Review

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Gene Therapy in Human Breast Cancer

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Abstract: Gene therapy, being a novel treatment for many diseases, is readily applicable for the treatment of cancer patients. Breast cancer is the most common cancer among women. There are many clinical protocols for the treatment of breast cancer, and gene therapy is now being considered within current protocols. This review will focus on the basic concepts of cancer gene therapy strategies (suicide gene, tumor suppressor gene, anti-angiogenesis, immunotherapy, oncolytic viruses and ribozyme/antisense targeting) and current approaches for breast cancer gene therapies.

Key Words: gene therapy, breast cancer, viral vectors, vector targeting

Introduction

Breast cancer is the major cancer in females in industrialized countries. Currently breast cancer therapy consists of surgery, chemotherapy, radiotherapy and hormone therapy. All have side effects and limitations. Moreover, they do not guarantee a complete eradication of tumor cells from the body and a prolonged lifespan. A new therapeutic approach for cancer is gene therapy. Human gene therapy (1) has been shown to be effective not only with cancer, but also with many other types of diseases.

This review will focus on recent approaches to cancer gene therapy in general and breast cancer in particular.

Cancer Gene Therapy

Cancer gene therapy can be defined as the delivery of gene(s) to cancer cells in the body. These genes must then enter the cancer cell and have a direct or indirect therapeutic effect in the person. The delivery of therapeutic genes involves the use of carrier vehicles, called vectors, which can be specifically targeted to the cancer tissue or cells. For the optimum therapeutic effect certain requirements must be fulfilled by the vector and the gene(s) to be delivered: i) the vector should meet certain stringent conditions such as having high transfection efficiency, ii) the vector must specifically target the tumor cells including metastatic cells, iii) for optimal gene expression there should be controllable genes that can be combined with suitable promoter-enhancer sequences.

Vectors for Gene Delivery

Gene delivery methods can be classified as viral vectors (2,3) and nonviral vectors (4,5). Table 1 shows the distribution of vectors currently being used in clinical gene therapy clinical trials. Although the table lists the vectors involved in all gene therapy trials, either in progress or completed, in more than 60% of these trials the target disease was/is cancer [official web site of the Journal of Gene Medicine (6)].

As seen from Table 1, viral vectors (71.8%) are more frequently used than nonviral vectors (24.4%). Viral vectors have higher transfection efficiency compared to nonviral vectors. Transfection efficiency is one of the main concerns in cancer gene therapy since the overall aim is to transduce as many cancer cells as possible with the therapeutic gene(s) (see Table 2). Retroviruses are the most common viral vector currently used for therapy in all diseases, including cancer. The most common retroviruses are derived from the Moloney murine leukemia virus. Some present difficulties, (e.g. viral titers and efficiency of gene transfer) can be overcome by the

Table 1. Vectors used and their distribution in clinical gene therapy trials (6)*

VECTORS		PROTOCOLS	
Name		Number	%
Retrovirus	V	212	35.5
Adenovirus	V	164	27.4
Lipofection	NV	77	12.9
Naked / Plasmid DNA	NV	59	9.9
Pox virus	V	37	6.2
Adeno-associated virus	V	13	2.2
Gene gun	NV	5	0.8
RNA transfer	NV	5	0.8
Herpes simplex virus	V	3	0.5
N/C	-	23	3.8
Total	-	598	100

Abbreviations: V for viral vector and NV for nonviral vector.
* Table is rearranged and printed with permission from the Journal of Gene Medicine

development of pseudotyped retroviruses (7). Adenoviruses are the second most commonly used vectors. Although useful, they cause immunological and inflammatory reactions that may render repeated administrations impossible. The use of adenoviral vectors of different serotypes may help to solve this problem. Improvements are still being made on adenoviral vectors, as a third generation "gutless" adenoviral vector has been introduced that is devoid of all viral genes (8). However, it is dependent upon helper viruses for proper functioning. There are currently several efforts to combine the advantages of both retroviruses and adenoviruses via a chimeric virus (9). In addition, a less frequently used vector, the Adeno-associated virus (10), integrates at a locus on human chromosome 19, which does not code for any important gene (11). Herpes simplex virus (12) based vectors are also being employed in several approaches to gene therapy.

Although nonviral vectors are less frequently used they have some very critical advantages over viral vectors. Lipofection, liposome mediated gene transfer (13-16), is the third most common vector system in gene therapy clinical trials. Additionally, the transfer of naked DNA (17) by physical means is another method for gene transfer. Comparisons between viral vectors and nonviral vectors are shown in Table 3. While viral vectors have many disadvantages over nonviral vectors, the fact that viral vectors have a higher transfection efficiency makes them preferable.

Strategies in Cancer Gene Therapy

Strategies in cancer gene therapy can be classified into several approaches, as there are different targets and rationales involved.

a) Delivery of suicide genes

Suicide genes can be defined as genes that are able to convert a nontoxic prodrug into a toxic drug. Thus, only the cells that are transfected will be killed. There are several suicide gene/prodrug remedies currently being used in therapeutic protocols. These include Herpes simplex virus thymidine kinase (HSV-tk)/acyclovir (ACV) or ganciclovir (GCV), and the bacterial or fungal cytosine deaminase (CD)/5-florocytosine (5-FC) (18). Suicide gene therapy also initiates a bystander effect. This arises when the intracellularly produced toxic drug kills the target cancer cell and also neighboring cells. This phenomenon increases the percentage of cells killed above the transfection efficiency. The bystander effect occurs when the toxic drug is transferred via gap junctions (18,19) or by simple diffusion (20). The bystander effect is very much dependent on the gene/prodrug combination and the target cells (21).

b) Delivery of tumor suppressor genes

Many tumor suppressor genes are lost during oncogenesis (22). Re-establishing the therapeutic effect of p53 (23) and increasing the quantity of cyclin dependent kinase (CDK) inhibitors are common approaches in cancer gene therapy. When evaluating the therapeutic effect of p53, $p21^{WAF1/CIP1}$ (p21), and $p16^{INK4a}$ (p16) were compared in cancer cells; p16 and p21 gave a higher tumor suppressor effect than p53 (24). Moreover, when the effectiveness of cyclin dependent kinases were compared, it was seen that cells transfected with p16, $p18^{INK4c}$ (p18) and $p27^{KIP2}$ (p27) induced the greatest amount of cell death and induction of apoptosis. In addition, the re-establishment of p27 and p16 were the most effective in inhibiting cancer growth (25). However, in terms of p27, it was also reported that a mutant p27 induced a stronger tumor growth suppression and G_1-S blockage in all cancer cell lines tested compared with the wild type (26).

c) Inhibition of angiogenesis (anti-angiogenesis)

Angiogenesis is a good target for cancer gene therapy because solid tumors can not grow beyond 2-3 mm without sufficient oxygen and nutrition. Some common

Table 2.   Comparison of viral vectors (1, 2, 3, 13, 14, 15)

Vector	Advantages	Disadvantages
Retrovirus (RV)	- No viral proteins produced - Low immunogenicity - High transfection efficiency *ex vivo* - Stable gene integration	- Low transfection efficiency *in vivo* - Risk of insertional mutagenesis due to random integration into the genome - DNA carrying capacity < 8 kb - Only dividing cells are transfected - Moderate titers (10^6-10^7 particles/ml)
Lentivirus (LV)	- Transfects dividing and nondividing cells - Their cell tropism can be increased via pseudotyping - Stable gene expression - Easy transduction of the hematopoietic stem cells	- DNA carrying capacity < 8 kb - Because related to HIV, safety concerns - Truly resting cells can be resistant to infection - Moderate titers (10^6-10^7 particles/ml)
Adenovirus type-1 (AV)	- Very high transfection efficiency - Transfects dividing and nondividing cells - Very high viral titer (10^{10} particles/ml) - High gene expression	- Transient expression - Immune responses to viral proteins - DNA carrying capacity < 8 kb - Potential interactions with wild type viruses - Repeated dosing may be ineffective due to neutralizing antibodies
Adenovirus type-2 (AV)	- Less immunogenic than type-1	- Low transfection efficiency compared to type 1 - Low transgene expression - DNA carrying capacity < 8 kb
Adenovirus type-3 (AV)	- Large insert size up to 36 kb - No viral proteins coded - Devoid of immune reactions	- Helper viruses are needed
Adeno-associated virus (AAV)	- Transfects dividing and nondividing cells - Sustained expression - Minimum immune response - Has not been associated with a disease so far	- DNA carrying capacity < 5 kb - Random integration and less efficient than retrovirus
Herpes simplex virus (HSV)	- Insert size up to 40 kb - Infect both dividing and nondividing cells - High titers (10^8-10^9 particles/ml)	- Toxicity in many cell types - Promoter inactivation after ~1 week postinfection - Transient expression
Pox virus	- High transduction efficiency - Insert size up to 25 kb - Easy to grow in high titers	- Induces host immune response

Table 3.   General advantages and disadvantages of vector systems

	Viral Vectors	Nonviral Vectors
Capacity for DNA	Limited	Unlimited
Expression	Variable	Transient
Transfection Efficiency	High	Inefficient
Immunogenicity	High	Low (toxic)
Manufacturing	Difficult	Easy

anti-angiogenic genes are vascular endothelial growth factor (VEGF) receptors, angiostatin, certain cytokines and tissue inhibitors of matrix metalloproteinases (27). The most common anti-angiogenesis agents involve the use of antisense strand and ribozymes against angiogenic gene products of tumor cells.

d) Immunotherapy

Immunotherapy depends upon the enhancement of the immune recognition of tumor antigens through their association with molecules such as the cytokines (28). The cytokines that have been evaluated in cancer immunotherapy are IL-1, 2, 4, 5, and 12, INF-α, β, and γ, GMCSF and TNF (29). Since the systemic application of cytokines is not possible for extended periods of time in patients, due to numerous toxic side effects, the utilization of cytokine genes in cancer gene therapy is currently a new approach in cancer gene therapy.

e) Use of oncolytic viruses

Oncolytic viruses are not designed to carry genes to the target cancer cells, but rather to lyse these cells at post-infection. Several oncolytic viruses, which are currently undergoing clinical trials, involve the mutant adenovirus ONYX-015 (dl1520), the HSV-1 mutant G207, the human reovirus (strain type 3 Dearing), vesicular stomatitis virus (VSV) (30), and Epstein-Barr virus and Newcastle disease virus (31). The mutant adenovirus ONYX-015 has a nonsense mutation and a deletion in the region coding for E1B-55 kb, which is known to bind to the transactivation domain of wild type p53 and block its transcriptional activity (32). Thus, this virus can only replicate in p53 mutant cells, where the entry into the S phase is permitted regardless of E1B-55 kb status. However, normal cells are not in danger since they have a functional p53 that can lead the cell to arrest and/or apoptosis upon viral infection. Although the results with ONYX-015 are very promising, there are arguments regarding the dependency of the virus on the cells p53 status (33). While the controversy on ONYX-015 is continuing, studies have progressed to phase II clinical trials (34) and many improvements on the current vector are now being reported (35).

f) Ribozyme/antisense targeting

The main target of this type of therapeutic strategy is at the DNA expression level, decreasing the availability of messenger RNA for translation. Ribozyme targeting (cleaving of selected messenger RNA) (36) and antisense strategies (interfering with transcription) (37) are new concepts for gene therapy applications, especially concerning the inhibition of oncogenes. Their capability of being engineered to target specific sequences makes them very attractive for specific cancer gene therapy.

Targeting Tumor Cells *in vivo*

For cancer gene therapy, there are two major strategies in targeting vectors to metastatic and/or nonmetastatic cancer cells: i) Vector targeting, ii) transcriptional targeting.

Vector targeting can be sub-divided simply into physical targeting and biological targeting. Physical targeting depends upon the route of application of the vector system, such as intra-cancer tissue. Currently almost all vectors applications involve physical targeting.

What is more of a challenge is the biological targeting of the vector itself. In the case of viruses, the surface of the vector can be modified such that the tumor cell tropism is enhanced (31). The same strategy is also applicable for nonviral vectors. Either through surface modifications or through the formation of virus-liposome (virosomes) chimeras (38), nonviral vector targeting can be achieved. For example, targeting the endothelial cells rather than the cancer cells offers a potential approach for gene delivery (anti-angiogenesis) (39).

Transcriptional targeting aims at the level of gene expression, which can be controlled by suitable promoter/enhancer elements that are unique to the cells being targeted (40). In this perspective, promoters based on tumor biology, tissue specificity promoters, and potential inducibility are being studied (41).

Concepts of viral targeting (42), including protease targeting (43) and several retroviral targeting strategies (44), were discussed previously.

Developments in Breast Cancer Gene Therapy

There have been several studies on the vector targeting of vectors for gene expression in breast cancer cells. It has been demonstrated that HER-2 and HER-4 targeting, using retroviral vectors in breast cancer, resulted in an effective mechanism of delivery through ligand-directed receptor interactions (45). Antibodies have also been used to target exogenous DNA to breast cancer cells via erbB2 (46). Another targeting approach employed the upstream regulatory sequences from the human α-lactalbumin and ovine β-lactoglubin genes in an attempt to control the expression of the HSV-tk gene (47). The same researchers repeated their work using the Candida albicans CD gene/5-FU system and obtained parallel results (48). When the heat shock protein 70 (HSP70) promoter was used to control the expression of the HSV-tk gene, the *in vitro* results showed that heat shock treated cells were 50,000 times more sensitive to GCV than the control group (49). There are many demonstrations of the transcriptional targeting of breast cancer cells via a hybrid promoter, which was engineered from the estrogen responsive elements, and also hypoxia responsive elements (50).

In evaluating the suicide gene therapy approach, cells infected with a retroviral vector carrying the *Vericella zoster* virus thymidine kinase (VZV-tk) were challenged

with the prodrug; a very effective killing both *in vitro* and in athymic nude mice was observed (51). When HSV-tk was delivered via an adenoviral vector, using increasing doses, the outcome was toxicity. However, when the gene was put under the control of an inducible tetracycline promoter, the induction caused an increased sensitivity to GCV (52). A retroviral construct, which has the ability to be self-deleted in cells possessing functional p53, carrying the HSV-tk gene showed effective self deleting and cell killing in both normal and breast cancer cells respectively, *in vivo* and *in vitro* (53).

Delivery of tumor suppressor genes to breast cancer cells was also tested in several experimental settings. Studies have shown that the transfection of the wild type p53 gene into the cells having mutated or malfunctional p53 had no effect on chemosensitivity; however, radiosensitivity was greatly influenced (54). Other studies involving adenoviral vectors as the tools of gene delivery have shown that tumor regression was intensified with the addition of doxorubicin (55). These results conflict with the results of other studies that reported the combination with doxorubicin, VP-16 and paclitaxel did not increase death during gene therapy, but combinations with exogenous p53 and radiotherapy showed increased cancer cell death rates *in vitro* (56). When another tumor suppressor gene, p21, was put under the control of the inducible tetracycline promoter, the *in vitro* cell growth was significantly inhibited and tumor volumes in mice were much smaller (57). Various human cancer cell lines, including three breast cancer cell lines, were tested for the effects of transecting with p16. They showed marked increased levels of p16 during cell cycle arrest, but this occurred only in cells expressing functional endogenous pRb (58). Certain mouse model studies showed inhibition of tumor growth when exogenous p16 expression was provided (59). Tumor suppressor gene delivery strategies have been combined with other several therapeutic regimens (radiotherapy and chemotherapy) to increase cancer killing potential. Certain *in vitro* studies showed p27 to be more effective than p16 (60), and other in vivo studies revealed increased apoptosis in the tumor cells and increased tumor regression when p27 was administered intravenously via an adenoviral vector (61). From these results, a new fusion gene construct of p16 and p27 was more potent than either molecule separately (62). Other than directly re-establishing the function of tumor suppressor genes, certain genes unique to breast cancer, such as BRCA1, have been transferred using a vector, which was shown to be effective in reducing tumor cell growth and also increasing the proportion of cells going to cell cycle arrest (63).

When the anti-cancer strategy is anti-angiogenesis, gene therapy usually involves the use of nonviral vectors for breast cancer gene therapy (64). The results of liposomal delivery of endostatin and angiostatin separately to breast cancer cells growing in nude mice showed inhibition of tumor growth (65). Delivery of TIMP-2, via an adenoviral vector, caused more apoptotic cells that were less vascularized (66). However, TIMP-4, when administered intramuscularly, resulted in a stimulatory effect on mammary tumorigenesis (67).

Remedies for the immunotherapy of breast cancer have been attempted. One agent is TNFα, which is toxic when it diffuses directly into the blood. A mutant TNFα, when delivered via an adenoviral vector and administered directly into tumor cells, induced a potent antitumor effect (68). In a phase I trial of IL-2 on 8 breast cancer and 15 melanoma patients, the results were not satisfactory because single injections of the adenoviral vector resulted in inflammation in the majority of patients and reduction in tumor size in only a minority of patients (69). While the *in vivo* results of adenovectors carrying IL-12 showed a dose dependent tumor growth inhibition, prolonged survival and a rejection of subsequent challenges in a metastatic model of breast cancer (70). In contrast, complete tumor regression was not observed when IL-12 was delivered via an adenoviral vector. However, when animal models bearing bilateral tumors were injected with the IL-12 bearing vector and a chemokime, compete regression at the injection site within the tumor and total tumor regression in 60% of the animals on the opposite site tumor were observed (71). Other immunotherapy strategies via adenoviral vectors were also discussed previously (72).

In an experimental setting, the effect of adenoviruses expressing either ribozymes or antisense constructs against HER-2/neu in breast cancer models was investigated. The results demonstrated that in both high versus low HER-2/neu expressing in vivo cell line models tumor cell growth and antitumor effects of the ribozyme-expressing adenoviral vectors were greater than its antisense expressing equivalent (73).

Conclusion

Cancer gene therapy is at a point where the need for an optimal gene delivery vector has become the rate limiting step. The strategies are well established with many different approaches; at least the approaches and strategies have developed faster than optimal vectors. Viral vectors are one step ahead in the race for the optimal vectors, since they have naturally gained the best mechanism for gene delivery to other cells during the course of evolution. There are many reports on the development of new viral vectors for gene delivery, in addition to the ones currently used. A striking fact is that among the viral vectors utilized in cancer gene therapy applications, adenoviral vectors are much more frequently utilized than retroviral vectors, although Table 1 indicates the opposite. However, one must keep in mind that the table refers to all gene therapy applications. Retroviral vectors are more frequently used when the targeted disease requires a gene correction, rather than just a cell killing.

The situation for breast cancer is not different from that of other cancers. Each therapeutic strategy (suicide gene, tumor suppressor gene, anti-angiogenesis gene, immunotherapy, oncolytic viruses and ribozyme/antisense targeting) has been successfully used in breast cancer experimentation. There is currently extensive research seeking to optimize these systems for therapeutic treatment in breast cancer patients. Various reports also show that gene therapy can be used as another "drug" in combined therapy using current therapeutic regimes. The introduction of exogenous tumor suppressor genes can be combined with either chemotherapy and/or radiotherapy, as in the case of p53 (74) and p14, which are shown to reduce drug resistance (75). Moreover, not only can these different therapeutic approaches be combined to provide optimum therapy, it is thought that there will be a decrease in toxic side effects for the patient. Such examples could also include suicide and immunotherapy strategies in cancer patients (76).

The mechanism of gene therapy, in theory, is very attractive because one can target all cancer cells in the body and attempt to destroy them. In addition, the introduced gene vectors will dormantly travel in the blood circulation after intravenous administration and continue to search for cancer cells to destroy over a finite period of time. In the future, it is probable that people will be treated with a special gene vector carring a specific gene which will be designed against his/her own specific cancer. Thus today's advances could lead to the development of new specific types of cancer vaccines and cancer therapies in the near future (77).

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