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Gene therapy for carcinoma of the breast

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Abstract

In view of the limited success of available treatment modalities for breast cancer, alternative and complementary strategies need to be developed. The delineation of the molecular basis of breast cancer provides the possibility of specific intervention by gene therapy through the introduction of genetic material for therapeutic purposes. In this regard, several gene therapy approaches for carcinoma of the breast have been developed. These approaches can be divided into six broad categories: (1) mutation compensation, (2) molecular chemotherapy, (3) proapoptotic gene therapy, (4) antiangiogenic gene therapy, (5) genetic immunopotentiation, and (6) genetic modulation of resistance/sensitivity. Clinical trials for breast cancer have been initiated to evaluate safety, toxicity, and efficacy. Combined modality therapy with gene therapy and chemotherapy or radiation therapy has shown promising results. It is expected that as new therapeutic targets and approaches are identified and advances in vector design are realized, gene therapy will play an increasing role in clinical breast cancer treatment.

Keywords

breast cancer; gene therapy; immunotherapy; carcinogenesis; suicide gene therapy

Introduction

Breast cancer is the most common female malignancy in the United States. Breast cancer affects one in nine women in the United States. Yearly, 46 000 women die from breast cancer, despite early detection methods and advanced conventional treatments.[1]

The elucidation and the molecular mechanisms underlying neoplastic transformation and progression have resulted in the understanding that breast cancer can be regarded as a genetic disease, deriving from the accumulation of a series of acquired genetic lesions. As the limits of existing treatment regimes for breast cancer are recognized, it is evident that novel therapeutic modalities may be beneficial for the successful treatment of carcinoma of the breast.

In this regard, a number of gene therapy approaches for carcinoma of the breast have been developed. These approaches can be divided into six broad categories: (1) mutation compensation, (2) molecular chemotherapy, (3) proapoptotic gene therapy, (4) antiangiogenic gene therapy, (5) genetic immunopotentiation, and (6) genetic modulation of resistance/sensitivity. Early-phase clinical trials have validated the safety and feasibility of these various gene therapy strategies in the context of breast cancer. As of early 2003, *The Journal of Gene Medicine* has tracked completed, ongoing, and pending clinical trials involving gene therapy

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for the treatment of cancer such as breast carcinoma (http://www.wiley.co.uk/genetherapy/clinical/). In the following, we want to highlight these different gene therapy strategies for carcinoma of the breast.

Genetic basis of breast carcinoma

Carcinogenesis is a multistep process characterized by genetic alterations that influence key cellular pathways involved in growth and development. Two major forms of genetic alteration in carcinoma of the breast have been suggested: chromosomal alterations/loss of tumor suppressor function and amplification of oncogenes.

The *loss of heterozygosity* (LOH) is the most common type of somatic alteration found in human breast tumors.[2,3] LOH refers to the loss of one allele in a patient's tumor for which the normal cellular DNA marker is heterozygous. This loss is significant when it occurs at the locus of a tumor suppressor gene (TSG), and is accompanied by an inactivating mutation in the second allele (the 'two-hit' hypothesis formulated by Alfred Knudson in reference to retinoblastoma).[4,5] LOH in the carcinoma of breast has been detected on several chromosomes such as 1, 3, 4–11, 13, 16–18, 22 and X.[6-9] The LOH may correspondent to losses or inactivation of TSG. TSGs refer to those genes whose loss of function results in the promotion of malignancy.[9] They are usually negative regulators of growth or other functions that may affect invasive and metastatic potential, such as adhesion and regulation of protease activity. In some cases, there may not be a mutation of the TSG, but rather some other mechanism that interferes with its expression or function. This may include methylation of the gene promoter that suppresses its transcription, an increased rate of proteasomal degradation, or abnormalities in other proteins that interact with the gene product.

Although discussion of cancer genetics with familial presentations is beyond the focus of this review, the rapid progression in characterization of breast cancer genes in the past decade has been monumental and allowed the identification in 1990 of the *BRCA-1* gene on the long arm of chromosome 17, q21.[10,11] In 1995, the *BRCA-2* gene was isolated on chromosome 13, q12–13.[12] These mutations in BRCA1 and BRCA2 (breast cancer 1 and 2) are responsible for approximately 80–90% of all hereditary breast cancers, whereas they are not very frequent in sporadic breast cancers.[13] The development of BRCA1[10,11] or BRCA2[12,14] tumors has been attributed mainly to the loss of DNA repair function ensured by these proteins. Indeed both proteins are involved in DNA double-strand breaks repair.[15] However, BRCA1 expression is reduced in most sporadic cancers, suggesting other mechanisms that control BRCA1 expression and inactivation, such as promoter methylation or protein ubiquitination.[16] The knowledge of human TSGs is expanding rapidly. A detailed explanation of the diverse roles TSGs play in normal cells cannot be given in this review; however, briefly the role of p53 and the retinoblastoma gene (*Rb*) will be explained.

The *Rb* was the first TSG to be discovered.[17] In breast cancer, mutation or loss of Rb has been observed in up to 30% of cases, and it has been associated with a greater progression.[18] The TSG *p53* was the first TSG linked to hereditary breast cancer. Beginning with the detection of a mutated form in lung and colon cancers some 15 years ago, *p53* has become, perhaps, the most studied TSG.[19] p53 is a nuclear phosphoprotein important in cell cycle regulation, repairing DNA damage, apoptosis to eliminate and inhibit the proliferation of abnormal cells, and inhibition of angiogenesis. It is one of the most commonly mutated genes in all human cancers.[20] Mutations of p53 are estimated to occur in over 50% of all human cancers and in up to 20–30% of sporadic breast cancer.[21] Independent of estrogen receptor (ER) status, mutation of *p53* increases the relative risk of relapse by 33%; the gene has been ranked as a category II prognostic marker in breast carcinoma. Thus, p53 and Rb play a central role in

mammalian cell proliferation. They are both regulated at protein level by oncogenes and other TSGs.

Although no consistent pattern of oncogenic mutation has yet emerged in breast cancer, the second most common type of cytogenetic alteration is thought to be gene amplification, which involves the formation of extrachromosomal, self-replicating units. Thus, more of the protein encoded by the gene is present; hence, its function is enhanced.[9] To date, the most certain and commonly amplified and functional genes for breast cancer tumorigenesis (dominant oncogenes) include the growth factor receptor c-erbB-2/HER-2 (neu) and the nuclear transcription factor c-myc.[22] HER-2(neu) is located on chromosome 17q and encodes a transmembrane tyrosine kinase (TK) receptor protein. HER-2(neu) is a key component for regulating cell growth. Amplified HER-2(neu) was originally encountered in the breast cancer cell line MAC117.[23] HER-2(neu) amplification occurs in 15–30% of breast cancers.[24] An extensive DNA sequence survey did not reveal mutations in the HER-2(neu) coding region, which is consistent with the concept that increased dosage of the wild-type gene has a role in tumorigenesis.[23] The *c-myc* oncogene has been localized to chromosome 8q24 and encodes a nuclearphosphoprotein that acts a transcriptional regulator involved in cellular proliferation, differentiation, and apoptosis. It is amplified and overexpressed in 15–25% of breast cancer tumors.[25] Further, c-myc has been associated with a worse prognosis or more aggressive clinical features.[26]

Mutation compensation

TSG replacement strategy for breast cancer

A century ago, the German biologist Theodor Boveri suggested that ‘cells of tumors with unlimited growth would arise if inhibiting chromosomes were eliminated’.[27] The multiple genetic alterations found in ‘inhibiting chromosomes’ are now known as TSGs. In this regard, cancer is thought to arise due to the functional defect or absence of one or more TSGs. Hence, TSGs can induce apoptosis or suppress tumor cell growth; most often gatekeeper genes have been the most attractive targets for developing gene replacement strategies so far. Several TSGs have already been shown to induce apoptosis or cause cell cycle arrest when introduced to breast cancer cells, including Rb,[28,29] p16,[30,31] p27,[32] p21,[33] p53,[29] mda7,[34,35] BRCA-1,[36] BRCA-2,[37] Maspin,[38] and Testin.[39]

Most clinical trials to date involve replacement of the TSG most commonly altered in cancer, p53. The introduction of a viral ‘wild-type’ p53 in human breast cancer cells has been shown to be sufficient to restore the normal balance of cell proliferation and cell death of the breast cancer cell. In addition, p53 insertion has been shown to be associated with a *bystander effect*, which means that not only p53-transduced cells are killed, but also that surrounding nontransduced cells are killed by the transduction of the neighbor cells.[40,41] Several proposed mechanisms include antiangiogenesis,[42] secretion of soluble proapoptotic proteins,[43] and immune upregulation.[44,45] A bystander effect is highly desirable for a therapeutic gene, because it reduces the level of transduction efficiency required for successful gene therapy.[46] Transfer of p53 into lung cancer by an adenoviral vector was initially demonstrated by Zhang et al.[47] In this regard, an *in vivo* study of p53 gene transfer in mouse xenograft breast cancer models displayed significant suppression of tumor growth.[48]

Another TSG for replacement strategy for breast cancer is *Rb*.[17] Restoration of Rb expression in cells reduced the tumorigenicity in a breast cancer model in nude mice.[29] In addition, enhanced breast cancer suppression could be demonstrated via replication-deficient adenovirus vectors expressing an N-terminal truncated retinoblastoma protein.[41]

Registered clinical trials of TSG gene replacement therapy for breast cancer include p53, Rb, and mda7.[49]

Ablation of oncogene function – antisense technology

This strategy involves the use of antisense molecules that can specifically inhibit the expression of pathogenic genes. Antisense oligodeoxynucleotides are short ssDNA molecules that modify gene expression by blocking the transfer of genetic information into protein.[50] The phosphorothioate oligomer (P = S antisense oligodeoxynucleotide), the gold standard in antisense technology, contains a sulfur instead of an oxygen atom in the phosphodiester backbone. This modification increases the stability of the oligomer and resistance to nucleases. Once inside the cell, antisense oligodeoxynucleotides inhibit mRNA processing through several possible mechanisms, including inhibition of transcription or splicing, RNase-mediated mRNA cleavage, or translation arrest.

With regard to antisense inhibition of oncogene function, early studies demonstrated inhibition of lymphoma growth by administration of naked antisense DNA to c-myc.[51] In addition to c-myc,[52] other genes relevant to breast cancer have been successfully targeted by antisense oligonucleotides such as αV integrin,[53] ribosomal protein P2,[54] c-erbB-2,[55] methylenetetrahydrofolate reductase (MTHFR),[56] p21,[57] protein kinase C-alpha (PKC-α),[58,59] Bcl-2,[60] telomerase reverse transcriptase and telomerase,[61] plasma membrane calcium ATPase,[62] type I insulin-like growth factor receptor (IGF-IR),[63] and c-fos.[52] Compared with preclinical studies, far fewer clinical studies of oligonucleotides have been reported. Currently in clinical development is LY900003 (Affinitak, ISIS-3521; Eli Lilly and Company, Indianapolis, IN) presenting an antisense oligonucleotide to specifically block PKC-α. Although its single-agent activity in breast cancer is modest, its potential role may be in concert with traditional chemotherapy.[58]

Ablation of oncogene function – applications of RNAi and ribozymes

Another closely related area is ribonucleic (RNA) interference (RNAi) technology.[64,65] RNAi has been demonstrated to be a powerful intracellular mechanism for sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNAs homologous to the gene. In this regard, it has been recently shown that specific downregulation of c-myc by RNAi was sufficient to inhibit the growth of breast cancer MCF-7 cells *in vitro* and *in vivo*.[66]

In addition to application of RNAi, another approach to ablation of oncogenes in breast cancer are ribozymes. They are RNA molecules capable of acting as enzymes even in the complete absence of proteins.[67,68] They have the ability to catalyze the cleavage and formation of covalent bonds in RNA strands at specific sites. Initially ribozymes were used for the treatment of human immunodeficiency virus (HIV).[67,68] Among several ribozymes, the hammerhead ribozyme is the simplest and best characterized. The hammerhead motif, approximately 30-nucleotide long, is the smallest endonucleolytic *cis*-acting ribozyme structure found in natural circular RNAs of some plant viroids. Hammerhead ribozymes became appealing when it was shown that it is possible to produce *trans*-acting ribozymes directed against RNA sequences of interest. Since then, gene-tailored ribozymes have been designed, produced and given to cells to 'knock down' the expression of specific genes.[69] In this regard, adenovirus-mediated ribozyme targeting of HER-2/neu inhibited *in vivo* growth of breast cancer cells in a mouse model.[70] Further, ribozyme-mediated cleavage of the human survivin mRNA and inhibition of antiapoptotic function of survivin in MCF-7 cells could be demonstrated.[71]

Recently, it has been shown that ribozymes may affect not only mRNAs in cancer cells but also those in normal cells since those genes are necessary for a growth factor-dependent signal transduction and a cell cycle in normal cells. To overcome this problem, it has been endeavored

to construct an allosteric trans-maxizyme, a dimer of minimized ribozymes (minizymes), to target two distinct oncogenes, cyclin D1 and hst-1, being overexpressed in breast cancer cells.[72]

Ablation of oncogene function – alternate strategies

In addition to these genetic ablation strategies, another strategy aims at disrupting normal cellular localization of growth receptors. Of particular interest is the proto-oncogene *erbB-2*, which has been extensively studied in breast cancer. In this regard, the delivery of a gene encoding an anti-erbB-2 intracellular single-chain antibody (sFv) resulted in downregulation of cell surface erbB-2 levels and induction of apoptosis in erbB-2-overexpressing breast cancer cells.[73,74]

E1A has also been found to inhibit HER-2(neu) expression in both rodent and human breast cancer cells through transcriptional repression of the HER-2(neu) promoter.[75] The adenovirus type 5 E1A gene encodes a phosphonuclear protein (transcriptional factor) that is the first viral gene product expressed in host cells after adenoviral infection. This factor in turn activates viral gene transcription and reprograms the host's cellular gene expression to allow efficient propagation of adenovirus in the host cells.[76] It could be demonstrated that the *E1A* gene delivered via a cationic liposome suppressed tumor growth and prolonged the disease-free survival of tumor-bearing mice in an orthotopic model of breast cancer.[77] On the basis of these results, a phase I trial of *E1A* gene therapy was initiated, in which the adenovirus type 5 *E1A* complexed with dendritic cell (DC)-Chol cationic liposome (DCC-*E1A*) was injected into the thoracic or peritoneal cavity of patients with breast or ovarian cancer.[78]

Molecular chemotherapy/suicide gene therapy

The possibility of rendering cancer cells more sensitive to chemotherapeutics or toxins by introducing so-called suicide genes was suggested in the late 1980s.[79] Suicide gene therapy can be divided into two categories: toxin gene therapy and enzyme-activating prodrug therapy. Toxin gene therapy incorporates the transfection of genes that express toxic molecules; enzyme-activating prodrug therapy describes the transfection of genes able to express enzymes that can selectively activate specific prodrugs. The latter has been variously called virally directed enzyme prodrug therapy (VDEPT), gene-directed enzyme prodrug therapy (GDEPT), gene prodrug activation therapy (GPAT), or suicide gene therapy.[79]

GDEPT is a two-step treatment for cancer. In the first step the gene for the enzyme is delivered to the breast cancer cell. In the second step a nontoxic prodrug is administered, which is activated into a toxic metabolite by the foreign enzyme expressed in the tumor. The enzymes proposed for GDEPT for breast cancer can be divided into two categories. The first category consists of ‘foreign’ enzymes of nonmammalian origin, such as viral TK,[80,81] bacterial cytosine deaminase (CD), and carboxypeptidase G2 (CPG2).[79,82,83] The second category comprises enzymes of human origin, such as cytochrome *P*450 isophorms.[79,84] Perhaps the two best examples of this strategy are thymidine kinase (TK) and CD, which convert ganciclovir (GCV) and 5-fluorocytosine, respectively, into their toxic drug forms.

In theory, GDEPT-mediated toxicity would be limited to cells that have been transduced. However, this may not prove to be as severe a limitation as initially believed, due to a phenomenon called *bystander effect* (see also TSG replacement strategy). The *bystander effect* in GDEPT can be defined as the cytotoxic effect on nontransduced cancer cells following prodrug administration, when only a fraction of the tumor mass is genetically modified to express an activating enzyme.[79,83] Experimental data from murine and human tumor models suggest different, but not mutually exclusive, mechanisms that explain the bystander effect, including transfer of phosphorylated gancliclovir between tumor cells via gap junctions,

transduction of endothelial cells within the tumor, and induction of antitumoral immunity.[85-89]

Combination gene therapy with suicide genes and cytokines has been proven to be useful in a variety of experimental tumor models.[90,91] Breast cancer cells grown as xenografts in BALB/c mice were injected with an adenoviral vector carrying the granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2) or HSV-1 thymidine kinase (HSV-TK). When both cytokine genes were combined with HSV-TK, a substantial reduction in tumor growth was observed in comparison with HSV-TK alone.[85]

Tumor specificity of molecular chemotherapy can be further enhanced using protein translational control.[92] Previous studies have shown increased expression of the translation initiation factor/proto-oncogene *eIF4E* to occur in breast cancer.[93] In this regard, the suicide gene, *HSV-Tk*, was modified with a complex 5′ upstream-untranslated region (5′-UTR) that limits efficient translation to cells that express high levels of the translation initiation factor eIF-4E, relative to normal cells.[92] These results indicate that translational targeting of suicide gene expression in breast cancer cells *in vitro* is effective and may provide a platform for enhanced cancer gene therapy specificity *in vivo*.

Several reports indicate that a double transfer of suicide genes is able to enhance the efficacy of GDEPT system. Transfection of cancer cells with two different suicide genes allows the activation of two distinct types of prodrugs. In this regard, results reveal that, for murine mammary tumors, the antitumoral effect mediated by two different suicide gene sytems, CD and cytochrome *P*450 2B1, is more efficient to each single system alone.[84] This approach is based on the rationale that the permeable toxic metabolites resulting from the CD/5-FC system enhance the overall bystander effect, and therefore a synergistic antitumoral effect can be achieved.[79,94]

To date, there are two clinical trials using GDEPT as a treatment for breast cancer.[95,96] The first clinical trial in genetic prodrug activation therapy for breast cancer was 1999. This phase I clinical trial of erbB-2-directed suicide gene expression was designed to test the safety and efficacy of a tumor-specific genetic prodrug activation therapy targeted by use of the human erbB-2 gene promoter.[96] The results of this study, the first targeted gene therapy for breast cancer and the first to use the CD system in human subjects, were encouraging for the development of genetic prodrug activation therapies that exploit the transcriptional profile of cancer cells. The approach was shown to be safe and resulted in targeted expression of the CD gene in 90% of cases. However, no significant tumor regression was reported.[96] The second clinical trial involving GDEPT was developed as the first trial of direct intratumoral injection of MetXia-*P*450 in patients with cutaneous tumor deposits from advanced breast cancer or melanoma.[96] MetXia-*P*450 is a novel recombinant retroviral vector that encodes the human cytochrome *P*450 type 2B6 gene (CYP2B6). 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 all, 12 patients with breast cancer ($n = 9$) and melanoma ($n = 3$) received three dose levels of MetXia-*P*450. One (8%) patient with breast cancer had a partial response and received 7 months of oral cyclophosphamide. Four (33%) patients had stable disease for > 3 months and the rest had progressive disease. Preliminary immunologic analyses were suggestive of an antitumor response in two patients (partial response in one patient and stable disease in one patient). Gene transfer was detected at all dose levels, and the initial suggestion of an antitumor response indicates that MetXia-*P*450 should undergo further clinical assessment.[96]

Proapoptotic gene therapy for breast cancer

Impaired apoptosis signaling is common in cancer cells, including breast cancer, and may play an important role in tumor initiation and progression.[97-100] Resistance of cancer cells to apoptosis is especially deleterious because it not only enhances the spontaneous growth of tumors but also renders them resistant to host defense mechanisms as well as various forms of therapy.[100]

While not classical TSGs, the identification of genes involved in the induction of apoptosis offers an additional strategy for breast cancer gene therapy. In this complementary approach, genes which do not only cause apoptotic cell death but also transmit additional death signals to adjacent tumor cells are delivered, allowing the so-called *bystander effect* (see above Suicide gene therapy and tumor suppressor replacement therapy). Proapoptotic strategies in breast cancer include suicide gene therapy, functional replacement of TSGs, BCL-2 family proteins, death receptor and ligand systems and pathways.

BCL-2 family

The best-studied mediators of apoptosis form a growing family of cysteine proteases, caspases, which function as ultimate effectors in the 'classical' apoptotic process.[101] *BCL-2* was the first oncogene recognized to function by inhibiting apoptosis.[102] The genes of the BCL-2 family have emerged as key regulators of apoptosis. The overexpression of antiapoptotic BCL-2 family members such as BCL-2 and Bcl-XL proteins appear to play an important role in breast cancer.[103] BCL-XS is a dominant-negative repressor of Bcl-2 and Bcl-xL, both of which inhibit apoptosis. Bcl-xs gene therapy has been shown to induce apoptosis of human mammary tumors in nude mice.[104] The proapoptotic Bik has also been linked to the development of breast and colorectal cancers when downregulated. The proapoptotic Bik induced significant apoptosis in four breast cancer cell lines *in vitro* as well as in orthotopic tumor tissues in nude mice.[105] *In vitro* cytotoxicity of breast cancer cells through tumor-selective expression of the BAX gene could also be demonstrated.[106]

Death receptor and ligand systems and pathways

There are a number of death receptor/ligand systems that can function to regulate apoptosis in breast cancer cells. These include tumor necrosis factor-α, tumor necrosis factor-related apoptosis-inducing ligands (TRAIL), and Fas ligand (FasL). It could be demonstrated that the introduction of the human *TRAIL* gene into breast cancer cells and subcutaneous human breast cancer xenografts in nude mice using an adenoviral vector leads to the rapid production and expression of TRAIL protein, and subsequent death of the breast tumor cells.[107,108] However, it has been reported that some cancer cell lines, such as the breast cancer cell line MCF-7, display strong resistance to adenovirus delivery of TRAIL.[109] Conventional flow cytometry analysis demonstrated that TRAIL-resistant MCF7 cells exhibit substantial levels of TRAIL decoy receptor-4 expression on the surface.[109]

One other major apoptotic pathway is mediated by the Fas receptor, which recruits FADD[110] through the death domain by crosslinking with FasL[111] or anti-Fas antibody.[112] FADD is then recruited to the death-inducing complex (DISC) along with pro-caspase-8, which activates downstream caspases and leads to apoptosis.[113,114]

Among the caspases, in many tumors caspase-3 is a key molecule in the execution of Fas-mediated apoptosis.[114] Interestingly, overexpression of Fas concomitant with FasL transduction induced apoptosis in MCF-7 cells despite the absence of caspase-3, indicating that in MCF-7 cells Fas-mediated apoptosis occurs through a cascade that does not appear to be mediated by caspase-3 activation.[114]

Heat shock proteins

Intensive apoptosis research during the last decade has resulted in the identification of several other proteins, which may similarly promote tumorigenesis by suppressing apoptosis.[98,100] Indirect experimental evidence suggests that the major stress-inducible Hsp70 (also known as Hsp72 or Hsp70i) may be such a cancer-relevant antiapoptotic protein.[98] Heat shock protein 70 is an antiapoptotic chaperone protein highly expressed in human breast tumors and tumor cell lines. In this regard, it could be demonstrated that the mere inhibition of its synthesis by adenoviral transfer or classical transfection of antisense Hsp70 cDNA (asHsp70) results in massive death of human breast cancer cells, whereas the survival of nontumorigenic breast epithelial cells or fibroblasts (WI-38) is not affected. Despite the apoptotic morphology as judged by electron microscopy, the asHsp70-induced death was independent of known caspases and the p53 tumor suppressor protein. Furthermore, Bcl-2 and Bcl-X_L, which protect tumor cells from most forms of apoptosis, failed to rescue breast cancer cells from asHsp70-induced death. These results show that tumorigenic breast cancer cells depend on the constitutive high expression of Hsp70 to suppress a transformation-associated death program. Neutralization of Hsp70 may open new possibilities for treatment of cancers that have acquired resistance to therapies activating the classical apoptosis pathway.[100]

In addition, the adenovirus type 5 E1A protein has been demonstrated to elicit antitumor effects through the induction of apoptosis, inhibition of cell cycle progression, induction of differentiated epithelial phenotypes, repression of oncogene expression and function, and sensitization to chemotherapeutic agents and radiation. These unique properties have led to use of the *E1A* gene in adenoviral and lipid-based gene therapy systems, and it has demonstrated antitumor effects in breast cancer xenograft model systems.[115-117] Further, adenovirus-directed expression of dominant-negative ER receptor induces apoptosis in breast cancer cells and regression of tumors in nude mice.[118]

Antiangiogenic gene therapy

It is now well established that tumor growth and spread are angiogenesis-dependent processes. Therefore, inhibition of angiogenesis is likely to be an effective anticancer approach for breast carcinoma.[119] Recent knowledge in tumor angiogenesis may have clinical implications in diagnosis and treatment. Quantification of microvessel density in tumor specimen correlates either metastasis or recurrence in many malignancies such as breast cancer and lung cancer.[120] Tumors *in situ*, having a size smaller than 3 mm in diameter, exist in a prevascular state and are limited in their ability to grow without perfusion from the blood supply.[121]

In the context of breast cancer, liposomes complexed to plasmids encoding angiostatin and endostatin demonstrated to inhibit breast cancer in nude mice.[122,123] However, in spite of promising early results, it may be unlikely that antiangiogenic therapy could control disseminated breast cancer in humans alone. Therefore, it has been suggested that better results could be achieved by combining antiangiogenic therapy with other strategies, both conventional and gene transfer-based. Both the hormonal and chemotherapic approaches could, in theory, complement antiangiogenic therapy, as their antitumoral action is mediated by different mechanisms. In a recent study, a transgenic mouse model of breast cancer was used to show that the association of angiostatin with tamoxifen could give better results than either approach used alone.[124] Further, the potential efficacy of intramuscular delivery of the endostatin gene for treatment of metastatic breast cancer to the brain could be demonstrated.[123]

Angiogenesis is a field that has only been established in the last 30 years. The systemic administration of recombinant proteins that inhibit angiogenesis has demonstrated regression of tumors in mouse models.[125,126] The inhibitors angiostatin and endostatin have been shown

to be safe. However, studies have revealed that repeated administration of recombinant proteins is of necessity to achieve a therapeutic level. Under this consideration and the difficulty to manufacture and purify recombinant proteins in large quantities for clinical use, it has become evident that gene therapy offers a rational approach as the patients could produce the inhibitors directly in their own cells.

Genetic immunotherapy

It was Coley[127] at the beginning of the last century who revealed that the power of the immune response could be harnessed and applied to the specific elimination of cancerous cells. If breast cancer cells are susceptible to control by the immune system, then the success of breast cancer may depend on novel cancer immunotherapy strategies that take the tumor 'escape' mechanism into account.

Immunotherapy can generally be divided into two functional approaches: *passive immunotherapy* and *active immunotherapy*. The former approach describes the administration of pre-formed elements of the immune system, such as tumor antibodies, antitumor cytokines, or tumoricidal effector cells to patients, with the intention to directly kill the cancer cell; the latter approach endeavors to stimulate the patient's immune response to generate an antitumor immunity by using tumor vaccines and immunostimulatory cytokines.

Gene therapy has the potential to deliver immunotherapeutic modalities in both a more effective and a less toxic way. The administration of genes encoding therapeutic proteins can allow for more 'natural' sustained protein levels *in vivo*, reducing problems with cytokines that are toxic at high concentrations but exhibit short circulating half-lives. These are some reasons for the enthusiastic development of genetic immunotherapy strategies.

Genetic immunotherapy by nonspecific immune stimulation

This strategy endeavors to provide immune factors that directly affect tumor cell survival or elicit host immunity. The advantage of this strategy is that it does not require the identification of tumor-associated antigens.

Immune-stimulatory cytokines and chemokines

The danger theory suggests that alarm signals induced by pathogen injury promote a potent immune response.[128] In this regard, transfer of genes encoding immunostimulatory molecules directly into breast cancer cells may be an effective means of activating the numbers of immune effector cells. Initial studies focused on systemic injection of cytokines such as IL-2.[129] This treatment resulted in some serious dose-limiting toxicities.[129] To overcome this problem, direct *ex vivo* transduction of tumor cells with cytokine-encoding genes has been explored in mouse models of mammary carcinoma. After irradiation, transduced cells may be used as a vaccine, providing a scenario in which tumor antigens are available in an environment of locally high concentrations of the immunostimulatory molecules.[130-132]

Bubenik *et al*.[133] proposed the idea of delivering biologically active cytokine at a tumor site without manipulating with tumor cells *ex vivo*. For this purpose, the use of autologous or allogenic genetically modified normal cells for local secretion of immunostimulatory molecules was suggested. In this regard, intratumoral injection of IL-2-secreting syngeneic/allogeneic fibroblasts transfected with DNA from breast cancer cells prolonged the survival of mice with intracerebral breast cancer metastasis.[134] The rationale for this type of vaccine is that genes specifying an array of weakly immunogeneic, unique tumor antigens associated with the malignant cells will be expressed in a highly immunogenic form by the transfected cells.[134] Of note, the immunotherapeutic properties of transfected fibroblasts modified to secrete IL-18 or GM-CSF were less efficacious.[134] The antitumor response was mediated

predominantly by T-cell subsets ($CD8^+$ and natural killer (NK)/LAK cells).[134,135] In addition to IL-2, a variety of other cytokines have been explored, such as the GM-CSF, interferon-alpha, IL-4, ILl-12, IL-18, and IL-23.[136-139]

Immune costimulatory receptors/ligands

There is considerable evidence that breast cancer cells may escape immune control by the downregulation of expression of major histocompatibility (MHC) molecules,[140,141] costimulatory molecules, and other ligands. Ordinarily, MHC class II presentation stimulates $CD4^+$ activation, resulting in the secretion of cytokines, which along with MHC class I presentation recruit $CD8^+$ cytotoxic T cells and result in cell death.[142] Thus, gene therapy is an approach to introduce such deficient receptors and ligands into breast cancer cells to enhance host immune system recognition and antitumor activity.[143]

Active specific immune stimulation (genetic vaccines)

Since the 1980s, antigens associated with cancer cells have been identified and cloned.[144-146] These cancer-associated antigens have been applied to the immunotherapy of cancer by vaccination. Genetic vaccines have the advantage over these types of vaccines in that the antigens themselves have not to be prepared, purified, and formulated. Hence, only genes are required. Most tumor antigens are products of nonmutated host genes (self-antigens), and their utility as immunotherapy targets resides primarily in the fact that they are aberrantly expressed by tumor cells. In this regard, aberrant expression of Her-2/neu/erbB-2 overexpression has been shown to provide a growth advantage for breast cancer cells.[147] Other potential targets currently under investigation for vaccination in breast carcinoma include the carcinoembryonic antigen (CEA),[148] MAGE-1,[149] MUC-1,[150] hTERT,[151] Fos-related antigen 1 (Fra-1),[152,153] tumor cell-associated extracellular matrix metalloproteinase inducer (EMMPRIN)[154] and B7-H4.[155,156]

Recently, combination vaccine strategies have been investigated. These strategies endeavor to enhance the effectiveness of genetic vaccination in breast cancer by utilizing various cytokines and costimulatory molecules as molecular adjuvants.[157] Thus, these approaches are a combination of ‘conventional’ and ‘genetic’ immunotherapeutic strategies. In this regard, the GM-CSF has been investigated as an vaccine adjuvant for breast cancer because it has been reported to enhance antigen processing and presentation by dendritic cells.[158] In another approach, it could be demonstrated that priming mice with plasmids encoding Her-2/neu and boosting with cytokines coexpressed in the same or in other plasmids enhances efficacy against breast cancer.[157]

Gene-modified DC vaccines

DCs are powerful antigen-presenting cells that play a central role in generating and directing immune responses through the processing of antigens and presentation of epitopes in the context of surface MHC molecules to interact with T cells.[159] In addition, DCs express an array of costimulatory molecules and cytokines that are required to sustain and direct the immune response.[160,161] Major efforts at developing antitumor vaccines have focused on harnessing DCs to effectively present tumor antigens to the immune system. A number of clinical trials have examined the efficacy of epitope-modified DCs, most often by ‘pulsing’ DCs *ex vivo* with synthetic peptides based on antigens,[159] mutant oncogenes, or immunoglobulin idiotypes expressed by tumors.[159,162-165] There is a wide range of studies, which involves DCs targeting breast cancer antigens. We will focus only on those that involve gene transfer approaches, whereby the idea is to directly modify potent antigen-presenting cells (especially DCs) by genetic modification *in vitro* and then to administer modified APCs or DCs as a vaccine. For modification, genes encoding cytokines, breast cancer antigens, and molecules involved in antigen presentation were used.

In this regard, vaccination with DCs that are modified by adenoviruses encoding nonfunctional tumor antigens, such as nonsignaling HER-2/*neu*, displayed a regression of breast cancer in BALB-*neu*T mice.[159] Dendritic cells transduced with a Tat fusion protein (from the HIV) containing the extracellular domain of Her2/neu induced $CD8^+$ cytotoxic T lymphocytes that specifically lysed Her2/neu-expressing breast cancer cells.[159] In addition, Tat mammaglobin transduced dentritic could induce both $CD4^+$ and $CD8^+$ mammoglobin-specific T cells for breast cancer therapy.[166,167]

In vivo transfer of genes encoding antibodies to known tumor antigens

Recombinant antibodies and their fragments currently represent over 30% of all biological proteins undergoing clinical trials for diagnosis and therapy.[168] Genetically engineered antibody molecules composed of a constant part of human antibody molecule and variable domain of monoclonal antibody against the antigen of interest have generally a long half-life in the human body due to the absence of neutralizing immune response and at the same time maintaining all biological functions of natural antibodies.[169] In this context, the best example is Herceptin. Direct binding of this humanized monoclonal antibody to Her-2-positive tumor cells leads to significant tumor growth inhibition. Second, tumor rejection strategy is based on the development of antibody-dependent cellular toxicity. In this case, binding of NK cells to the Fc surface receptor with the opsonized breast cancer cells leads to activation of killing mechanisms directly.[169]

Rationale for combination therapy

Many breast cancer patients fail conventional therapy because their tumors are resistant to DNA-damaging agents such as chemotherapy and radiation therapy. For breast cancer multidrug resistance, different underlying mechanisms have been discussed, such as the cellular overproduction of P-glycoprotein (ABCB1, MDR1), multidrug resistance-associated protein 1 (MRP1, ABCC1), breast cancer resistance protein (BCRP, ABCG2, MXR, ABCP), amplification of oncogenes, deletion or amplification of topoisomerase II and modulations in the apoptotic function.[170,171] These mechanisms have been shown to cause drug resistance, although evidence linking the different mechanisms to drug resistance *in vivo* is limited.[170, 171] Overexpression of antiapoptosis genes and downregulation of apoptosis contribute to pathogenesis of chemoresistance, which has been regarded one of the main causes of failure in present-day chemotherapy and radiotherapy of breast cancer.

The rationale for TSG combination therapies lies in the observation that p53 is often nonfunctional in radiotherapy- and chemotherapy-resistant tumors. Based on the link between p53 and apoptosis and the low toxicity of p53 in initial trials, it may be suggested that a combination with other anticancer treatments may be beneficial. In this regard, tumor suppression and doxorubicin therapy sensitization of localized and metastatic breast cancer by adenovirus p53 could be demonstrated.[172] Currently, there is a phase II clinical trial of docetaxel and doxorubicin in combination with local administration of Ad5CMV-p53 (RPR/INGN-201) in locally advanced breast cancer (Sponsor: Introgen Therapeutics, Inc.) under investigation. P53 function is involved in the cellular response to DNA-damaging events following not only chemotherapy but also radiotherapy. After radiotherapy of tumor cells, p53 promotes cell cycle arrest, initiation, and transcription of DNA repair complexes, and the induction of apoptosis.[173,174] For example, DNA damage by radiotherapy promotes p53-dependent transcription of p21 and arrest of cells at the G1 checkpoint.[175] Loss of this checkpoint has been associated with decreased tumor cell apoptosis and clinical response to radiotherapy.[176] Successful preclinical studies suggesting that p53 gene replacement might confer radiation sensitivity to some tumors led to the initiation of a phase II clinical trial of adenoviral-mediated p53 gene transfer to localized non-small-cell lung cancer (NSCLC) in

conjunction with radiation therapy.[177-180] However, to date this combination has not been endeavored in breast cancer.

Many tumors reveal gene amplification of oncogenes, growth factors, or receptors. Of particular interest is the proto-oncogene *erbB-2*, which has been extensively studied in breast cancer. Current data show that overexpression of epidermal growth factor receptors is widely correlated with resistance to radiotherapy and to other forms of adjuvant therapy.[181] Adenovirus-mediated overexpression of dominant-negative epidermal growth factor receptor-CD533 as a gene therapeutic approach radiosensitized human breast cancer cells.[182-184]

Although most antisense oligonucleotides are tested *in vivo* as monotherapy, combination treatment of antisense oligonucleotides and conventional chemotherapy has also been investigated. Preclinical studies demonstrated that downregulation of specific gene products with antisense oligonucleotides sensitizes breast cancer cells to chemotherapeutic agents, resulting in an additive or synergistic anticancer effect. In preclinical trials, these antisense targets include mouse double minutes 2 (MDM2),[185] erbb2,[186] PKC-α,[187] and Bcl-2.[188] In addition, clinical trials have tested antisense oligonucleotides in combination with chemotherapeutic agents. In this regard, results of a phase I trial with ISIS 2503, an antisense inhibitor of H-ras, in combination with gemcitabine and a phase I trial of a BCL-2 antisense (G3139) and weekly docetaxel in patients with advanced breast cancer and other solid tumors, indicated that antisense therapy in combination with chemotherapeutic agents was well tolerated and clinically active in the group of heavily pretreated patients.[189,190]

Strategies using apoptotic genes like Bax may also be beneficial if combined with other agents. Gene-mediated Bax-α expression has been shown to increase cellular radiosensitivity in MCF-7 breast cancer cells and may potentially have a therapeutic application by enhancing radiation sensitivity in breast cancer cells.[179,191,192]

To overcome P-gp-mediated drug resistance, recently anti-MDR1 hammerhead ribozymes have been developed and delivered to breast cancer cell lines by a retroviral vector containing RNA polymerase III promoter, resulting in reversal of chemoresistance.[193]

Combination of radiotherapy and suicide gene therapy has also been proposed as an advantage. It is hypothesized that the radiation-induced cellular membrane damage may facilitate the transfer of cytotoxic nucleotide analogs from HSV-tk-expressing cells to neighboring nontransduced cells. This combined approach has been effective in various tumor models[194] and has been shown to delay local tumor growth and prolong survival in a mouse prostate cancer model[195,196] and a mouse mammary tumor model.[197] These results indicate that multiple courses of HSV-TK therapy, in combination with radiation, improve the therapeutic efficacy of this approach and may provide therapeutic implications for the treatment of human breast cancer and other solid tumors. It was also demonstrated that the combined therapy had a considerably better antimetastatic effect compared to HSV-tk gene therapy alone; this phenomenon was attributed to the induction of a potent local and systemic immune response, as evidenced by the abundance of CD4+ cells in the primary tumor.[196,197]

Another area likely to be investigated intensely in the coming years is the combination of immunotherapy with established conventional tumor therapies. In this regard, enhancement of antitumor immune response of granulocyte/macrophage-colony stimulating factor-secreting whole cell vaccines in Her-2/neu mice by administration of cyclophosphamide, doxorubicin, or paclitaxel could be demonstrated.

However, chemosensitization and radiosensitization of breast cancer by gene therapy is just beginning. Considering gene therapy for breast cancer, replacement of gene function of breast cancer tumors harboring a particular defect leading to restoration of drug sensitivity would be

the ultimate proof for a key biological function of that factor in the drug response. Chemo- and radiotherapy are an effective therapeutic modality for diverse human malignancies such as breast cancer. Gene therapy strategies have been demonstrated *in vitro* and in clinical trials to increase the tumoricidal effects of chemo- as well as radiotherapy in a variety of tumors

Conclusion

A variety of gene therapy approaches have been evaluated for treatment of breast carcinoma. The majority of clinical trials focus on the p53 TSG. The preferred way of administration is the intratumoral injection of an adenovirus p53 vector. Despite the observed higher transgene expression with the adenoviral vectors, clinical evidence of tumor regression occurred only in a small minority of patients.

With respect to suicide gene therapy, a variety of enzyme/prodrug systems have been investigated in pre-clinical studies for breast cancer. However, clinical trials for breast cancer gene therapy focusing on transducing tumors to express herpes-simplex virus thymidine kinase (HSV-tk) and systemically administering the prodrug (GCV) have demonstrated low efficacy.

With regard to immunotherapy for breast cancer, identification of novel tumor antigens as well as better delineation of the nature of antigens and antigenic determinants that elicit the most effective antitumor immune responses will particularly impact the development of genetic vaccines.

It is clear that gene therapy of breast cancer is a very difficult undertaking. The results of breast cancer gene therapy clinical trials to date have demonstrated little toxicity. However, the rate of clinical response has been low to date and efficient transgene expression remains to be challenge. In this regard, future research needs to focus not only on novel strategies and transgenes, but also on the development of novel gene transfer vectors to help overcome this inefficiency. The immediate future of breast cancer gene therapy would suggest increasing success in using cancer gene therapy as adjunct therapy in local control of many cancers. Thus, the envisaged future practice for breast cancer treatment may involve a multimodality approach, integrating curative or debulking resections, followed by adjuvante therapies including concurrent or sequential gene therapy, chemotherapy, and radiotherapy.

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