Abstract

The gene therapy strategy of mutation compensation is designed to rectify the molecular lesions that are etiologic for neoplastic transformation. For dominant oncogenes, such approaches involve the functional knockout of the dysregulated cellular control pathways provoked by the overexpressed oncoprotein. On this basis, molecular interventions may be targeted to the transcriptional level of expression, via antisense or ribozymes, or post-transcriptionally, via intracellular single chain antibodies (intrabodies). For carcinoma of the breast, these approaches have been applied in the context of the disease linked oncogenes erbB-2 and cyclin D1, as well as the estrogen receptor. Neoplastic revision accomplished in modal systems has rationalized human trials on this basis.

Keywords: antisense, dominant-negative, gene therapy, oncogene, ribozyme

Introduction

The gene therapy approach of mutation compensation involves correction of the genetic lesions that are etiologic for neoplastic transformation [1•,2–4]. For the dominant oncogene type of genetic lesion, such genetic correction approaches are designed to functionally ablate dysregulated patterns of gene expression. In considering this type of gene therapy approach for breast cancer, knowledge of the genetic mechanisms responsible for initiation and progression of malignancy is of relevance. In this regard, breast cancer, like other types of human cancer, develops via a succession of genetic alterations [5–7]. Inherited or somatic genetic changes that occur in oncogenes, tumor suppressor genes, the DNA repair machinery, and cell cycle checkpoints lead to low-risk or high-risk forms of in situ carcinoma, and subsequently to invasion and metastasis. For colorectal carcinoma, such changes have been well enumerated by Vogelstein and coworkers [8,9].

A similar analysis of these genetic changes in breast cancer represents a daunting task, because of the greater genetic heterogeneity that characterizes carcinoma of the breast and because few of the relevant genes appear to cause familial syndromes. In this regard, potentially, functional analysis of gene products that are known to be involved in breast cancer (eg p53, Rb, p16, BRCA-1, BRCA-2, ATM), combined with further characterization of other loci implicated by allelic loss or gene amplification, will lead to the identification of pathways that are important to the pathogenesis of many or all breast cancers. Such an understanding of the molecular pathogenesis of breast cancer will allow rational application of novel gene therapeutic and pharmacologic strategies for adjuvant therapy and the early treatment of recurrence.

On this basis, it may be understood that genetic ablation gene therapy strategies may be most successful when such strategies are designed to correct one, or more, of the spe-
cific genetic changes known *a priori* to be present in tumor cells. Many of these changes, including gene amplification, allelic loss, p53 mutation, and increased expression of certain oncogenes, are found in preinvasive lesions such as ductal carcinoma *in situ* of the breast [10–15]. Early correction of these defects could prevent subsequent progression of invasion and metastasis. Indeed, such gene therapy approaches for precancer have been attempted in the context of carcinoma of the lung, and are clearly relevant in the context of carcinoma of the breast also [16]. In addition, targeting of the same alterations in more advanced tumors could prove effective in supplementing current therapies: surgery, radiotherapy, and chemotherapy.

**Genetic abnormalities in breast cancer**

Two major forms of genetic alteration in breast cancer are loss of specific chromosome arms and gene amplification. Loss of heterozygosity analysis of polymorphic DNA markers have implicated chromosomes and subregions of chromosome arms that probably harbor tumor suppressor genes [7]. Nonetheless, in only a few cases have specific genes relevant to allelic loss been identified. Karyotype analysis and chromosome *in situ* hybridization approaches, such as comparative genomic hybridization or fluorescent *in situ* hybridization, point to amplified chromosomal loci likely to harbor oncogenes, and facilitate loss of heterozygosity studies by identifying regions of the genome that are under-represented in tumors [6,17–21]. These studies have shown that breast cancers are unusual among human tumors because of their great degree of genetic heterogeneity, suggesting that breast cancer in reality results from multiple genetic changes. Although characterization of the many unidentified genes that are relevant to allelic loss and gene amplification will undoubtedly suggest additional gene therapy strategies for breast cancer, current knowledge of a few such targets already offers the possibility of effective intervention.

The genetic heterozygosity of breast cancer may thus predicate gene therapy approaches that are targeted to multiple dysregulated alleles. In this regard, the genetic heterogeneity of breast cancer is reflected in the various oncogenes previously implicated. Genetic alteration involving known oncogenes is restricted to six loci that undergo gene amplification. No known genes, including the ras family members, have been shown to undergo base mutation or translocation in primary human breast cancer. Gene amplification occurs at the following specific loci at the approximate frequencies indicated: erbB-2 (chromosome 17q12, 20% of tumors), c-myc (8p24, 20%), PRAD1/CYD1 (11q13, 15%), the fibroblast growth factor receptors (8p12, 10–15%), BEK (10q26, 10–15%), and the insulin-like growth factor receptor (IGF) (15q24-25, 2%). It also involves unidentified genes at chromosomes 13q31, 17q22-24, and 20q12-13.2 [19,20]. In addition to these, other potential oncogenes that are expressed in the absence of genetic alteration include H-ras, erbB-1/epidermal growth factor receptor (EGFR), erbB-3, and others. Thus, a variety of candidate oncogenes have been identified that might be approached via genetic ablation strategies.

With the exceptions of c-myc and PRAD1/CYD1 (encoding the kinase-associated cyclin D1), gene amplification in breast cancer commonly involves one of several growth factor receptors, as noted above. Although the signal transduction mechanisms of these diverse molecules are currently under study, it is likely that common elements of the signaling machinery are involved. For example, signaling by p185*erbB-2* utilizes downstream elements such as phospholipase C-γ, phosphatidylinositol 3-kinase, guanosine triphosphate-activating protein, and the adapter protein SHC [22]. Gene therapeutic modulation of the basal signal transduction apparatus could therefore prove effective in a majority of breast cancer cases. Thus, despite the molecular heterogeneity, common points of dysregulation can provide a limited set of rational targets.

**Genetic ablation strategies for breast cancer**

In this regard, the dysregulation of oncogenes by mutation, gene amplification, gene rearrangement, or overexpression contributes to oncogenesis by removing controls on normal cell cycle regulation. One approach to gene therapy for breast cancer is to ‘knockout’ dominant oncogenes and thereby reduce the growth or invasive potential of the tumor. Inhibition or ablation of oncogenic function can occur at three levels. First, the translation of the oncogene can be targeted. This strategy involves the use of antisense molecules to sequester and/or functionally ablate oncogene messenger RNA. Second, the function of the gene product can be targeted. This approach uses polypeptides containing dominant interfering mutations (‘dominant-negative’) to downregulate signal transduction in tumor cells. Third, the nascent oncogenic protein can be prevented from reaching its proper intracellular location. This approach uses intracellular antibodies (‘intrabodies’) to pre-empt the cellular localization machinery and sequester growth factor receptors inside the cell.

**Antisense molecules**

With regard to antisense inhibition of oncogene function, early studies demonstrated inhibition of lymphoma growth by administration of naked antisense DNA to c-myc [23]. Somewhat more recently, a myc antisense phosphorothioate oligonucleotide that has enhanced stability has been shown to produce cytostatic effects in estrogen-dependent and estrogen-independent breast cancer cell lines [24]. As an alternative approach to delivering antisense molecules, inhibition of K-ras expression and its normally potent tumorigenicity was achieved in nonsmall cell lung cancer cells *in vitro* through the use of a mammalian plasmid vector that produces antisense RNA oligo-
The resulting heterodimers failed to show high affinity EGF mutants of EGFR into cells expressing wild-type receptor. This was accomplished by transfection of cytoplasmic domain which is required for intracellular signaling. This was receptor function. One approach disrupts dimerization, amplified and/or overexpressed in breast cancer. Two different ErbB-1/EGFR, ErbB-2, and ErbB-3), which appear to be approach are the receptor tyrosine kinases (eg mutant proteins. Especially attractive targets for such an approach are the receptor tyrosine kinases (eg ErbB-1/EGFR, ErbB-2, and ErbB-3), which appear to be dominant-negative mutations. In addition, antisense approaches have been developed targeting the ErbB-2 oncprotein. In those studies, antisense oligonucleotides delivered in vitro to breast cancer cell lines could accomplish downregulation of ErbB-2 with modest inhibition of cellular proliferation [30]. In addition, the adenoviral protein E1A has also been shown to select dominant ErbB-2 downmodulation with reversion of the transformed phenotype [31–33].

In the context of clinical trials, a messenger RNA antisense approach for breast cancer is being entered into human trials in a protocol by Arteaga and Holt [34*], which uses disabled mouse mammary tumor virus to drive the tissue-specific expression of antisense oligonucleotides to c-fos and c-myc in late-stage breast cancer patients with lung, meningeal or peritoneal metastases. These investigators have shown that delivery of this vector encoding antisense to c-fos results in inhibition of breast tumor growth and increased survival in a mouse model. Similarly, the E1A approach has been translated into the clinical context [35].

The use of antisense oligonucleotides must overcome several obstacles in order to be clinically useful. These include attaining stable intracellular levels by frequent administration or by constant production internally, and the need to inhibit oncogenes that are amplified or expressed at high levels. As for other therapeutic approaches, the molecule must be delivered to nearly every tumor cell in order to be effective in inhibiting growth or invasive potential. Thus, the enthusiasm for antisense approaches to human gene therapy trials has been limited.

**Dominant negative mutations**

A second approach to ablation of oncogene function is expression of dominant interfering or ‘dominant negative’ mutant proteins. Especially attractive targets for such an approach are the receptor tyrosine kinases (eg ErbB-1/EGFR, ErbB-2, and ErbB-3), which appear to be amplified and/or overexpressed in breast cancer. Two different mechanisms have proved effective in blocking receptor function. One approach disrupts dimerization, which is required for intracellular signaling. This was accomplished by transfection of cytoplasmic domain mutants of EGFR into cells expressing wild-type receptor. The resulting heterodimers failed to show high affinity EGF binding, receptor endocytosis, or biological signaling [36].

Alternatively, growth factor binding to the receptor can be prevented by expression of a mutant growth factor, or by sequestering the growth factor extracellularly. NIH3T3 cells that produced a mutant platelet-derived growth factor molecule showed a reverted phenotype with a reduced growth rate in culture, and reduced invasive potential [37]. Extracellular sequestering was demonstrated for IGF-1. Cells were transfected with IGF binding protein-3, which bound IGF-1 in the extracellular space. This inhibited growth of Balb/c cells even in the presence of high concentrations of insulin and IGF-1 [38]. In addition, this type of approach has been applied in the context of the estrogen receptor, whereby neoplastic reversion has been achieved [39].

**Intracellular single-chain antibodies**

In addition to antisense strategies and dominant-negative mutations, another strategy aims at disrupting normal subcellular localization of growth factor receptors. We have recently developed an approach that prevents growth factor receptors from reaching the cell surface. By transfecting human ovarian cancer cells with a gene encoding an anti-ErbB-2 single-chain variable fragment antibody (sFv), we were able to demonstrate downregulation of cell surface ErbB-2 and a corresponding specific growth inhibition of cells overexpressing the receptor [40,41*]. This sFv-mediated oncogene downmodulation triggered apoptotic cell death in cells that overexpress the receptor [42]. Furthermore, it could be shown that breast cancer cells that overexpressed ErbB-2 were also eradicated in this manner [43]. Interestingly, the level of ErbB-2 that characterized breast cancer tumor targets was predictive of their response to this genetic intervention. The ability to accomplish selective abrogation of oncogenes by the use of intracellular sFvs opens a wide variety of possibilities in breast cancer investigation and therapy. These results were corroborated by Hynes and coworkers, who demonstrated ErbB-2 downregulation with impairment of receptor activation in breast cancer cells [44] and reversion of the transformed phenotype in ErbB-2 overexpressing cells [45].

**Strategies for gene delivery**

As for all mutation compensation approaches, the delivery context is an important predicate of the viability of the genetic intervention. In this regard, contexts whereby target cells can be manipulated ex vivo may allow the achievement of a level of specificity and efficiency of gene delivery that is commensurate with a meaningful therapeutic outcome. Thus, in the context of carcinoma of the breast it may be rational to employ such an ex vivo approach to achieve genetic purging of bone marrow, as has been attempted in a variety of neoplastic contexts [46,47].

Alternatively, locoregional and disseminated disease require direct in vivo delivery of the knockout gene. This
delivery context imposes a greater stringency, and current generation vectors are limited in their ability to achieve this goal [48,49,50,51]. In this regard, such in vivo gene delivery approaches have been limited to disease contexts whereby tumor is sequestered in a compartment context. On this basis, the relatively limited presentation of breast cancer in this manner, combined with vector limitations in this regard, have restricted direct clinical application of genetic ablation strategies for carcinoma of the breast. This recognition has led to the exploration of vector approaches that possess the capacities for efficient, and cell-specific gene delivery in vivo. Such systems, based on both viral and nonviral schemes, have been attempted [52–54]. Thus, despite the fact that genetic ablation strategies offer the possibility of a tumor cell-specific effect, this utility cannot be realized until vector approaches can achieve a requisite level of efficiency in the stringent in vivo gene delivery context.

Acknowledgement
We wish to thank Patty Parker for her administrative assistance.

References
Articles of particular interest have been highlighted as:
- of special interest
- of outstanding interest

1. Grushcow J, Curiel DT: Gene therapy for carcinoma of the breast. Cancer Gene Ther 1995, 4:190–202. An excellent subject review is presented.

2. Curiel DT: Gene therapy approaches for treatment of cancer. In: Cecil Textbook of Medicine, 20th ed. Edited by Claude Bennett J, Plum F. WB Saunders Company, 1996:98–117.

3. Rosenfeld ME, Curiel DT: Gene therapy strategies for novel cancer therapeutics. Curr Opin Oncol 1996, 8:72–77.

4. Bilbao G, Curiel DT: Gene therapy for cancer therapeutics. Oncol Endocr Metab 1997, 3:1287–1284.

5. Mars WM, Saunders GF: Chromosomal abnormalities in human breast cancer. Cancer Metast Rev 1990, 9:35–43.

6. Cox LA, Chen G, Lee EY: Tumor suppressor genes and their roles in breast cancer. Breast Cancer Res Treat 1994, 32:19–38.

7. Devilee P, Cornelisse CJ: Somatic genetic changes in human breast cancer. Biochem Biophys Acts 1994, 1198:113–130.

8. Parsons R, Li GM, Longley MJ, et al: Hypermutability and mismatch repair deficiency in RER+ tumor cells. Cell 1993, 75:1227–1236.

9. Cho KR, Vogelstein B: Suppressor gene alterations in the colorectal adenoma-carcinoma sequence. J Cell Biochem 1992, 16G (suppl):137–141.

10. Liu E, Thor A, He M, et al: The HER2 (c-erbB-2) oncogene is frequently amplified in in situ carcinoma. Oncogene 1992, 7:1027–1032.

11. Elledge RM, Allred DC: The p53 tumor suppressor gene in breast cancer. Breast Cancer Res Treat 1994, 32:39–47.

12. Zhuang ZP, Merino MJ, Chuqui R, Liotta LA, Emmertbuck MR: Identi cal allelic loss on chromosome 11q13 in microdissected in situ and invasive human breast cancer. Cancer Res 1995, 55:467–471.

13. Radford DM, Fair KL, Phillips NJ, et al: Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p, 17q. Cancer Res 1995, 55:3399–3405.

14. Munn KE, Walker EA, Varley JM: Frequent alterations of chromosome 1 in ductal carcinoma in situ of the breast. Oncogene 1995, 10:1653–1657.

15. Weinstat-Salisow D, Merino MJ, Manrow RE, et al: Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. Nature Med 1995, 1:1257–1260.

16. Zou Y, Zong G, Ling YH, et al: Effective treatment of early endobronchial cancer with regional administration of liposome-p53 complexes. J Natl Cancer Inst 1998, 90:1130–1137.

17. Trent J, Yang J-M, Emerson J, et al: Clonal chromosome abnormalities in human breast carcinomas. Thirty four cases with metastatic disease. Genes Chrom Cancer 1993, 7:194–203.

18. Thompson F, Emerson J, Dalton W, et al: Clonal chromosome abnormalities in human breast carcinomas. Twenty eight cases with primary disease. Genes Chrom Cancer 1993, 7:185–193.

19. Kallioniemi A, Kallioniemi OP, Piper J, et al: Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. Proc Natl Acad Sci USA 1994, 91:2156–2160.

20. Guan X-Y, Meltzer PS, Dalton WS, Trent JM: Identification of cryptic sites of DNA sequence amplification in human breast cancer by chromosome microdissection. Nature Genet 1994, 8:155–161.

21. Pandis N, Jin Y, Gorunova L, et al: Chromosome analysis of 97 primary breast carcinomas: identification of eight karyotypic subgroups. Gene Chrom Cancer 1995, 12:173–185.

22. Dougall WC, Qian X, Peterson NC, et al: The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. Oncogene 1994, 9:2109–2123.

23. McManaway ME, Neckers LM, Loke SL, et al: Tumor-specific inhibition of lymphoma growth by an antisense oligonucleotide. Lancet 1990, 335:808–811.

24. Watson PH, Pon RT, Shiu RP: Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. Cancer Res 1991, 51:3996–4000.

25. Mukhopadhyay T, Tainsky M, Cavender AC, Roth JA: Specific inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA. Cancer Res 1991, 51:1744–1748.

26. Georges RN, Mukhopadhyay T, Zhang Y, Yen N, Roth JA: Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct. Cancer Res 1993, 53:1743–1746.

27. Zhou P, Jaing W, Zhang Y, et al: Antisense to cyclin D inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. Oncogene 1995, 11:571–580.

28. Burkef P, Chemicky CL, Rininsland F, Ilan J, Ilan J: Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo. Proc Natl Acad Sci USA 1996, 93:7263–7268.

29. Kenney NJ, Saeki T, Gottardis M, et al: Expression of transforming growth factor α antisense mRNA inhibits the estrogen-induced production of TGFα and estrogen-induced proliferation of estrogen-responsive human breast cancer cells. J Cell Physiol 1993, 156:497–514.

30. Bertram J, Killian M, Brysch W, Schlingensiepen K-H, Kneba M: Reduction of erbB-2 gene product in mammary carcinoma cell lines by erbB-2 mRNA-specific and tyrosine kinase consensus phosphorothioate antisense oligonucleotides. Biochem Biophys Res Commun 1994, 200:661–667.
An interesting treatment study is presented.

A highly novel approach to achieve mutation compensation is presented.

via an intracellular antibody directed against the erbB-2.

A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization.

Intracellular expression of single-chain antibody mediates targeted tumor cell killing via an intracellular antibody directed against the erbB-2.

A highly novel approach to achieve mutation compensation is presented.

A novel gene therapy strategy for elimination of prostate carcinoma cells from human bone marrow.