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**Title:** The Identification of Genes Mediating Chemo-Sensitivity in Human Mammary Cells

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**ABSTRACT:** Our working hypothesis is that the loss of function of a number of diverse checkpoint molecules will lead to the proliferation of chemo-resistant tumor cells, and that the various molecules altered will be specific for any given class of chemotherapeutic agent. Besides the p53 pathway, relatively little is known regarding other checkpoint molecules whose loss of function results in chemo-resistance. This is probably due to the fact that loss of function of these molecules occurs through very subtle genetic alterations which are not easily detected by any current molecular techniques. Research performed under this award is focusing on identifying genes whose loss of function results in the proliferation of tumor cells that are resistant to the action of two different types of drugs (i.e. Adriamycin and taxol) that are used to treat women with breast cancer. This is being performed with a novel system that allows for the isolation of genes encoding selectable recessive phenotypes. Identifying molecular pathways that are altered in chemo-resistant malignant cells will be a critical step for the future design of novel therapeutic strategies that aim to restore chemo-sensitivity to chemo-resistant breast tumor cells.
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INTRODUCTION

Circumventing drug resistance is a critical step to increase the treatment success rate for women with breast cancer. The object of this grant is to improve our understanding of cellular genes whose loss of function results in the emergence of chemo-resistant tumor cells. It is becoming apparent that loss of function of checkpoint molecules regulating cell cycle progression and apoptosis are important in both tumor cell evolution and in the emergence of chemo-resistant malignant cells. Of the relatively small number of checkpoint molecules identified at this time, the best understood is the p53 tumor suppressor. Loss of p53 function through targeted deletion (in the case of the p53 knock-out mouse) renders cells deficient in their ability to undergo cell cycle arrest and/or apoptosis upon exposure to DNA damaging agents (1,2). Consistent with these in vitro observations, patients with hematological malignancies (3) and cancers of the stomach (4), lung (5) and breast (6) are less likely to respond successfully to chemotherapeutic agents as compared to patients with wild type p53 alleles. It is however obvious that p53 gene alterations do not explain all cases of chemo-resistance in human malignancies, as well as in breast cancer patients. Also, there is in vitro evidence which shows that breast tumor cells can become resistant to the action of chemotherapeutic drugs in the absence of p53 gene mutations (7).

Our working hypothesis is that loss of function of a number of diverse checkpoint molecules will lead to the proliferation of chemo-resistance tumor cells, and that the various molecules altered will be specific for any given class of chemotherapeutic agent. Besides the p53 pathway, relatively little is known regarding other checkpoint molecules whose loss of function results in chemo-resistance. This is probably due to the fact that loss of function of these molecules occurs through very subtle genetic alterations which are not easily detected by any current molecular techniques. Research being performed under this award is focusing on the identification of genes whose loss of function results in the proliferation of malignant epithelial cells that are resistant to the action of two classes of drugs; DNA damaging agents (e.g. adriamycin) and inhibitors of microtubule formation (e.g. taxol). This is being performed with a novel system based on a recently described technique (8) that allows for the isolation of genes encoding selectable recessive phenotypes. Identifying molecular pathways that are altered in chemo-resistance tumor cells will be critical for the future design of novel therapeutic strategies restoring chemo-sensitivity to chemo-resistant breast tumor cells.
BODY

Overview of the in vitro knock-out system.

An in vitro knock-out approach is being used to identify genes that are important for mediating the cytotoxic action of specific chemotherapeutic drugs. The strategy used is based on a technique described in the journal Cell by Dr. Stanley Cohen’s laboratory that was used to identify cellular genes whose loss of function results in the transformation of immortalized NIH3T3 cells (8). We have modified the previously used technique for two main reasons. First of all, we have not been able to transfect human epithelial cells with the retroviral gene search vector at a high enough frequency to insure the knock-out of a reasonable number of expressed cellular genes. This has failed using a number of packing cell lines, including the Bing, Bosc and Phoenix lines. Second, we believe that the use of an inducible promoter based system, instead of a constitutive one, will dramatically increase the ease of identifying chemo-resistant clones that are derived from the disrupted cellular allele.

Figure 1 outlines our novel in vitro knock-out approach. This approach relies on a gene search insert and a cell lines that contains high levels of the muristerone transcription factor, VgRXR. The gene search insert encodes a G418 resistance/B-galactosidase fusion protein. The fusion cDNA is downstream of a functional splice acceptor (SA) site, but is not downstream of a functional promoter. Therefore, expression of the fusion product is dependent on the integration of the gene search insert into transcribed sequences supplying a splice donor site. The first step in this process is to transfect the gene search insert into a target cell line containing high levels of the muristerone transcription factor, VgRXR. After transfection, cells are cultured in the presence of G418 to select for those cells harboring integration of the gene search insert into expressed cellular sequences. After two weeks of selection, those cells that are G418 resistant are pooled and expanded.

Once a library of knock out cells have been generated, 1x10^7 cells derived from at least 5,000 G418 resistant clones will be exposed to muristerone. Muristerone is an inducer of the VgRXR transcription factor. Treatment of cells with this hormone will induce antisense transcripts complementary to sequences upstream of the gene search vector and thus theoretically reduce the expression of protein encoded by the other allele. Twenty-four hours later, these muristerone treated cells will be exposed to a specific concentration of chemotherapeutic drug (this will be determined experimentally for each drug by drug titration assays) that kills 99.999999% of the parental target cell line. We plan to use two drugs with diverse mechanisms of action. The first drug, adriamycin, is a DNA damaging agent while the second drug taxol is an inhibitor of microtubule formation. Both of these drugs are used in the clinic to treat women with breast cancer.

Once drug resistant colonies have been isolated and expanded, the subsequent series of experiments will verify that the acquisition of drug resistance is mediated by the antisense transcript produced by the gene search vector, and not by spontaneous acquisition of drug resistance or by direct insertional inactivation by the transfected gene search construct. This will be performed by comparing the sensitivity of cells from the resistant colonies to drug +/- muristerone. Those cells that remain resistant to the cytotoxic action of these drugs in the presence of muristerone, but not in its absence, will have likely acquired drug resistance through the antisense knock-out procedure. Standard recombinant DNA techniques will then be used to identify the specific cDNA whose loss of function results in the clonal proliferation of chemo-resistant cells. Finally, the isolated cDNA will be subcloned into a eukaryotic expression vector and transfected into chemo-resistant epithelial cells. Those cDNAs that reverse the drug resistance phenotype of the cells will be sequenced and characterized by data base searches.
Results from work performed in this first year of this award.

The success of this project is dependent on meeting at least two main technical objectives. The first is to generate a potent muristerone inducible line to insure that high levels of the antisense transcript will be produced in each cell harboring the gene search insert. The second is to optimize a transfection procedure such that a high number of G418 resistant colonies will be generated. This will insure that a reasonable number of expressed cellular genes are being knocked-out. Work performed in the first year of this award has focused on accomplishing these two tasks.

Generation of a potent inducible line: A muristerone inducible system (9) was chosen for our in vitro knock-out approach (see figure 2). The muristerone regulated transcription factor used in this system is a heterodimer derived from the drosophila ecdysone receptor (contains a modified VP16 transactivation domain) and the mammalian retinoid x receptor. Treatment of cells with the ecdysone analogue, muristerone, induces heteromeric complex formation of the two subunits, sequence specific binding of the complex to particular response elements (GRE's) and activation of downstream target genes. In order to generate an inducible line, human epithelial cells were transfected by the calcium-phosphate precipitation method using an expression construct (VgRXR, Invitrogen) encoding both subunits of the muristerone inducible transcription factor and a selectable marker (zeocin resistance). After transfection, cells were treated with 200 ug/ml zeocin for two weeks. Zeocin resistant colonies were isolated and expanded into cell lines. To test for inducibility, lines were transfected with a construct possessing a reporter gene (the B-galactosidase gene) located downstream of 5 hormonal responsive elements. After transfection, cells were split into two dishes. Twenty-four hours later, one of these dishes received muristerone (final concentration of 1mM). The concentration of muristerone used was the minimum concentration found to give the highest level of B-galactosidase induction by muristerone in parental cells transiently transfected with the VgRXR and B-galactosidase reporter constructs (pIND-LacZ) (data not shown). Extracts were prepared twenty-four hours later from treated and untreated dishes and the level of B-galactosidase activity determined. The amount of B-galactosidase activity in extracts from untreated cells were all very similar (data not shown). However, the amount of B-galactosidase activity in extracts from muristerone treated cells differed dramatically between clones. Figure 3 shows the fold induction of reporter activity after muristerone treatment in the 14 different VgRXR transfected lines. Cells derived from clone 9 gave the highest fold induction of the 14 clones analyzed (approximately 10 fold).

The ability to reduce protein expression from the wild-type allele via muristerone induced antisense transcripts produced from the knock-out insert may be limiting in a line that gives only a 10 fold level of induction. We therefore set out to improve muristerone inducibility in clone 9 cells. We decided to transfec these cells with the a VgRXR expression construct containing both a zeocin and puromycin selectable marker. This construct was generated by subcloning the puromycin resistance gene from pPuro (a Pvu II-BamHI fragment) into the BstE II site of VgRXR. This vector (VgRXR-puro) was then transfected into cells derived from clone 9 of the initial VgRXR transfectants and cells were cultured in the presence of zeocin (200 ug/ml) and puromycin (0.5 ug/ml) for two weeks. Zeocin/puromycin resistant colonies were isolated and expanded into cell lines. Testing for muristerone inducibility was performed as described above using the same B-galactosidase reporter construct. Figure 4 shows the fold induction of reporter activity after muristerone treatment in 20 different VgRXR-puro transfected clone 9 cells. Cells derived from clone 9 gave the highest fold induction of the 14 clones analyzed (approximately 50 fold). To verify the fold induction in these cells (9-8 cells) using another reporter, cDNA encoding the mdm2 proto-oncogene was subcloned into the pIND vector. 9-8 cells were then transiently transfected with pIND and pIND-mdm2 and cells were processed as described above. Western blot analysis was then performed using extracts prepared from untreated and muristerone treated
transfected cells. Figure 5 shows dramatically higher levels (at least 10 fold) of mdm2 protein in 9-8 cells transiently transfected with the muristerone inducible mdm2 construct (pIND-mdm2). Considering these cells possess endogenous levels of mdm2 and the calcium phosphate method can only transfect approximately 10% of these cells (data not shown), the total level of mdm2 inducibility in the transfected cell population is probably over 100 fold.

The level of muristerone induced B-galactosidase activity or the amount of mdm2 protein produced in 9-8 cells (between 50-100 fold) is close to that which is produced when either B-galactosidase or mdm2 is cloned downstream of a strong constitutive promoter (e.g. CMV promoter, SV40 promoter). These type of promoters have been used in the past to produce antisense transcripts against target genes and block expression of proteins encoded by these genes (see references 10, 11 and 12 for examples). We therefore feel that these 9-8 cells should be able to produce enough antisense transcripts from the knock-out insert to significantly reduce the expression of protein encoded by the other wild-type allele.

Generation of a high number of G418 resistant colonies in gene search insert transfected cells:
Besides the generation of a highly inducible cell line, the second area where substantial progress was made during the first year of this award was the generation of a knock-out cell library. An acceptable knock-out library will depend on the efficient transfection of the 9-8 cells with the gene search vector and the generation of a large number of G418 resistant cells. We performed many small scale transfection experiments to optimize the generation of G418 resistant colonies. For example, comparisons between transfection procedures (e.g. electroporation vs lipofectamine vs calcium phosphate), percent confluency prior to transfection, amount of DNA used in the transfection and concentrations of G418 used in selection were all performed to determine the best method for generating the highest number of G418 resistant clones. A method was developed from all of these studies that was able to generate approximately 130 G418 resistant colonies per transfection point (2 x 10⁷ cells). After scaling up to 50 transfection points, we have been able to generate a knock-library of approximately 6,500 G418 resistant colonies. To verify insertion of the G418 resistant/B-galactosidase fusion sequence from the gene search insert into expressed cellular sequences, northern blot analysis on RNA isolated from some of these colonies was performed. Using a probe to the G418 resistant/B-galactosidase fusion sequences, we were able to detect transcripts that were larger than the predicted size of the G418 resistant/B-galactosidase fusion sequences alone (figure 5). Northern blot analysis has been performed using RNA from 5 more lines (data not shown) and the average number of fusion transcripts detected in all lines analyzed is approximately three. These results suggest that integration of the gene search vector has occurred downstream of expressed cellular sequences in the 9-8 cells. Considering that our library is derived from 6,500 G418 resistant clones and each of these clones has at least 3 integration events, we estimate that our cell library contains the disruption of approximately 20,000 expressed cellular genes in 9-8 cells. We do realize that this estimate is based on some assumptions (i.e. transcripts detected are from different genes-not splice variants from the same gene, our northern blot analysis can detect all transcripts derived from integration of the gene search insert into expressed cellular sequences). Nonetheless, because it has been estimated that there are 100,000 genes and 10% of these are expressed in any given cell type, it appears that we have been able to disrupt a reasonable percentage of genes in 9-8 cells.

Progress in relation to the statement of work proposed in the initial application.

Because we have moved away from the retroviral system, it is difficult to compare exactly our progress in relation to the statement of work proposed in the initial application. However, we have been able to generate both a highly inducible line and a cell knock-out library in this line in the first
12 months of the proposal. We are now ready to start performing drug titration assays to find the optimum dose of taxol and adriamycin that kills 99.999999% of cells from the knock-out library. Once this dose is defined we will then pretreat cells with muristerone, expose cells to drug and select for drug resistant cells harboring the gene search vector. Because these experiments were planned to be performed between 16-20 months in the initial application, we are currently 3 months ahead of the schedule planned in the statement of work section outlined in the initial grant proposal.

**CONCLUSIONS**

We have made substantial progress in the first year of this proposal in developing a novel in vitro knock-out approach that will allow us to identify genes that mediate chemo-sensitivity to chemotherapeutic drugs in human epithelial cells. Although this project is high risk and still at the early stages of development, it has enormous potential to identify factors that are important in mediating the cytotoxic action of chemotherapeutic drugs. If successful, this project will lead to long term studies that will try to dissect the function of identified proteins and work that will examine the possibility that these factors may be altered in chemo-resistant mammary tumors. These studies will hopefully lead to novel therapeutic strategies that will restore chemo-sensitivity to chemo-resistant tumor cells in women with breast cancer.
REFERENCES

Figure 1. Generation of an in vitro cell knock-out library

Gene Search Insert

Transfect into cells containing stable expression of the muristerone-inducible transcription factor, VgRXR, and select for the integration in expressed cellular sequences by exposing cells to G418

Pool G418 resistant colonies

Expose cells to muristerone to produce antisense and then to drug

Isolate resistant clones and identify those that are resistant to drug in the presence of muristerone, but not in its absence

Legend. Method for using a random knock-out procedure to identify genes that mediate the cytotoxic action of chemotherapeutic drugs.
Figure 2. The muristerone inducible transcriptional activation system

Legend. Schematic representation of the mechanism by which the expression of genes downstream of the muristerone inducible promoter are regulated in the absence (-) or presence (+) of this hormone.
Figure 3. Fold induction of β-galactosidase activity in muristerone treated VgRXR transfected lines

Legend. Zeocin resistant, VgRXR transfected lines were transfection with a construct containing the β-galactosidase reporter gene under the control of 5 hormonal responsive elements. Extracts were prepared from muristerone treated and untreated cells and β-galactose activity measured. Fold induction was calculated as follows: 
\[
\frac{\text{β-gal. activity (+ murs.)}}{\text{β-gal. activity (- murs.)}}
\]
Figure 4. Fold induction of B-galactosidase activity in muristerone treated VgRXR-puro transfected lines

![Graph showing fold induction against clone number]

Legend. Zeocin/puromycin resistant lines were transfection with a construct containing the B-galactosidase reporter gene under the control of 5 hormonal responsive elements. Extracts were prepared from muristerone treated and untreated cells and B-galactosidase activity measured. Fold induction was calculated as follows: $\frac{\text{B-gal. activity}(+ \text{ murs.})}{\text{B-gal. activity}(- \text{ murs.})}$
Figure 5. Induction of mdm2 protein in muristerone treated 9-8 cells transfected with pIND-mdm2

Legend. 9-8 cells were transfected with pIND or pIND-mdm2 by the calcium phosphate precipitation method. Twenty four hours after transfection, cells were split into dishes. One of these dishes was treated with muristerone (+) while the other one was not. Extracts were prepared 24 hours later and the amount of mdm2 protein measured by a western blot using the mdm2 Ab-1 monoclonal antibody (Calbiochem).
Figure 6. Northern analysis of clones isolated from the 9-8 cell knock-out library.

Legend. A northern blot containing RNA from parental 9-8 cells (P) and different G418 resistant colonies (C3 and C4) isolated from the 9-8 knock-out library was probed with a G418 resistance/B-galactosidase fusion DNA probe. After hybridization, blot was washed in 0.2X SSC/0.1% SDS at 65°C and exposed to x-ray film.