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Review Article

Advances and perspectives in gene-based therapy for breast cancer

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CONTENTS

Abstract

Breast cancer is one of the leading causes of cancer-related death in the developed world. Conventional treatments are not adequate for a majority of patients with metastatic disease; consequently, novel therapeutic strategies are in high demand. Gene therapy constitutes an experimental approach gaining increased attention as a putative future therapeutic strategy for cancer. Using this strategy, cancer cytotoxicity can be obtained by replacement of tumor suppressor gene function or by blockade of dominant oncogene function. Genetic prodrug activation therapy and genetic immunomodulation are also showing remarkable promise. This review presents an update on the experimental and clinical results obtained in the field of breast cancer gene therapy.

Introduction

Although a significant number of new anticancer agents have been developed and there has been some improvement in the overall survival rate for patients with breast cancer, the life expectancy for patients who develop metastases is limited. Therefore, there is an urgent need to develop novel systemic strategies for this major health problem. Gene therapy is the introduction and expression of recombinant genes in somatic cells for the purpose of treating a disease. This approach has been used in multiple clinical trials with different experimental gene therapies. This article reviews the most important strategies in breast cancer gene therapy: 1) the use of tumor suppressor genes to restore a normal functional gene to cancer cells; 2) the use of antisense oligonucleotides to prevent the expression of oncogenes; 3) the use of a suicide gene approach utilizing genes expressing enzymes that can selectively activate specific prodrugs; 4) the use of a multidrug resistance gene to protect bone marrow during chemotherapy by transducing a drug resistance gene into marrow stem cells; and 5) the use of immunomodulatory strategies. Following a review of the current delivery systems used for gene therapy, we will discuss recent achievements in these different gene therapy strategies for breast cancer, including details and results from several clinical trials (Table I).

Delivery systems for gene therapy

Current delivery systems for gene therapy include viral and nonviral vectors. Early gene therapy studies and clinical trials employed retroviruses as vectors. Although they have the advantage of permanent integration into cellular DNA, retroviruses have a limited carrying capacity of roughly 8-12 kb, are reproduced at lower titers and are vulnerable to serum complement inactivation. Also, retroviruses permanently infect epithelial and hematopoietic cells, which can be a safety concern. Lastly, with stable integration into the host genome, retrovirus vectors can transduce only tumor cells that are actively dividing (1, 2).

Adenoviruses have a 36-kb chromosome complement with 6 gene products early in the course of infection and 3 late gene products at the onset of DNA replication. *E1A*, the first early gene, encodes two proteins via alternative splicing that suppress or activate transcription of viral and cellular genes and regulate the cell cycle. Therefore, in gene therapy studies, the majority of adenoviral vectors

Table I: Published clinical trials of gene therapy for breast cancer.

Gene target	Mechanism	Delivery system	Delivery mode	Comments	Ref.
p53	Gene replacement	Adenovirus	Intratumoral	Different solid tumors (1 breast cancer patient)	25
p53	Gene replacement	Adenovirus	Intravenous	Safety of i.v. Ad5CMV-p53	26
p53	Gene replacement	Adenovirus	Intravenous	Neoadjuvant breast cancer	27
E1A	Growth factor	Liposome	Intratumoral	Minimal toxicity, modest response	43
E1A	Growth factor	Liposome	Intratumoral	Minimal toxicity	44
HSV-TK	Suicide gene therapy	Plasmid	Intratumoral	Targeting erbB-2 cancer cells using a tumor-specific erbB-2 promoter	53
CYP2B6	Suicide gene therapy	Retrovirus	Intravenous	Breast cancer and melanoma patients	54
BCL2	Antisense		Intravenous	Oblimersen + weekly docetaxel	66
RAS	Antisense		Intravenous	ISIS-2503 + gemcitabine	71
RAS	Antisense		Intravenous	ISIS-2503 + paclitaxel	72
MDR1	Drug resistance	Retrovirus	*Ex vivo*	Low transduction efficiency without cytokines	76
MDR1	Drug resistance	Retrovirus	*Ex vivo*	Good transduction efficiency with cytokines	77
MDR1	Drug resistance	Retrovirus	*Ex vivo*	Acceptable transduction efficiency with cytokines	78
IL-12	Immunomodulation	Retrovirus	*Ex vivo*	Good response rate	81
IL-2	Immunomodulation	Adenovirus	Intratumoral	Good safety	85
STn-KLH	Immunomodulation	Theratope®	Intramuscular	Phase III: CY + KLH *vs.* CY + STn-KLH	90
MUC1	Immunomodulation	Vaccinia virus (TG1031)	Intramuscular	Good biological efficacy	91
MUC1	Immunomodulation	Vaccinia virus (TG1031)	Intramuscular	48% stable disease	92
MUC1	Immunomodulation	Vaccinia virus (TG4010)	Intramuscular	No objective response	93

developed have a deletion of the *E1* early gene. This allows the virus to be infective but nonreplicative. Adenoviruses transduce epithelial cells with a high frequency but do not transduce bone marrow cells. The adenovirus is highly immunogenic and does not usually integrate into cellular DNA, making the expression of the transgene mostly temporary (3, 4).

Adeno-associated virus (AAV) has also been investigated as a potential vector for gene delivery. The AAV can integrate stably into the host cell genome at a site-specific area on chromosome 19 and it then remains dormant until infection with a helper virus allows its replication. Advantages of the AAV are that it is not implicated in any human disease and that integration into the host genome does not affect cell replication. However, the AAV has not been used clinically to date because it has a small capacity to hold DNA and it has not been produced in high titers (5, 6).

Another viral vector that has been used is the herpes simplex virus (HSV) (7, 8). HSV infects a wide range of cell types with prolonged expression and high titers. The vector is large and can carry multiple genes. However, because of its large genome, HSV is quite complex and can be difficult to manipulate. In addition, the virus itself could be cytotoxic. Because of these difficulties, few clinical trials have employed this virus as a vector.

Nonviral vector strategies have employed primarily naked plasmid complementary DNA (cDNA) either alone or coated onto gold particles, liposomes that contain cationic lipids and neutral or helper co-lipids and nonlipidic polycationic polymer complexes with DNA (9, 10). Nonviral vectors have potential advantages over viral systems: 1) cellular transfection does not require specific receptors; 2) nonimmunogenic formulations can be administered repeatedly; 3) the vector can package large DNA constructs; and 4) the vectors are safe and easy to produce. Unfortunately, however, nonviral vectors suffer from low transduction efficiencies and mediate only transient gene expression. In addition, release of plasmid DNA and/or components of the vector can mediate inflammatory and/or immune reactions.

Adenoviral *p53* therapy

The most extensively studied tumor suppressor gene is *p53*. The *p53* gene is located on chromosome 17p and

encodes a 393-amino-acid protein that is critical to tumor biology (11-13). The general purpose of *p53* appears to be maintenance of the genetic integrity of the cell and induction of apoptosis when DNA damage is too great to guarantee a normal progeny cell. It affects: 1) cell cycle regulation; 2) cellular apoptosis; and 3) DNA repair. All these functions are dependent on the regulation of different proteins, such as p21, which inhibits CDK4 and CDK6 and is necessary for G1 to S transition, BAX (positive regulator of apoptosis), Mdm2 (negative regulator of *p53* function), thrombospondin-1 (inhibitor of angiogenesis), GADD45 (facilitator of DNA repair) and IGF-BP3 (a growth regulator).

Mutations in the *TP53* gene are the most frequent genetic changes in human cancer and, depending on the method of detection, the frequencies of *TP53* mutations reported in invasive breast cancer range from 12% to 46% (14, 15). The majority of studies suggest that the presence of *p53* dysfunction correlates with more aggressive tumors, early metastasis and decreased survival rates (16, 17).

There are conflicting data regarding *p53* as a predictor of response to therapy. Preclinical studies have reported the introduction of a wild-type *p53* gene into breast cancer cells with a mutant *p53* genotype using a variety of delivery mechanisms, including retroviral vectors, lipid complexes and adenoviral vectors. Results demonstrated that expression of the transgene product provides a normally functioning wild-type p53 protein within the malignant cell and expression of the transgene product has been shown to induce tumor regression and improve survival in animal models (18-22). Preclinical studies also revealed enhanced efficacy when combined with chemotherapy or radiation therapy (23, 24).

A phase I dose-escalation study of single intratumoral injection of a replication-defective adenoviral expression vector containing wild-type *p53* was carried out in patients with metastatic melanoma or breast cancer with increased p53 protein immunoreactivity in pretreatment tumor biopsies. A total of 6 (5 melanoma and 1 breast adenocarcinoma) patients were treated at dose levels depending on tumor size/dose-escalation sequence. Five of 6 patients became positive for the transfer of wild-type *p53* into tumor tissue 2 days after injection of the vector. Of the 4 patients assayed, all developed anti-adenoviral antibodies. Adverse reactions associated with intratumoral injection were mild, with no obvious correlation between the incidence, severity or relationship of the events and drug dose (25).

In a recent paper, intravenous administration of Ad5CMV-p53 (INGN 201, Advexin®; Introgen Therapeutics), a replication-defective type 5 adenovirus (Ad5) vector that contains a cytomegalovirus (CMV) promoter and a wild-type *p53* gene construct, was investigated in patients with a variety of solid tumors, including 2 breast cancer patients (26). Results demonstrated that Ad5CMV-p53 can be safely and repeatedly administered up to 1 x 10^{12} vector particles (vp) i.v. daily for 3 consecutive days. The absence of severe toxicities, the presence of circulating adenovirus 24 h after administration, and detectable *p53* transgene within tumor tissue distant from the site of administration were seen.

A recently completed study explored Ad-p53 combined with chemotherapy (27). Patients with locally advanced breast cancer were treated with intratumoral injections of Ad5CMV-p53 on days 1 and 2 plus docetaxel 75 mg/m^2 i.v., doxorubicin 50 mg/m^2 i.v. on day 1 and prophylactic granulocyte colony-stimulating factor (G-CSF). Thirteen patients were enrolled, with a median age of 56 years (range = 39-71) and a median tumor size of 8.0 cm (range = 5-11 cm). Up to 6 cycles of Advexin® were given, and all patients had surgery. Eight patients (83%) had *p53* mutations. The toxicity profile for Ad-p53 in combination with the other agents was similar to that for Ad-p53 and chemotherapy alone, and no grade 3 adverse events were considered to be related to Ad-p53 (27). Twelve patients were evaluated for response and all 12 achieved an objective clinical response, although none of the patients achieved a pathological complete response. Eight patients (67%) had residual pathological foci of disease in the breast of < 10 mm. The mean size of the residual tumor in the breast was 1.78 cm. All specimens showed extensive T-lymphocyte infiltration (CD3, 80%; CD4, 30%; CD8, 70%).

Recently, a retrospective exploratory analysis was performed of patients with locally advanced breast cancer who were treated at the University of Texas M.D. Anderson Cancer Center with protocol MDA ID-97099 (27). The objectives of the analysis were to describe and compare the clinical and pathological response in size- and volume-matched patients who were treated with the combination of docetaxel and doxorubicin *versus* a group of patients who received the same regimen with local injection of a nonreplicating adenoviral vector (Ad5) containing the human wild-type *p53* transgene (Ad5CMV-p53) (see above). Data from 22 consecutive patients were collected who had an initial presentation that more closely matched that of the 12 patients who received combination treatment. The historic comparison demonstrated that there was a significantly improved clinical response with the combination regimen (41% *vs.* 100%; $p = 0.0006$). These data demonstrate that combination of Advexin®/docetaxel/doxorubicin has significant therapeutic activity and could represent an interesting approach in the treatment of primary breast cancer.

The *E1A* gene

The *E1A* gene functions as a tumor inhibitor by repressing oncoproteins and sensitizing cancer cells to chemotherapeutic and radiation treatments. The interaction of E1A, a nuclear phosphoprotein, with a wide range of cellular proteins in multiple signal transduction pathways (cell cycle, DNA damage, histone deacetylation) results in multiple biological activities. *E1A* was initially appreciated for its ability to repress transcription, leading to downregulation of HER-2/neu protein and resulting in loss of malignant phenotypes (28-30). The antioncogenic

activity of the *E1A* gene, however, is not limited to tumors that overexpress *HER-2/neu*. *E1A* also modulates the expression of other genes, resulting in differentiation of certain cancer cells. Studies of immune-mediated rejection of rodent cells transformed by human adenovirus serotypes 2 and 5 (Ad2/5) led to the discovery that Ad2/5-mediated *E1A* gene expression actively induces cellular susceptibility to immune-mediated killing, a process that is independent of cellular expression of *p53* (31-33). Other studies revealed that *E1A* expression also sensitizes cells to apoptotic cell death in response to chemotherapeutic agents through both *p53*-dependent and -independent mechanisms (34-36). Finally, *E1A* has also been shown to enhance antitumor activity in response to etoposide, cisplatin, paclitaxel and doxorubicin (36-39). Several studies have also demonstrated a significant tumor radiosensitization response to *E1A* therapy. Investigations using cationic liposomes mixed with plasmid DNA encoding for *E1A* have shown safety and efficacy in animal models, as well as preliminary safety and activity in clinical trials (40-42).

At the M.D. Anderson Cancer Center, a phase I trial was performed to assess biological activity (*E1A* gene transfer/transcription/translation and *HER-2/neu* expression) and to determine the maximum tolerated dose (MTD) of local administration of *E1A*-lipid complex. Eighteen patients with metastatic breast cancer (n=6) or recurrent or metastatic ovarian cancer (n=12) overexpressing *HER-2/neu* were treated using a local injection of *E1A* gene-liposome into skin lesions or pleural/peritoneal effusion (43). The treatment was well tolerated and no serious adverse events other than fever or pain at the injection site were reported. *E1A* gene expression in tumor cells was detected by immunohistochemical staining and by reverse transcriptase-polymerase chain reaction (RT-PCR). *E1A* gene expression was accompanied by *HER-2/neu* downregulation, increased apoptosis and reduced proliferation. Although clinical response was not an endpoint in this trial, stable disease at the injection sites and improved performance status were noted in 3 patients. One of these patients was a 39-year-old white woman who experienced recurrent metastatic breast cancer after high-dose chemotherapy and autologous transplantation. The recurrence of disease in her right thoracic cavity with accompanying pleural effusion was manifested by the presence of a nonproductive cough accompanied by pleuritic chest pain and elevated serum CEA and CA 27.29 levels. After receiving 2 cycles of *E1A* gene therapy, she showed a significant improvement in breathing, accompanied by disappearance of pleuritic chest pain. In addition, elevated CEA and CA 27.29 levels in serum and CEA level in the pleural fluid returned to normal over the 2 cycles.

In a small pilot study in 9 patients with recurrent, unresectable breast cancer and 9 patients with recurrent, unresectable head and neck squamous cell carcinoma (HNSCC), the *E1A* gene was administered using a lipid complex and minimal toxicity was observed (44). *E1A* transfection was confirmed in 14 of 15 tumor samples tested and downregulation of *HER-2/neu* was demonstrated in 2 of 5 patients who overexpressed *HER-2/neu* at baseline. Response to the injection was modest. The combination of the *E1A* gene with chemotherapy or radiation therapy is currently being investigated in clinical studies.

Suicide gene approach

Suicide gene therapy, also called gene-directed enzyme prodrug therapy (GDEPT), involves the delivery of genes encoding metabolic enzymes which convert systemic, innocuous prodrugs to toxic metabolites (45). This strategy is a two-step process. First, a vector delivers a gene into tumor cells, which leads to expression of an enzyme. Second, a prodrug that is activated selectively by the enzyme is administered. Because the activating enzyme is present only in tumor cells, these cells selectively accumulate high concentrations of active toxic drug, thereby avoiding systemic toxicity. However, not only transduced cells but also circumferential cells are reported to die with this gene therapy. This phenomenon is known as the “bystander effect”. The molecular mechanisms of these bystander effects have now been characterized, and can be divided into chemical and immunological bystander effects (46, 47). *In vivo*, both of these effects may be observed within the same experiment, but with different kinetics.

More than 20 suicide gene therapy systems have been identified and tested in a variety of cancers, although the two most advanced are HSV-1 thymidine kinase (TK) using the antiviral prodrug ganciclovir and *Escherichia coli* cytosine deaminase (CD) using the prodrug 5-fluorocytosine. Preclinical studies with the HSV-TK gene followed by ganciclovir administration in animal breast cancer models have confirmed expression of the TK gene product and shown evidence of tumor regression (48-50). Activity has been demonstrated with nonreplicating adenoviral vectors, AAVs and retroviral vectors (48, 49, 51). It has also been reported that double transfer of GDEPT suicide genes allows the activation of two distinct types of prodrugs. For murine mammary tumors, the antitumor effect mediated by two different suicide gene systems, CD and cytochrome P-450 2B1, is more efficient than either system alone (52).

To date, two clinical trials are using GDEPT as a treatment for breast cancer. A phase I clinical trial of erbB-2-directed suicide gene expression was designed to test the safety and efficacy of a tumor-specific prodrug activation therapy (53). In this study, the genetic prodrug activation was specifically targeted to *ERBB2*-overexpressing breast cancer cells by use of a therapeutic cassette that contains the *E. coli* CD gene driven by the tumor-specific erbB-2 promoter, thus allowing activation of 5-fluorocytosine to the active cytotoxic agent 5-fluorouracil only within tumor cells that express the oncogene. Twelve postmenopausal breast cancer patients with multiple, well-demarcated skin metastases received a direct intratumoral injection of a plasmid construct combined with

systemic administration of prodrug. The results of this study were encouraging; the approach was shown to be safe and resulted in targeted expression of the CD gene in 90% of cases. Suicide gene expression was specifically restricted to *ERBB2*-positive tumor cells. Tumor reduction was shown in 4 of 12 cases.

The second clinical trial was designed to test the safety of MetXia®-P450, a novel recombinant retroviral vector that encodes the human cytochrome P-450 type 2B6 gene (*CYP2B6*) in patients with cutaneous metastasis from advanced breast cancer or melanoma (54). Cytochrome P-450 enzymes are primarily expressed in the liver and convert the prodrug cyclophosphamide to an active phosphoramide mustard and acrolein. MetXia® was safe and well tolerated. In total, 12 patients with breast cancer and melanoma received three dose levels of MetXia®-P-450. One patient with breast cancer had a partial response, 4 patients had stable disease and the rest had progressive disease. A third clinical trial of retroviral HSV-TK gene transfer into breast cancer tumor tissues and treatment with ganciclovir is ongoing (Favrot).

Combination of radiotherapy and suicide gene therapy has also been proposed to be advantageous. It is hypothesized that radiation-induced cellular membrane damage may facilitate the transfer of cytotoxic nucleotide analogues from HSV-TK-expressing cells to neighboring nontransduced cells. This combined approach has been shown to delay local tumor growth and prolong survival in a mouse mammary tumor model (55). These results were attributed to the induction of a potent local and systemic immune response, as demonstrated by the abundance of $CD4^+$ cells in the primary tumor. However, to date this combination has not been examined in clinical trials.

Bcl-2 targeting

One of the functions of Bcl-2 protein is to block apoptosis (56), the final common pathway that leads to cell death in response to DNA damage or microtubule disturbance triggered by cytotoxic agents or radiation. Overexpression of Bcl-2 has been shown to confer multidrug resistance to chemotherapy in several cancers, including breast cancer (57, 58), in which Bcl-2 is overexpressed in 50-75% of cases (59, 60). A Bcl-2 antisense compound, oblimersen (G3139, Genasense®; Genta) is an 18-base phosphorothioate oligonucleotide complementary (antisense) to the first 6 codons of Bcl-2 mRNA, which leads to a selective decrease in concentrations of Bcl-2 mRNA and protein levels (61). Results from *in vitro* and xenograft models have demonstrated the activity of oblimersen in decreasing Bcl-2 mRNA and protein levels, inducing apoptosis and sensitizing tumors to chemotherapy. While effective when used alone in lymphoma models, the effect of oblimersen monotherapy in solid tumor models is modest. However, antitumor activity is significantly enhanced in the presence of external apoptotic signals provided by the addition of chemotherapeutic agents, such as docetaxel, dacarbazine or anthracyclines (62-65).

A phase I trial of oblimersen and weekly docetaxel in patients with advanced breast cancer indicated that it is well tolerated and clinically active even in heavily pretreated patients (66). Most of the oblimersen-related toxicities reported in this study were typical of those reported with other phosphorothioate oligonucleotides (PS oligos), and included fatigue, thrombocytopenia, aPTT prolongation and transaminase elevation. These toxicities are likely related to the polyanionic nature of the phosphorothioate backbones. In this early study, clinical efficacy was not the primary endpoint. Only 7 breast cancer patients were evaluated for response, 5 of whom had previous exposure to taxanes. Two patients achieved partial responses (lasting for 4 and 6.5 months) and 2 patients with nonmeasurable disease remained progression-free for 4 and 9 months. The durable tumor response (6.5 months) in the patient with paclitaxel-resistant disease was encouraging.

RAS targeting

One of the most frequent abnormalities identified in breast cancer involves the proto-oncogene *RAS*. The *RAS* family includes *HRAS, KRAS* and *NRAS.* The proteins encoded by members of the *RAS* family serve as critical components of the cell signaling pathway by acting as cell-surface receptors. They are involved in the control of cellular proliferation, differentiation and cell death (67, 68).

ISIS-2503 is a 20-base antisense drug that specifically inhibits the expression of H-Ras mRNA and protein (69, 70). Results of a phase I trial with ISIS-2503 in combination with gemcitabine in patients with advanced solid tumors indicated that antisense therapy combined with chemotherapy was well tolerated and clinically active (71). Nineteen patients were treated with a fixed gemcitabine dose of 1000 mg/m^2 on days 1 and 8 and two escalating doses of ISIS-2503 (4 and 6 mg/kg/day) as a 14-day continuous infusion starting on day 1. Cycles were repeated every 3 weeks. Only 1 patient with advanced breast cancer was enrolled in this study and a partial response was obtained (at dose level 2) in this patient, who had received extensive previous treatment (including stem cell transplantation). Subcutaneous metastases decreased by 80% and liver metastases shrank by 50% in this patient.

In a phase II trial, weekly paclitaxel 80 mg/m^2 in combination with ISIS-2503 at the recommended phase II single-agent dose (6 mg/kg/day by 14-day continuous i.v. infusion repeated every 21 days) was administered to 25 patients with metastatic breast cancer (72). Prior adjuvant therapy was permitted, including prior taxanes if tumor progression occurred more than 6 months later. Treatment was continued for 12 weeks and could continue until disease progression if toxicity was acceptable. Intrapatient dose escalation of ISIS-2503 to 10 mg/kg/day was allowed if toxicity permitted. Three patients had received adjuvant paclitaxel. Four patients had disease stabilization and 8 patients had a partial response.

Treatment was well tolerated, with no patient discontinuing treatment due to toxicity. Larger trials of this antisense agent are planned.

Manipulation of drug resistance

The multidrug resistance gene (*MDR1*) encodes the 170-kDa P-glycoprotein (P-gp), which acts as an adenosine triphosphate (ATP)-dependent transmembrane efflux pump for a diverse group of chemotherapeutic agents, as well as other compounds (73). Agents pumped out of the cell by P-gp170 include the anthracyclines, vincristine, etoposide and paclitaxel. *MDR1* is normally expressed on the atypical surface of secretory cells in the liver, colon and kidney, and the endothelium of the brain, testis and lung. Overexpression of the *MDR1* gene is one of the most common known mechanisms by which cancer becomes resistant to chemotherapy. To overcome P-gp-mediated drug resistance, anti-*MDR1* hammerhead ribozymes were recently developed and delivered to breast cancer cell lines using a retroviral vector containing an RNA polymerase III promoter, resulting in reversal of chemoresistance (74). However, the most important clinical application of *MDR1* gene transfer is represented by the insertion of the gene into normal marrow stem cells to produce a population of cells that can be selected for resistance to systemically administered chemotherapeutic agents. In one study, the percentage of the *MDR1* gene-modifying peripheral blood cells increased from 1% to 7% in reconstituted mice after drug treatment (75). An advantage of this approach is that it may permit higher doses of chemotherapy without the bone marrow suppression induced by chemotherapy.

So far, several protocols for *MDR1* have been approved for the treatment of patients with breast cancer (76-78). The M.D. Anderson Cancer Center performed retroviral gene transfer without using cytokines. *In vitro* transduction efficiency was 2.8% with the solution method and 5.6% with the stromal method, detected by *in situ* PCR. However, 3-4 weeks after transplantation, direct PCR assay of peripheral blood leukocytes in patients showed positive results in 0/10 with the solution method and 5/8 with the stromal method. These data show insufficient transduction efficiency without cytokines (76).

The National Cancer Institute (NCI) also reported the results of a clinical trial of retroviral *MDR1* gene therapy. In this trial, the *MDR1* gene was transferred into bone marrow mononuclear cells or peripheral blood stem cells stimulated by IL-3, IL-6 and stem cell factor (SCF). The *ex vivo* transduction efficiency was 0.2-0.5%. The investigators treated transplanted patients with paclitaxel (77). Another group at Columbia University also transferred *MDR1* genes into bone marrow mononuclear cells or peripheral blood cells stimulated by IL-3, IL-6 and SCF. They showed that 20-70% of erythroid burst-forming units (BFU-E) or colony-forming units granulocyte-macrophage (CFU-GM) colonies were positive for *MDR1* by PCR (78).

Immunomodulatory approaches

It is well known that a state of immune anergy or active immune suppression is often associated with cancer. Immunomodulatory defects involving effector cell function, natural killer (NK) cells, lymphokine-activated killer cells, tumor production of immune-inhibitory cytokines and diminished dendritic cell function have been well demonstrated in breast cancer. However, attempts to correct such defects in human clinical trials involving non-specific immune modulation with BCG (Bacillus Calmette-Guerin) have not demonstrated evidence of activity in breast cancer. Recently, specific gene-directed immunological approaches have been pursued and include: 1) transfer of cytokine genes; and 2) transfer of antigen molecule genes. Despite the therapeutic success achieved in numerous preclinical immune-gene therapy studies, only a few of these approaches have entered human trials for the treatment of breast cancer.

Cytokine genes

Cytokines can inhibit the development and progression of tumors. Therapy with immune-stimulating cytokines is often tested, but it is usually associated with dose-limiting toxicities. High local cytokine production has the advantage that it is most effective, without toxicities associated with large, systemic doses. For this approach, recombinant adenoviruses expressing a variety of cytokine genes have been tested in breast cancer models. An adenovirus expressing TNF-α has been used in murine models of breast cancer to induce complete regression of spontaneous mammary tumors (79). Although the vector was injected into the tumor, systemic levels of TNF-α were achieved and resulted in significant toxicity. Retroviral transfer of the TNF-α gene and *Neo* gene into tumor cells *ex vivo* and subcutaneous injection of tumor cells may activate the systemic immune response to tumor cells (80).

Retroviral transfer of the IL-12 gene into skin fibroblasts of patients *ex vivo*, followed by injection of the fibroblasts into tumor tissues, may activate a tumor-specific immune response. In a phase I study, 9 cases with advanced solid tumors, including breast cancer, were treated by Kang *et al.* (81). Reduction of tumor at injection sites was shown in 4 cases, and reduction of tumor at remote sites was achieved in 1 melanoma case. There were no side effects other than slight pain at the injection sites.

In animal models of breast cancer, a substantial number of responses were observed following intratumoral injection of adenoviral IL-2 gene therapy (82-84). The data suggested that IL-2 activity is mediated by induction and expansion of an antigen-specific T-cell response. Stewart *et al.* conducted a phase I trial in which an E1/E3-deleted adenovirus encoding IL-2 (AdCAIL-2) was directly injected into subcutaneous deposits of melanoma or breast cancer (85). Twenty-three patients were injected at 7 dose levels. The side effects noted were minor and included local inflammation at the site of injection in 60%

of patients. Postinjection biopsies demonstrated tumor necrosis and lymphocytic infiltration, with the predominant tumor-infiltrating cells being $CD3^+$ and $CD8^+$. Combined transfection of the IL-2 gene with the HSV-TK gene in a murine breast cancer model revealed greater antitumor activity with the combined IL-2/TK gene followed by ganciclovir compared with the IL-2 gene alone (86). The combination of direct tumor killing from the TK gene product and generation of tumor-specific immune responses may provide a local environment that optimizes the gene therapy effect.

Antigen molecule genes

Several breast tumor antigens have already been identified based on the biology of breast cancer (87). These include mucin-1 (MUC-1), HER-2/neu, carcinoembryonic antigen (CEA), p53, sialyl-Tn (STn), the melanoma-associated (cancer testis) antigens MAGE, BAGE, GAGE and XAGE, and the putative universal tumor antigens survivin and telomerase (hTERT). Of these antigens, MUC-1 has been most extensively tested as a cancer vaccine. MUC-1 is a high-molecular-weight glycoprotein rich in serine and threonine residues that are *O*-glycosylated. Expression of MUC-1 is increased in breast, ovarian and other adenocarcinomas, and altered glycosylation results in exposure of novel peptide epitopes and the expression of tumor-associated carbohydrate residues, such as Thomsen-Freidenreich and STn antigens.

Multiple clinical trials have tested the safety and bioactivity of vaccines that target MUC-1-derived peptide or carbohydrate epitopes. One group of trials tested Theratope®, a cancer vaccine that was designed by Biomira incorporating a synthetic STn antigen that emulates the carbohydrate seen on human tumors (88). Two phase II trials in 50 breast cancer patients compared the STn-KLH vaccine with and without a single low-dose infusion of cyclophosphamide used as an immunomodulator prior to initiation of treatment (89). Humoral immune responses were higher in patients who had received low-dose i.v. cyclophosphamide compared with patients who had received no cyclophosphamide or oral cyclophosphamide. There was a statistically significant survival difference between all patients treated with the STn-KLH vaccine (overall median survival = 19.1 months; n=50) and the retrospective control patients (overall median survival = 9.2 months; n=104). Furthermore, patients who received i.v. cyclophosphamide prior to the STn-KLH vaccine had median survival rates close to 3 times those of patients in a retrospective, frequency-matched control group who received conventional therapies (i.v. cyclophosphamide 26.5 months *vs.* 9.2 months in the control group). The trials reported minimal toxicity with local reactions at the injection site and some flu-like symptoms. On the basis of the phase II trial results, a phase III clinical trial of the STn-KLH vaccine has been proposed. The definitive phase III trial comparing the outcome of patients with metastatic breast cancer receiving vaccinations with Theratope® *versus* vaccination with the nonspecific immune stimulants keyhole limpet hemocyanin (KLH) and Detox-B stable emulsion (Detox-B) (now called Enhanzyn Immunostimulant) was closed to enrollment on March 30, 2001 (90). Over 1,000 women with metastatic breast cancer were enrolled in the program, 34% of whom were on concurrent hormone therapy. Although no differences in time to disease progression (TTP) or overall survival (OS) emerged in an intent-to-treat analysis, an exploratory analysis revealed a trend toward improved TTP and OS in those participants on hormone therapy. Median OS was greater in the subgroup of patients receiving hormone therapy who developed higher than median IgG titers specific for naturally clustered STn antigens (asialo-ovine submaxillary mucin [OSM]), with a median OS of 41.1 months *versus* 25.4 months. Follow-up continues.

Another group of trials tested TG1031 (VV-MUC1-IL-2), an attenuated recombinant vaccinia virus containing sequences coding for human MUC-1 and the immunostimulatory cytokine IL-2. Scholl *et al*. performed a phase I study in patients with metastatic breast cancer using TG1031 (91). A significant T-cell proliferative response against MUC-1 following vaccination was observed in 1 of 9 advanced metastatic breast cancer patients. In addition, evidence of MUC-1-specific cytotoxic T-lymphocyte (CTL) activity induced by VV-MUC1-IL-2 was seen. To confirm these results, an open-label, randomized study comparing two dose levels, 5×10^6 and 5×10^7 pfu, was carried out in 31 metastatic breast cancer patients (92). Two of 31 (6%) patients achieved a partial response, 1 in each treatment group. No complete responses were obtained and stable disease was the best overall response observed in 15 (48%) patients (7 in the 5×10^6 pfu group and 8 in the 5×10^7 pfu group). The TG1031 vaccine, based on a replication-competent vaccinia virus, suffered some regulatory issues. Patients were required to be isolated in a specialized hospital facility for 1 week, until 2 consecutive PCR evaluations of blood, sputum, urine and feces showed no evidence of viral dissemination. A safer and potentially more effective new vaccinia virus, TG4010, was therefore developed. The recombinant vector contained in TG4010 is based on MVA, a nonpropagative, highly attenuated vaccinia virus. Although retaining immunogenicity, MVA lost its ability to replicate in most mammalian cells. In patients with advanced breast cancer, TG4010 was tested as a single agent at two doses, but no objective clinical responses were obtained (93).

Conclusions

Significant advances in understanding the molecular biology of cancer have opened up a new field of study in which potential therapeutic gains may accrue from the introduction of genetic sequences into tumors or normal tissue. This approach has yielded dramatic therapeutic responses *in vitro* and in some animal models, but, as yet, has not had a major impact on human cancer treat-

ment. The generation of improved delivery systems and refinement of the mechanism of controlling gene expression from them will be a key step in the clinical development of gene therapy. In addition, integration of these new approaches into current therapeutic regimens using surgery, radiotherapy and chemotherapy is likely to yield the most immediate evidence of therapeutic efficacy. It is likely that future trials of gene therapy will continue to enroll significant numbers of patients with breast cancer.

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