmentation of radioiodide uptake and retention and subsequent effective tumor cell death in transfected NSCLC cell lines (Huang et al, 2001). Although there have so far been few reports on the treatment of lung cancer with NIS gene, it promises to be an effective approach for cancer treatment.

IV. Antiangiogenesis

Targeting angiogenesis is an attractive strategy to treat cancer. As progressive growth and metastasis of solid tumors is dependent on the formation of new blood vessels (Folkman, 1971), antiangiogenic therapy is a broad spectrum treatment for cancer. Two strategies used in the development of antiangiogenic agents involve therapy with endogenous inhibitors of angiogenesis as well as the inhibition of proangiogenic factors.

A. Endogenous inhibitors of angiogenesis

1. Endostatin

Endostatin, a 20-kDa C-terminal fragment of collagen XVIII (O'Reilly et al, 1997), is the leading member of a class of physiologic inhibitors of angiogenesis with potent antitumor activity. Boehm et al, (1997) have also reported that when three different mouse tumors were subjected to chronic, intermittent therapy with endostatin, there were no traces of acquired resistance. To establish a constant therapeutic concentration of circulating endostatin, investigations into endogenetic expression by a gene therapy approach have been prompted. Many viral vectors are actively under study in endostatin delivery. After systemic administration of a recombinant adenovirus to nude mice, persistent high serum levels of murine endostatin were achieved. The endostatin vector treatment not only resulted in significant reduction of the growth rates and volumes of Lewis lung carcinoma, but also completely prevented the formation of pulmonary micrometastases (Sauter et al, 2000). Intramuscular injection of adeno-associated viral vector expressing human endostatin led to a sufficient level of serum endostatin to inhibit angiogenesis and tumor growth (Shi et al, 2002). High-level endostatin was also detected in the vasculature of mice in which hematopoietic stem cells were implanted after being transduced by retrovirus encoding a secretable form of endostatin (Pawliuk et al, 2002). In addition, Lentiviral vector (Shichinohe et al, 2001) and Semliki Forest viral vector (Yamanaka et al, 2001) have been developed to express endostatin, and were first evaluated in T24 human bladder cancer cells and mice bearing B 16 brain tumor respectively. Some other nonviral transgene delivery approaches also involve endostatin transfer. Utilizing cationic vector, Nakashima et al, (2003) found that intravenous endostatin gene delivery significantly inhibited murine lung metastases. Intramuscular injection of polymerized endostatin plasmid inhibited syngeneic tumor growth and lung metastases in mice (Blezinger et al, 1999), and was also shown to inhibit murine metastatic brain tumor growth (Oga et al, 2003). When electroporation was used to enhance endostatin gene transfer into muscle tissues, the electrotransfer resulted in reduced numbers of experimental melanoma metastases in the lungs, while intratumoral electrotransfer significantly inhibited tumor growth (Cichon et al, 2002). Recently, engineered Bifidobacterium, a type of nonpathogenic anaerobic bacterial vector, was applied to bear endostatin by Li X et al, (2003), who demonstrated that vectors centered in tumors only, and inhibited local tumor growth after delivery by tail vein injection.

2. Angiostatin

Angiostatin is another specific endogenous inhibitor of endothelial cell proliferation. It is an internal fragment of plasminogen, isolated from the urine of mice bearing Lewis lung carcinoma (LLC) (O'Reilly et al, 1994). Tanaka et al, (1998) have demonstrated that retroviral and adenoviral vectors transducing angiostatin cDNA can be used to inhibit endothelial cell growth in vitro and angiogenesis in vivo. In a pulmonary metastatic breast cancer model, the delivery of Ad-angiostatin ($1x10^9$ pfu) to the lung significantly delayed tumor growth, as measured by the number of visible surface tumor nodules (Gyorffy et al, 2001). Intratumoral injection of a high-titer AAV-angiostatin vector effectively suppressed tumors and resulted in long-term survival in 40% of a group of treated rats, whereas the control AAV-GFP vector had no therapeutic benefits (Ma et al, 2002a). As angiostatin is an endogenous internal fragment of plasminogen, effective systemic gene therapy could be obtained by angiostatin gene transfer. Studies on liposome-coated plasmid carrying murine and human angiostatin showed that repeat intraperitoneal vector injection resulted in tumor growth suppression and delay in the onset of tumor growth to the same degree as intratumoral injection in a nude mice melanoma xenograft model (Rodolfo et al, 2001). Gene transfer of AAV-angiostatin via the portal vein led to significant suppression of the growth of both nodular and metastatic EL-4 lymphoma tumors established in the liver, and prolonged the survival time of the mice (Xu et al, 2003). Similar long-term therapeutic effects have also been demonstrated by Ma et al, (2002b), who used a single i.m. injection of AAV-angiostatin to effectively suppress human glioma growth in the brain of nude mice. The generation of angiostatin from endogenous plasminogen

by delivery of protease gene, such as mouse macrophage metalloelastase (Gorrin-Rivas et al, 2000; 2001) and porcine pancreatic elastase 1 (Matsuda et al, 2000), have been demonstrated as an effective alternative in different cancers.

3. TIMPs

TIMP-1, TIMP-2, and TIMP-3 are natural matrix metalloproteinase (MMP) inhibitors that prevent the degradation of extracellular matrix proteins (Anand-Apte et al, 1997; Moses et al, 1990; Takigawa et al, 1990). The in vivo efficiency of TIMP-2 has been evaluated in murine lung cancer LLC, and colon cancer C51 in syngeneic mice as well as in human breast cancer in athymic mice (Li et al, 2001). A single intratumoral injection of Ad-TIMP-2 significantly reduced tumor growth rates by 60-80% and tumor-associated angiogenesis index by 25-75%, and was accompanied by significantly prolonged survival. Lung metastasis of LLC tumor was inhibited by >90%. Pulmonary metastasis was significantly reduced in a murine melanoma metastasis model following 4 weeks of intramuscular injection with plasmid encoding TIMP-1 compared to controls treated with the plasmid DNA vector alone. Further therapeutic effects were realized by combination treatment with IL-2 (Shi et al, 2002). Gene transfer based on nontoxic cationic cholesterol derivatives indicated potent antitumor efficiency of TIMP-2 and TIMP-3 in HCC xenograft in nude mice (Tran et al, 2003). However, if TIMPs are to be utilized in antiangiogenesis therapy, close consideration should be given to a study suggesting an angiostatin-producing role for MMP-9 (Pozzi et al, 2002).

4. Combination strategies

Many of the endogenous inhibitors involved in cancer gene therapy succeed merely in slowing tumor growth, and need to be used in combination therapy for greater effectiveness (Shi et al., 2003). A combination approach has been attempted with tricistronic retroviral vectors encoding two inhibitors of angiogenesis expressed in a rat glioblastoma cell line: N-terminal fragment of rat prolactin and a secret form of human platelet factor 4 (Spf4). The results suggested that, in order to successful counteract tumor progression, antiangiogenetic strategy should be combined with other strategies (Ciafre et al, 2002). Another multigene therapy presented dormant and eradicated tumors by inhibition of angiogenesis using endostatin gene together with cytotoxic HSV-tk gene therapy (Pulkkanen et al, 2002). Adeno-associated virus-mediated gene transfer, when combined with ionizing radiation, enhanced inhibition of tumor growth (Shi et al, 2003). When assessing antitumor immune response against the recombinant protein of angiostatin and endostatin, Li et al, (2001, 2001) demonstrated that the host's immune response may potentiate the antitumor effects of antiangiogenic agents. Angiostatin gene therapy preceded by an in situ gene transfer of T-cell costimulator B7.1 eradicates pre-established tumors and a systemic challenge of cancer cells (Sun et al, 2001). More than an endogenous inhibitor, IL-12 is strongly immunomodulatory. When multigene therapy using angiostatin plus IL-2 was performed, a synergistic therapeutic effect was noted (Wilczynska et al, 2001).

B. Inhibition of proangiogenic factors

1. Endothelial cell-specific ligand/receptor tyrosine kinase systems

Keeping tumors from proangiogenic stimuli and interrupting the resultant angiogenesis can be achieved by gene therapy to damage endothelial cell-specific ligand/receptor tyrosine kinase systems. One of these systems consists of vascular endothelial growth factor (VEGF) and its two receptors flt1 and flk1/KDR, and another consists of angiopoietin-1 and its receptor tie2. The antisense strategy to inhibit transcription of VEGF (Im et al, 1999) and angiopoietin-1 (Shim et al, 2001) produced controlled tumor growth in vivo by inhibiting tumor angiogenesis.

The possibility of blocking VEGF and angiopoietin-1 function by gene delivery to produce a soluble form of their receptors has recently attracted attention. Hoshida et al, (2002) have demonstrated that the intratumoral administration of adenovirus-mediated soluble flt-1 (sFLT-1) gene results in a regional tumor suppression effect. Using intramuscular injection of adenoviral vectors expressing sFLT-1, they demonstrated subcutaneous growth inhibition in five out of six human lung carcinoma cell lines tested in nude mice (Takayama et al, 2000). A similar strategy was used by Mahasreshti et al, (2001), who showed that adenovirus-mediated sFLT-1 gene therapy inhibited s.c. ovarian tumor growth, and i.p. injection increased survival in a murine model of ovarian carcinoma. Mori et al, (2000) demonstrated that repeated intraperitoneal transduction of a soluble flt-1 gene using HVJ-cationic liposomes suppressed peritoneal metastases of some cancers, thereby contributing to a longer survival period.

In vivo studies of the soluble form of flk-1 (sFLK-1) showed that the growth of neuroblastoma cells was inhibited by retroviral mediated expression of sFLK-1 (Davidoff et al, 2001) or by inoculation with fibroblast which produced retroviral vectors encoding sFLK-1 (Davidoff et al, 2000). Tseng et al, (2002) evaluated the antitumor effects of the in vivo administration of an adenovirus vector encoding sFLK1 in 3 murine models of pancreatic adenocarcinoma. Intravenous injection of Ad-sFLK1 resulted in smaller tumor volumes in subcutaneous tumor models both in immunocompetent and SCID mice. The treatment also contributed to longer survival in the metastatic model. A recent investigation employed an AAV vector to transfer the sFLK1 gene. Intraportal injection of this vector preceded the intrarenal or orthotopic renal tumor implant, and resulted in growth restriction of tumors or tumor rejection (Davidoff et al, 2002).

After generating an adenoviral vector encoding soluble Tie2 gene, Lin et al, demonstrated that i.v. injection of this vector significantly inhibited the growth of subcutaneous primary tumors, as well as experimental or spontaneously occurring lung metastases (Lin et al,

1998). Hangai et al, (2001) produced a high plasma level of soluble Tie2 in mice by a single intramuscular injection of adenovirus expressing soluble Tie2. This treatment inhibited intraocular neovascularization, providing a potential approach to treat metastatic cancer using an angiogenesis inhibitor gene.

However, not all attempts to target the angiogenesis of cancer using gene therapy strategy have been effective. Some studies have produced negative results even when continuous, high levels of protein were produced (Eisterer et al, 2002; Pawliuk et al, 2002). Kuo et al, (2001) generated adenoviral vectors encoding angiostatin, endostatin, and the ligand-binding ectodomans of Flk1, Flt1, and neuropilin, and evaluated them in several different preexisting murine tumor models by systemic delivery. Ad-Flk1 and Ad-Flt1 resulted in approximately 80% inhibition of preexisting tumor growth in murine and human tumors. By contrast, adenoviruses encoding angiostatin, endostatin, or neuropilin were significantly less effective. Regulier et al, (2001) compared the adenoviral delivery of endostatin, proliferin-related protein (PRP), and interferon-inducible protein 10 (IP10) genes in a murine B16F10 melanoma model in immunocompetent mice. Ad-PRP or Ad-IP10 was significantly more efficient than Ad-endostatin, leading to complete tumor rejection and prolonged survival in a high proportion of treated animals.

2. Endothelial progenitor cells targets

The modification of bone marrow-derived cells with therapeutic genes has recently provided long-term targeted angiogenesis inhibition. Davidoff et al, (2001) transduced murine bone marrow cells with a retroviral vector encoding sFlk1. Tumor growth in mice challenged 3 months after transplantation with tsFlk-1-expressing bone marrow cells was significantly inhibited. De Palma et al, (2003) showed that when tumors were grown in mice into which bone marrow progenitors transduced with lentiviral vectors expressing genes from transcription-regulatory elements of Tie2/Tek gene were transplanted, these Tie2-expressing mononuclear (TEM) cells had a distinguishable phenotype and were present selectively at angiogenic sites. An HSV-tk & GVC approach targeting TEM cells resulted in substantial inhibition of angiogenesis and slower tumor growth without systemic toxicity. This experiment demonstrated that targeting exogenous genes to tumor angiogenesis could be achieved by transplantation of genetically-modified hematopoietic stem cells.

V. Immunotherapy

A. DNA vaccine

1. Tumor-associated genes

Carcinoembryonic antigen (CEA) is a cell surface tumor marker present in a variety of cancers, including lung cancer. The antitumor effects of an oral DNA vaccine encoding human CEA were obtained in mice, when boosted with an antibody-IL2 fusion protein. This vaccine broke peripheral T-cell tolerance toward CEA expressed by Lewis lung carcinoma stably transduced with CEA, resulting in eradication of subcutaneous tumors in 100% of mice and prevention of experimental pulmonary metastases in 75% of experimental animals in prophylactic settings (Niethammer et al, 2001). Song et al, (2000) demonstrated that intramuscular injection of a CEA plasmid without coinjection of IL-12 plasmid could not achieve complete resistance to a tumor challenge in wildtype mice by CEA-positive Lewis lung carcinoma cells, while injection of the IL- 12 plasmid alone was not protective. Luo et al, (2003) improved naked CEA DNA vaccine by absorbing it onto cationic microparticles, which are more immunogenic. Boosting with GM-CSF plasmid increased the vaccine's efficacy, resulting in a complete rejection of tumor cells in 50% of mice. Utilizing conventional and transgenic mice, Grosenbach et al, (2001) demonstrated that the use of cytokines and diversified prime and boost regimens could be combined with the use of recombinant pox virus vectors expressing signal 1, such as B7.1, and multiple costimulatory molecules to further amplify T-cell responses toward more effective vaccine strategies. Three different costimulatory molecule transgenes (B7-1, ICAM-1, and LFA-3) were used, and the two unique vectors rV-CEA-TRICOM (recombinant vaccinia vector) and rF-CEA-TRICOM (recombinant fowlpox vector). A similar conclusion was reached by Aarts et al, (2002), who evaluated a diversified vaccination protocol consisting of rV-CEA/TRICOM and rF-CEA/TRICOM on transgenic mice. A Phase I clinical trial on colorectal cancer using naked DNA immunization against the CEA showed that the vaccination was tolerated well. The success of the treatment, which has proved to be effective in a number of patients treated solely by immunizations, clearly depends on the stage of the disease. The treatment is most efficient in patients with minimal disease or no metastases (Mincheff et al, 2001). In patients with metastatic carcinoma, clinical study has shown that ALVAC-CEA B7.1, a canarypox virus encoding the gene for CEA and for B7.1, is safe and stabilizes the disease for up to 13 months (von Mehren et al, 2001). This approach may be a promising strategy for lung cancer vaccines, as immunofluorescence assay showed that no cell surface expression of CD80 protein was detected at all in 31 human NSCLC cell lines (Wroblewski et al, 2001).

MUC1 is a cell surface glycoprotein, expressed in most epithelial tissues and normal lung tissue, and has been shown to be preserved in most NSCLC cell lines and tumors. However, it is not expressed in normal lymph nodes. Vaccination of mice with naked DNA of MUC1 produced long-term tumor growth suppression (Johnen et al, 2001), and also suppression of the development of lung metastases, in which natural killer cells are the major effector cells (Kamata et al, 2002). When a similar vaccine was given in a tumor-bearing mice model, it was insufficient to suppress tumor growth. However, the addition of activated but nonprimed dendritic cells (DCs) obtained from syngeneic mice markedly suppressed tumor growth, and prolonged survival time (Kontani et al, 2002).

2. Tumor vasculature targets

The development of vaccines targeting tumor vasculature is a new strategy for cancer immunotherapy. Recently Niethammer et al, (2002) presented an oral FLK-1 DNA vaccine that targets stable, proliferating endothelial cells in the tumor vasculature, which effectively protected mice from lethal challenges with melanoma, colon carcinoma and lung carcinoma cells, and reduced the growth of established metastases in a therapeutic setting. Angiogenesis in the tumor vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis, though there was a slight delay in wound healing. The investigation of a cross-reaction between microvessels in solid tumors and xenogeneic endothelial cells has shed light on DNA vaccine for cancer therapy (Wei et al, 2000). Several xenogeneic molecules identified as involved in this cross-reaction were explored to treat cancer in a vaccine formulation, including chicken homologous matrix metalloproteinase-2 (Su et al, 2003), ligand-binding domain of chicken homologous integrin β3 (Lou et al, 2002), Xenopus homologous vascular endothelial growth factor (Wei et al, 2001), and xenogeneic epidermal growth factor receptor (Lu et al, 2003). These have all demonstrated potential for antitumor therapy in vivo.

B. Tumor cell-based immune modulation

1. Cytokines and co-stimulatory molecules

Gene therapy with cytokine and lymphocyte surface molecules (B7.1 and CD40 ligand) has been applied in clinical studies of tumors.

In a spontaneous metastasis model of LLC-f5 model, particle-mediated IL-12 gene transfer into skin distant from the tumor site elicited antimetastatic effects equivalent to local gene transfer, although its effect on primary tumors was not as evident (Oshikawa et al, 2001). Interleukin-12-transduced Lewis lung carcinoma (LLC/IL12) cells were found to have diminished tumorigenicity in syngeneic C57BL/6 mice, depending on their level of IL-12 production, and both CD4+, CD8+ T cells and natural killer (NK) cells were involved. In addition, LLC/IL12 apparently had a much stronger antitumor effect against established LLC/wt tumors than LLC transduced with B7-1 or GM-CSF cDNA (Sumimoto et al, 1998). On the other hand, it has been reported that costimulatory molecule B7.1 is required for initial tumor sensitivity to IL-12 gene therapy (Heise et al, 2001). This observation may offer the prospect of developing an effective multiple cytokine gene therapy. Dietrich et al, (2003) demonstrated antitumoral and antimetastatic effects of continuous particle-mediated cytokine gene (IL-12, IL2, IFN-γ/B7.1) therapy in an LLC model, but a significantly enhanced survival and reduced tumor growth was dependent on the sequence and order. To present synergistic activities, hetero-dimeric IL-12 could be expressed either in a single-chain form, or maintained as a heterodimer in which the p40 subunit is fused to IL-2. Gillies et al, (2002) showed that IL12/IL2 bi-functional cytokine fusion protein induced extremely high levels of interferon-γ, similar to the synergy normally seen with the combined application of the individual cytokines. In addition, these bifunctional molecules have been shown to have striking anti-tumor activity as either gene therapy or as a fusion protein. A comparison of the antitumor effects of IFN-α and IL-12 revealed that interferon-α induces tumor-specific immune responses while interleukin-12 stimulates non-specific killing (Eguchi et al, 2003).

Kusumoto et al, conducted a Phase 1 clinical trial to determine the safety and antitumor activity of an autologous GM-CSF-secreting (granulocyte-macrophage colony-stimulating factor) melanoma cell vaccine that was engineered ex vivo with recombinant replication-incompetent adenovirus harboring a human GM-CSF gene. One of the 9 enrolled patients responded to the vaccination by an apparent reduction in the size of a metastatic tumor in the lung. It was shown that infiltration of inflammatory cells, such as T cells (CD3+, CD8+), macrophages and dendritic cells (CD83+), were involved in the activation of antitumor immune response (Kusumoto et al, 2001). Several studies on animal models also demonstrated that autologous tumor cell vaccine secreting GM-CSF is effective in preventing and treating established and metastatic tumors (Nagai et al, 1998; Lee et al, 2000; Kinoshita et al, 2001; Maini et al, 2003). Its efficiency could also be enhanced by the cosecretion of IL-6 (Kinoshita et al, 2001) and IL-2 (Lee et al, 2000). Maini et al, (2003) showed, in a murine renal cell carcinoma (RCC) model, that lung irradiation plus vaccination with autologous tumor cells producing recombinant interleukin-2 (IL-2), interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) reduced the number of lung metastases by over 90%. It appears that NK cells and granulocytes are predominantly involved in the antitumor action. Most recently, a Phase I clinical trial was conducted by Salgia et al, (2003), which demonstrated that vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augmented antitumor immunity in some patients with metastatic non-small-cell lung carcinoma.

CD40 is a member of the tumor necrosis factor receptor (TNF-R) family of cell surface proteins expressed in B cells, dendritic cells, human thymic epithelial cells, human endothelial cells, and several carcinoma cell lines. Interaction between CD40 and CD40 ligand (CD40L; CD154) is important for cross talking between T cells and B cells, an essential requirement for B-cell immunoglobulin class switching (Banchereau et al, 1994) Imaizumi et al, (1999) demonstrated that stimulation of CD40 molecules on the surface of alveolar macrophages with CD40L-expressing clones of Lewis lung cancer cells enhanced the production of NO, TNF-α, and IL-12, and also improved tumoricidal activity under the stimulation of IFN-γ. Noguchi et al, (2001) showed that murine lung cancer cells (3LLSA) transduced with the CD40L gene (3LLSA-CD40L) were rejected in syngeneic C57BL/6 mice, but grew in CD40-deficient mice to the same extent as control tumor cells. Coinoculation of interferon (IFN)-γ-transduced 3LLSA with 3LLSA-CD40L cells enhanced antitumor immunity efficiently in vivo. Tada et al, (2003) have shown that the expression of CD40L in tumors in murine lung carcinoma (A11) cells could produce

antitumor effects by facilitating the interaction between DCs and tumors, enhancing the maturation of DCs, inducing secretion of cytokines, and consequently producing T-cell-dependent systemic immunity. These findings suggest that CD40L gene therapy approaches for the treatment of lung cancer should be pursued.

2. (1,3) Galactosyl epitopes (·Gal)

The role of (1,3) Galactosyl epitopes (·Gal) in exnograft rejection has been closely studied (Sandrin and McKenzie 1994). Unfer et al, (2003) have demonstrated that immunity to ·Gal provided protection in mice against challenge with genetically modified colon cancer cells expressing ·Galactosyl-transferase. These results demonstrate the potential for a cancer gene therapy that uses the innate immunity to Gal antibody in humans to destroy tumors as xenografts.

3. Dendritic cell-based vaccine

Antigen presentation by dendritic cells (DC) is crucial for the induction of primary T cell-mediated immune responses in vivo. To further augment a cellular immune response against tumor antigens, attempts have been made to increase antigen presentation capacity by genetically modifying DCs with cytokine genes or tumor-associated antigen genes (Sharma et al, 2003; Eppler et al, 2002). In two murine lung cancer models adenoviral IL-7-transduced DCs (DC-AdIL-7) were administrated intratumorally. Compared with other intratumor therapies such as AdIL-7, DC-AdIL-7 therapy was more effective in achieving systemic antitumor responses and enhancing immunogenicity, and in induction of splenocyte GM-CSF and IFN-γ, although both treatments resulted in complete tumor eradication (Miller et al, 2000). Its potential is now being evaluated in clinical trials. In a metastatic liver cancer model, local delivery of DCs transduced with the IL-12 gene was able not only to inhibit colorectal tumor growth in vivo, but also to mount systemic antitumor immune responses, evidenced by enhanced production of IFN-γ by T lymphocytes isolated from both spleen and draining lymph nodes (Satoh et al, 2002). Liu et al, (2002) demonstrated that DCs transfected with AdV-CD40L (DC(CD40L)) could stimulate enhanced allogeneic T-cell proliferation and Mut1-specific CD8(+) cytotoxic T-cell responses in vitro. Vaccination of Mut1 peptide-pulsed AdV-CD40L-transfected DC (CD40L) induced an augmented antitumor immunity in vivo by complete protection of mice (8/8) from challenge of both low and high doses of Lewis lung carcinoma cells. However, more investigation into the role of DC maturation, as well as its timing and sequence, is needed before it can be used in clinical applications.

VI. Conclusion

For successful gene therapy to lung cancer or other cancers, gene delivery systems play a key role. It is well recongized that at current developing stage of cancer gene therapy, gene delivery technology is still a major obstacle to success of the cancer therapy, although major improvements in all areas of vector development have been achived. Further work on technology issues is necessary. Much has yet to be learned before safe, efficient, stable, economic, convenient gene delivery systems with an appropriate regulation system either targeting specific tissues or cells to obtain long-term gene expression or targeting tumor directly is developed.

As the molecular biology of lung cancer pathogenesis and progression becomes increasingly understood, and as techniques for gene cloning and identification improve, a number of possible approaches to lung cancer gene therapy are emerging, which have demonstrated promise in pre-clinical tests. Only some of these approaches have been mentioned here. Clinical trials indicate that different types of combined modalities may have to be tailored to deal with specific sub-populations or individuals. In other words, an optimal outcome will probably depend on a combination of several genes or combination of gene therapy and conventional treatments. The crux is how to best combine these novel approaches so that they produce such an optimal outcome. The diverse nature of lung cancer suggests that molecular staging of individual cases will provide the best direction for combined modality treatment. Most importantly, although they are not always a reliable indicator of clinical outcome, carefully tested and controlled studies on animal models should be conducted to optimize the protocols before clinical trials are made.

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