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13. ABSTRACT *(Maximum 200*
Polymorphic epithelial mucin (PEM), a product of the MUC1 gene, is aberrantly expressed by human breast cancer cells. It is a potential target for immune-mediated attack. The induction of immunity to breast cancer associated mucin could have important implications for the treatment of patients with breast cancer. Two approaches are being evaluated to augment the immunity to PEM. The studies are being carried out in transgenic mice that express human mucin as "self," mimicking as closely as possible the disease in humans. The first involves the introduction and expression of cytokine genes into mouse breast cancer cells modified to express human breast cancer associated mucin. The second involves the introduction and expression of genes specifying co stimulatory molecules required for T cell activation into the modified cells. Significant progress toward the accomplishment of these goals was made during the initial period of support. Immunization with cells modified to secrete IL-12 induces long-term immunity to breast cancer in syngeneic mice. Clones of cells expressing varying amounts of B7.1, a co stimulatory molecule have been identified. Their immunogenic properties are being evaluated. Future plans include studies to determine if expression of both cytokines and co stimulatory molecules by the same cell type will result in a cellular immunogen of greater efficacy.

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Edward P. Volk

PI - Signature Date

9/23/97

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INTRODUCTION

This investigation was prompted by the finding that polymorphic epithelial mucin (PEM), a product of the MUC1 gene, is aberrantly expressed by human breast cancer cells. PEM is a heavily glycosylated macromolecule present on the surface of both normal and malignant cells. The glycosylation pattern is altered in malignant cells. As a consequence, the altered PEM becomes immunogenic. PEM expressed by breast cancer cells has been identified as a weakly immunogenic, tumor associated antigen (TAA). The challenge is to induce immunity to PEM in patients with breast cancer.

In a mouse model system, we proposed to test methods found previously to augment the immunogenic properties of murine TAAs to induce immunity to human breast cancer-associated mucin. The successful application of these techniques will form the basis of an analogous approaches in breast cancer patients. The methods represent an immunotherapeutic approach that can be applied to patient care. The long-term objective of the study is the development of a vaccine useful in the treatment of patients with breast cancer.

We hypothesize that presentation of human breast cancer associated mucin in the microenvironment of immune-augmenting cytokines by cells modified to express co stimulatory molecules required for T cell activation will lead to induction of cellular immunity to breast cancer cells in patients with the disease.

The investigation is being carried out in an animal model of breast cancer that mimics as closely as possible breast cancer in humans. The studies were made possible by the development in Professor Joyce Taylor-Papadimitriou's laboratory of human MUC1 transgenic Sac II mice (H-2^k) that express human PEM in a tissue-specific manner. The transgenic mice are, therefore, naturally tolerant of human PEM. E3 cells, a derivative of 410.4 mouse breast carcinoma cells (BALB/c origin, H-2^d) were genetically modified to express human PEM. Thus, E3 cells express human

PEM and MUC1 transgenic Sac II mice express PEM as "self." (Dr. Taylor-Papadimitriou a collaborator on the project, is a subcontractee. Her progress report is included, below.)

During the first eight months of the project, Professor Taylor-Papadimitriou and I proposed to accomplish the following:

1. To genetically modify E3 cells for cytokine-secretion, by introducing genes for various cytokines into E3 cells, to select the cells, and to assay for cytokine secretion, and

1a. To introduce genes for B7 (a co-stimulatory molecule for T cell recognition) into E3 cells, to select the genetically modified cells, and to assay for B7-expression

Afterward, we planned to begin experiments that compare the survival of MUC1 transgenic mice immunized with cytokine-secreting E3 cells, challenged with viable E3 cells, and to characterize the anti tumor response, and to determine in MUC1 transgenic mice the contribution of B7 to the immunogenic properties of mouse breast cancer cells modified to express human PEM.

Significant progress toward these and related objectives has been accomplished. One manuscript on a related subject supported by the award is "in press," a second has been submitted for publication and a third is in preparation. The results are described in detail, below.

BODY

Background

The MUC1 gene codes for a membrane mucin (PEM) expressed at the apical surface of most glandular epithelial cells. Expression is dramatically up-regulated in breast and ovarian cancer cells. The extracellular domain of mucin consists of

tandem repeats of 20 amino acids with multiple O-glycans covalently bonded to the amino acid core. In breast cancer, the composition of the carbohydrate side chains is altered, resulting in the exposure of normally cryptic peptide epitopes. Aberrantly expressed breast cancer-associated mucin can become highly immunogenic, leading to the induction of T cell-mediated immunity to the malignant cells. Thus, methods to increase the immunogenic properties of PEM could have important therapeutic implications in the treatment of breast cancer patients.

Increasing the immunogenicity of breast cancer-associated mucin.

The protein core of PEM (the product of the MUC1 gene) expressed by breast cancer cells is aberrantly glycosylated, exposing a part of the protein core. The exposed core is potentially immunogenic. However, like other neoplasms, there is an immune selection in the host for malignant cells that express weakly immunogenic TAAs. (It is likely that cells that express highly immunogenic TAAs are recognized by the host's immune system and rejected.) Breast cancer cells that express weakly immunogenic PEM fail to be recognized, evade destruction by the immune system and proliferate without apparent inhibition.

Numerous techniques have been used to increase the immunogenicity of malignant cells. More recent attempts include the introduction of genes specifying immune-augmenting cytokines, or of a costimulatory molecule such as B7.1, required for T cell-recognition. Tumor cells modified to form cytokines, such as IL-2, or to express co stimulatory molecules, such as B7.1, are being tested in Phase I clinical trials for patients with different types of cancer. As a consequence, there is an increase in the activation and proliferation of cytotoxic T lymphocytes (CTLs) and Natural Killer/ Lymphokine Activated Killer (NK/LAK) cells directed toward the tumor.

These approaches are now evaluated in mice with breast cancer. Background studies were carried-out in 410.4 cells, a mouse neoplasm of mammary epithelial cells arising in BALB/c mice (H-2^d). E3 cells are a derivative of 410.4 cells that were transfected with the human MUC1 gene. They express human PEM. Both 410.4

cells and E3 cells have been transfected with genes specifying immune-augmenting cytokines and the murine B7.1 gene (described, below). The survival of mice with breast cancer is enhanced if the animals are treated by immunization with cells that secrete cytokines, or express B7.1. In addition, a plasmid vector containing mB7.1 cDNA under control of the MUC1 promoter has been developed to promote tissue-specific expression. To assess the effect of B7.1 expression on normal epithelial cells expressing MUC1, a double transgenic mouse expressing B7.1 under the MUC1 promoter is being developed by Professor Taylor-Papadimitriou, a co-awardee and collaborator.

Progress in Dr. Cohen's laboratory.

Development of a colony of MUC1 transgenic mice.

Professor Taylor-Papadimitriou kindly provided us with a breeding pair of MUC1 transgenic Sac II mice (H-2^k). After a six week period of isolation in our animal colony, the mice, found to be disease-free, were released by our Veterinarians to the general animal colony. The Sac II mice were then bred with BALB/c mice (H-2^d) to generate H-$2^{k/d}$ F1 hybrids for use in the experiments. Presently, we have 72 Sac II transgenic X BALB/c F1 mice on-hand. Breeding is continuing until we have sufficient numbers of mice to begin our immunotherapy experiments. The initial studies are designed to determine the immunotherapeutic properties of E3 cells, genetically modified for cytokine-secretion in syngeneic transgenic mice with breast cancer.

Confirmation that E3 cells are syngeneic in Sac II transgenic X BALB/c F1 mice

Our initial experiments were designed to determine if E3 cells were syngeneic in Sac II transgenic X BALB/c F1 mice. The test was performed by measuring the transplantability of the genetically modified cells. (Incompatible cells are rejected.) Our results indicated that E3 cells, as well as 410.4 cells, formed tumors and grew progressively in Sac II transgenic X BALB/c F1 F1 mice. The data, described in greater detail, below, indicate that both cell types are considered as "self" by the F1 mice.

Background experiments in BALB/c mice with 410.4 adenocarcinoma.

During the time we were breeding larger numbers of Sac II transgenic X BALB/c F1 mice, we undertook a series of necessary background experiments designed to determine the immunogenic properties of 410.4 cells modified for cytokine-secretion in syngeneic BALB/c mice. The following results were obtained:

Project Objective 1. To introduce genes for various cytokines into E3 cells, and as controls, into 410.4 cells, to select the genetically modified cells and to assay for cytokine secretion..

a. Cytokine secretion by transfected breast carcinoma cells.

In preparation for determinations of the immunotherapeutic properties of E3 adenocarcinoma breast cancer cells and for comparison, 410.4 cells, both E3 and 410.4 cells were successfully transfected with vectors carrying genes specifying immune-augmenting cytokines. (During the same period, Professor Taylor-Papadimitriou transfected E3 cells with a vector specifying the co-stimulatory molecule, B7.1. The data are presented, below.) The results are as follows:

Transfection of Murine Breast Cancer Cells with Vectors Specifying Cytokine Genes

	Cell Type	
Vector	410.4 cells	E3 cells
pZipNeoIFN-g	transfected	(in selection)
pZipNeoIL-4	transfected	transfected
pZipNeoIL-2	transfected	transfected
MFG-mIL-12	transfected	transfected*.

*IL-12 Secretion by 410.4 Breast Cancer Cells Transfected with MFG-mIL-12

		IL-12/10^6 cells/48 hrs
Cells transfected with MFG-mIL-12	(410.4-IL-12)	600 pg ± 15
Cells transfected with SV-(X)	(410.4-SV-X)	0.0 pg
Non transfected 410.4 cells		0.0 pg

Lipofectin was used to facilitate DNA up-take. The formation of IL-12 by the transduced cells was determined by an analysis of supernatants collected from transduced or mock (exposed to Lipofectin alone) transduced 410.4 cells. An ELISA (Endogen) specific for the p70 heterodimer of IL-12 was used in the analysis.

b. Comparison of the growth of 410.4 and E3 cells in MUC1 transgenic Sac II X BALB/c mice F1 mice.

As stated previously, both 410.4 and E3 cells of BALB/c origin are syngeneic in both BALB/c and Sac II X BALB/c F1 mice. Before investigating the immunotherapeutic properties of cytokine-secreting E3 cells, a preliminary experiment was carried out to determine the growth and time to tumor-development of 410.4 cells and E3 cells in the F1 mice. The following results were obtained:

	Cell Type	
	410.4	E3
No. cells injected	Time to first appearance of tumor, (days)*	
10^6	8 ± 2	8 ± 2.5
10^4	22 ± 3	24 ± 2

Legend:

Varying numbers of 410.4 or E3 cells from in vitro culture were injected into breast tissue of Sac II transgenic X BALB/c F1 female mice. The time until the first appearance of a palpable neoplasm was determined. The rate of subsequent tumor growth (2-dimensional measurements) is now under evaluation.

c. The growth of 410.4-IL-12 cells in BALB/c mice.

410.4 cells are an adenocarcinoma cell line syngeneic in BALB/c mice. The cells were modified for IL-12 secretion by transduction with a vector (MFG-IL-12) specifying IL-12 (410.4-IL-12 cells). After confirmation of IL-12 secretion, 10^6 410.4-IL-12 cells, and, for comparison, an equivalent number of unmodified, non cytokine-secreting 410.4 cells, were injected into breast tissue of naive BALB/c female mice. As indicated, (Figure 1), tumor growth occurred in both instances. However, the rate of tumor development in mice injected with 410.4-IL-12 cells was less than that of mice injected with the unmodified 410.4 cells.

2. Tumor growth in BALB/c mice injected with a mixture of 410.4-IL-12 and 410.4 cells.

The potential immunotherapeutic properties of 410.4-IL-12 cells were determined in BALB/c mice, syngeneic with the neoplasm. In the experiment, BALB/c mice were injected s.c. with a mixture of 1 X 10^6 410.4 cells and an equivalent number of 410.4-IL-12 cells. As controls, the mice received an injection s.c of 10^6 410.4 cells or 10^6 410.4-IL-12 cells alone. As indicated (Figure 2), the rate of tumor growth in mice injected with the mixture of 410.4 cells and 410.4-IL-12 cells was not significantly different ($P < .05$) that that of mice injected with 410.4 cells alone. Mice injected only with 410.4-IL-12 cells developed tumors as well, but the rate of tumor growth in these mice was significantly ($P < .01$) less than that of mice injected with either 410.4 cells or the mixture of 410.4 cells and 410.4-IL-12 cells.

Thus, 410.4-IL-12 cells retained their tumorigenic properties in syngeneic BALB/c mice. Mice injected with a mixture of 410.4 cells and 410.4-IL-12 cells, like mice injected with 410.4-IL-12 cells alone, developed progressively growing neoplasms.

We conclude that modification of 410.4 cells for IL-12-secretion was not sufficient to eliminate the cells tumorigenic properties.

3. Survival of BALB/c mice injected with 410.4-IL-12 cells.

410.4 cells modified for IL-12-secretion (410.4-IL-12 cells) exhibited tumorigenic properties in BALB/c mice. To determine if the cells were rejected after an initial period of growth, or if progressive tumor development would lead eventually to the animals' death, immunocompetent BALB/c mice were injected s.c. with 10^6 410.4-IL-12 cells, or with an equivalent number of unmodified 410.4 cells. As indicated (Figure 3), the period of survival of BALB/c mice injected with 410.4 cells was significantly ($P < .01$) less than that of mice injected with 410.4-IL-12 cells, however, in both instances, progressive tumor growth occurred until one hundred percent of the animals succumbed to the disease.

4. Survival of BALB/c mice immunized with (irradiated) 410.4-IL-12 cells following a challenging injection of 410.4 adenocarcinoma cells.

The potential immunogenic properties of 410.4-IL-12 cells against the growth of 410.4 adenocarcinoma cells were determined in BALB/c mice. Since our prior results indicated that the injection of 410.4-IL-12 cells resulted in the formation of slowly growing, progressive neoplasms in the mice, the cells were subjected to 5000 rads X-irradiation before injection to prevent cell-growth. We wished to determine if the antigenic properties of the irradiated cells were preserved.

In the experiment, naive mice were immunized by injection s.c of 10^6 410.4-IL-12 irradiated cells. (The cells received 5000 rads X-irradiation (from a ^{60}Co source) immediately before injection.) The mice received two subsequent injections at weekly intervals of an equivalent number of irradiated 410.4-IL-12 cells. One week

after the last injection, the mice were challenged by an injection s.c. of 10^6 viable 410.4 cells. As controls, other naive BALB/c mice were immunized according to the same injection schedule with an equivalent number of irradiated (5000 rads) 410.4 cells or irradiated (5000 rads) 410.4 cells transfected with pSVNeo(X) (specifies neomycin-resistance, but not IL-12) before the challenging injection of 410.4 cells. The results (Figure 4) indicate that none of the mice immunized with 410.4-IL-12 cells developed tumors of 410.4 cells. The mice survived indefinitely. In contrast, mice immunized with (non cytokine-secreting) irradiated 410.4 cells or with 410.4 cells transfected with pZipNeoSV(X) before the challenging injection of viable 410.4 cells developed progressively growing neoplasms that led eventually to the animals' death.

Thus, the immunogenic properties of 410.4-IL-12 cells were preserved following irradiation. The cells exhibited immunotherapeutic properties against the growth of viable 410.4 breast adenocarcinoma cells in syngeneic mice.

5. Long-term immunity to 410.4 cells developed in mice immunized with irradiated 410.4-IL-12 cells.

The results of the prior experiment, presented in Section 4, above, indicated that immunizations with irradiated 410.4-IL-12 cells protected BALB/c mice against the development of tumors of 410.4 adenocarcinoma. One hundred percent of immunized mice challenged by an injection of viable 410.4 cells appeared to have rejected the cancer cells and survived indefinitely (more than 90 days).

The possible long-term immunotherapeutic properties of 410.4-IL-12 cells were determined by rechallenging the surviving mice with a second injection of viable 410.4 adenocarcinoma cells, 90 days after the first immunization. The results (Figure 5) indicate that all of the mice that began immunization 90 days previously with the irradiated 410.4-IL-12 cells, and survived the challenge of viable 410.4 cells, survived after a second injection of 410.4 cells. Under similar circumstances, one hundred percent of naive BALB/c mice injected with an equivalent number of 410.4

cells alone developed progressively growing neoplasms that led eventually to the death of the animals.

6. Growth of 410.4-IL-12 cells in BALB/c nude mice.

As described, 410.4-IL-12 cells retained their tumorigenic properties in syngeneic, immunocompetent BALB/c mice, however, the rate of tumor development was less than occurred in mice injected with unmodified 410.4 cells. Irradiated 410.4-IL-12 cells were immunotherapeutic toward the breast cancer cells as indicated by the finding that immunizations with the irradiated cells led to long-term immunity toward unmodified 410.4 cells in BALB/c mice.

To determine the relative growth rates of 410.4-IL-12 and 410.4 cells, 410.4-IL-12 cells, and, for comparison, 410.4 cells transfected with pZipNeoSV(X) were injected s.c. into BALB/c nude mice, and the rate of tumor growth of the two cell-types was compared. As indicated (Figure 6) both 410.4-IL-12 and 410.4 cells formed progressively growing tumors in the immune-deficient mice. Unlike tumor growth in BALB/c mice, the rate of tumor growth of 410.4 and 410.4-IL-12 cells was the same in the immune deficient animals.

CONCLUSIONS

We interpret these findings as follows:

1. Like other neoplasms, 410.4 breast adenocarcinoma cells expressed weakly immunogenic TAAs.
2. Modification of 410.4 cells for IL-12 secretion modified, but did not eliminate the cells' tumorigenic properties.
3. The antigenic properties of 410.4-IL-12 cells were retained if the cells were subjected to 5000 rads X-irradiation.
4. Immunization of BALB/c mice with irradiated 410.4-IL-12 cells conferred long-term immunity to the breast adenocarcinoma.
5. IL-12-secretion followed transfection of E3 cells with MFG-mIL-12.

Other Related Work in Dr. Cohen's Laboratory Supported by DAMD 17-96-1-6178

Background.

The human MUC1 gene is mapped to chromosome 1q21. It specifies polymorphic epithelial mucin (PEM). Expression of the MUC1 gene is up-regulated in breast carcinoma cells. The MUC1 gene is a site of frequent dysregulation in breast cancer cells, resulting in the formation of altered gene products that can potentially act as tumor associated antigens. Modification of the "normal" glycosylation pattern frequently occurs, resulting 'exposure' of the tandem amino acid repeat of the molecule. The immunity is directed toward the protein core, the polymorphic, variable number of tandem repeat region (VNTR).

The finding that there is polymorphism of the CA repeat (microsatellite) sequence within intron 6 of the MUC1 gene enabled us to examine both the microsatellite and the VNTR region of the MUC1 gene in paired DNA samples from the neoplastic and nonneoplastic cells of the same breast cancer patient. The objective was to determine if the MUC1 gene was a site of unusual alteration in human breast cancer cells. To accomplish this goal, we investigated paired DNA samples from the normal and malignant cells of 118 breast cancer patients for alterations of the MUC1 gene and the microsatellite region within the gene. The results have important implications for our understanding of the genetic mechanisms leading to the origin of TAAs specified by the MUC1 gene in breast cancer cells.

The data along with details of the methods used are presented in the enclosed manuscript (galley proofs) supported in part by the award. The paper, by MR Waltz, SM Pandelidis, W. Pratt, D. Barnes, DM Swallow, SJ Gendler and EP Cohen, is entitled, "A Microsatellite within the MUC1 Locus at 1q21 is Altered in the Neoplastic Cells of Breast Cancer Patients," (Cancer Genetics Cytogenetics, in press").

The results of the study indicated that the frequency of alteration at the microsatellite within the MUC1 locus was significantly ($P < .001$) higher than the frequency of alteration at two other genetic loci (D1S104 and APO-A2) not known to be involved in breast cancer. An analysis of the VNTR region within the MUC1 gene revealed an amplification of one allele in 34 of 54 informative (heterozygotes) cases (63%). However, there was no significant association between alterations at the microsatellite within the MUC1 locus and the VNTR region within the same locus, indicating that independent genetic mechanisms were responsible for the changes. Thus, the MUC1 gene at 1q21 is a genetically unstable region. The instability is consistent with the high frequency of expression of the gene-products in breast cancer cells.

Progress in Professor Taylor-Papadimitriou's laboratory.

Background

Polymorphic epithelial mucin (PEM) is a product of the MUC1 gene, a site of frequent genetic alteration. It is expressed in more than 90% of human breast cancers. PEM is a potential target antigen for the Active Specific Immunotherapy of breast cancer.

The major aim of this related project is to evaluate methods for augmenting the immunogenicity of PEM specified by the MUC1 gene to develop an immunogen that induces effective immune responses against the neoplasm, leading to tumor rejection.

Breast cancer cells, like other neoplasms, are poor antigen presenting cells. They lack essential co-factors required for antigen-presentation. One approach toward augmenting the cells' immunogenic properties is to modify the cells to express B7.1, a co stimulatory molecule required for T cell activation.

Work Content

Development of a transgenic mouse expressing human PEM.

A transgenic mouse expressing the product (PEM) of the human MUC1 gene has been developed. The genetically modified animals express human PEM, and are naturally tolerant to the molecule. In the transgenic mice, PEM is expressed by the same epithelial tissues as in humans. The transgenic mice are therefore the closest model system for analyzing approaches for the induction of an immune response to human PEM. Like patients, the transgenic mice consider human PEM as "self." A map of the construct used to generate the founder mice is presented in Figure 7.

Transgenic mice that were modified to express the MUC1 gene are Sac II mice (H-2^k). E3 cells modified as described above express human PEM. They are of BALB/c origin (H-2^d) and are maintained, therefore, in SacII X Balb/c F1 mice (H-$2^{k/d}$).

Techniques used to prepare MUC1 Transgenic Mice

Previous studies indicated that a 1.4kb sequence 5' to the MUC1 gene was sufficient to drive tissue specific expression of a reporter gene such as the CAT gene or bacterial lacZ gene by breast cancer cells maintained *in vitro*. In order to determine if this portion of the promoter is able to drive expression *in vivo*, transgenic mice were modified using the 1.4kb promoter sequence to drive the MUC1 cDNA.

The construct was made in a promoterless mammalian expression vector pNASSβ (Clontech). This vector originally specified the bacterial β-galactosidase gene located within NotI restriction sites. The 1.4kb MUC1 promoter was initially cloned into the vector and used to drive expression of the β-gal gene *in vitro*. Expression was observed in ZR75 cells, a breast carcinoma cell line that usually expresses

MUC1, but not in HT1080, a fibrosarcoma cell that does not ordinarily express MUC1.

The β-gal gene was then replaced with full length MUC1 cDNA. This construct was then used to produce the MUC1 transgenic mice.

A total of 17 possible founders was initially identified. Seven of these mice failed to transmit the transgene to their litters. The remaining ten founders transmitted the transgene to their offspring, but offspring from six of these failed to show expression of the human MUC1 gene.

In offspring from two of the four founders that transmitted the transgene, expression of the MUC1 gene was seen only in a restricted range of epithelial tissues (Figure 8). Tissues such as the salivary gland, kidney, uterus, fallopian tubes, lungs, stomach and testis showed MUC1 expression, whereas little expression was found in the pancreas and mammary gland. Furthermore, up-regulation of expression was not seen in the mammary gland at lactation. Non-epithelial tissues such as the muscle, heart and spleen were negative for expression of the MUC1 gene.

However, in lines from the two remaining founders, expression was observed in the tissues mentioned above as well as in the pancreas. Up-regulation of expression was also found in the mammary gland at lactation.

Generation of E3 and 410.4 mouse adenocarcinoma cells for the expression of B7.1, a costimulatory molecule required for T cell activation.

E3 and 410.4 cells were transfected with a plasmid (murB7piLN) specifying the murine B7.1 gene. A map of the plasmid used in the experiment is presented in Figure 9. The vector includes both the murine B7.1 gene and a gene conferring neomycin resistance, used for selection. For use as controls, 410.4 and E3 cells

were transfected with analogous vectors specifying antibiotic resistance, without the gene for B7.1.

Figure 10 is an outline of the scheme used to derive 410.4 and E3 cells modified to express B7.1 in combination with antibiotic resistance genes, used for selection, or of 410.4 and E3 cells modified to express antibiotic resistance genes alone.

Table 2 outlines the clones of B7.1 transfectants that were developed. Three clones expressing both mB7.1 and MUC1 but with different levels of expression of mB7.1 were investigated. Ben3 7.1 expressed the highest level of mB7.1 and was used in the majority of the experiments. Neither parental 410.4 cells nor cell lines expressing only selectable markers with or without MUC1 show detectable levels of mB7.1 when analyzed by flow cytofluorometry using the fusion protein CTLA4-Ig or a monoclonal antibody to mB7.1.

The tumorigenic properties of B7.1 modified adenocarcinoma cells were determined in TG18 transgenic X BALB/c F1 mice and related to the level of expression of B7.1 by the modified cells. The experiment was carried out TG18 transgenic X BALB/c F1 mice injected with clones expressing different levels of B7.1.
The results are described in Table 3.

Thus, the period of survival of MUC1 transgenic mice injected with breast adenocarcinoma cells modified for MUC1 and the expression of high levels of mB7.1 was significantly ($P < .01$) greater than that of mice in each of the other groups.

REFERENCES

Waltz, M.R., Pandelitis, S.M., Pratt, W., Barnes, D., Swallow, D.M., Gendler, S. and Cohen, E.P. A microsatellite within the MUC1 locus at 1q21 is altered in the neoplastic cells of breast cancer patients. Cancer Genetics and Cytogenetics. In Press.

FIGURE 1

Growth of 410.4-IL-12 cells in Balb/C mice.

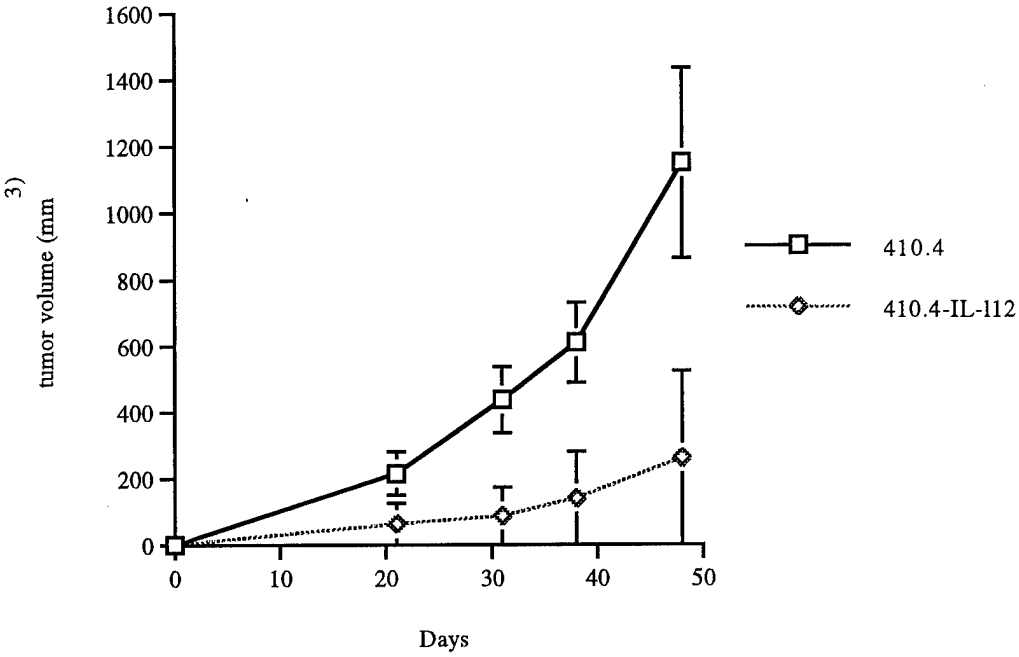


FIGURE 2

Tumor growth in BALB/c mice injected with a mixture of 410.4 and 410.4-IL-12 cells.

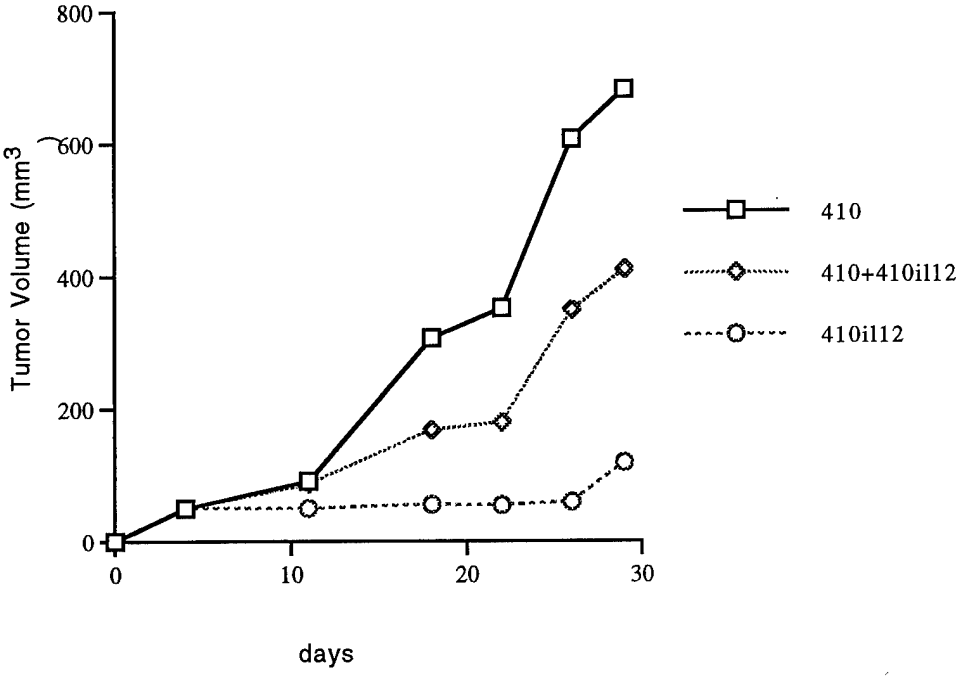


FIGURE 3

Survival of BALB/c mice injected with 410.4-IL-12 cells.

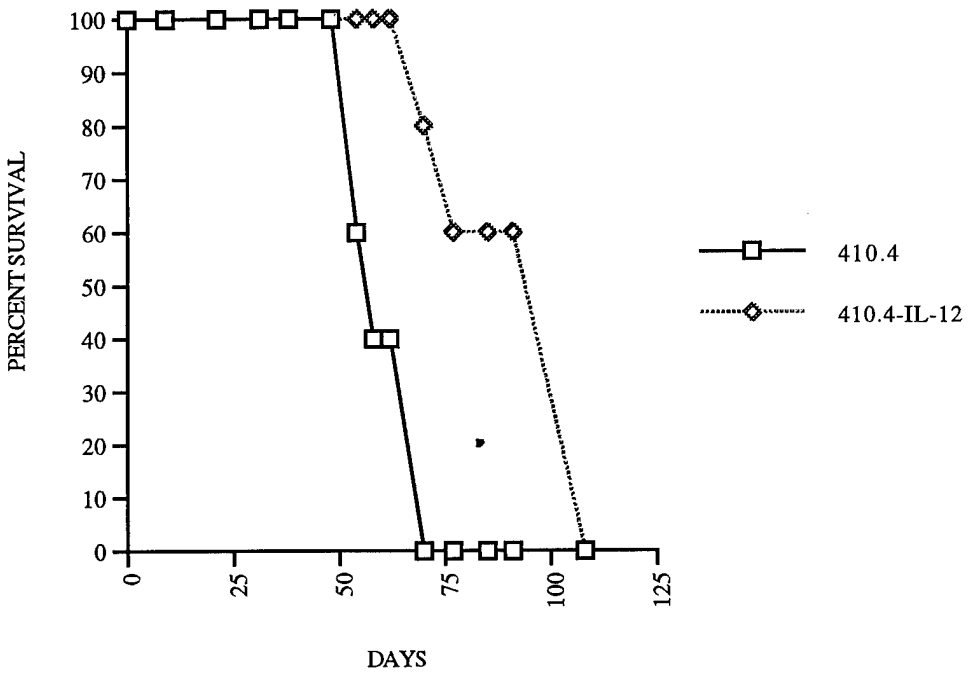


FIGURE 4

Immunization with X-Irradiated 410.4-IL-12 Cells Protects Mice against the Growth of 410.4 Breast Cancer Cells

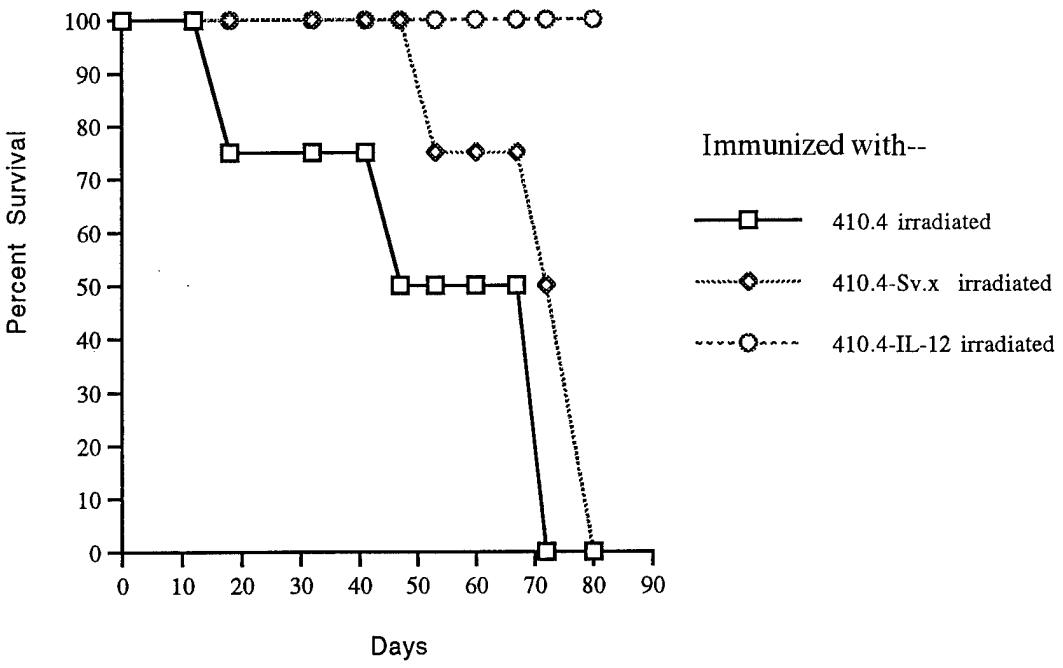


FIGURE 5

Tumor growth in mice with breast cancer surviving after treatment with 410.4-IL-12 cells rechallenged with 410.4 breast cancer cells.

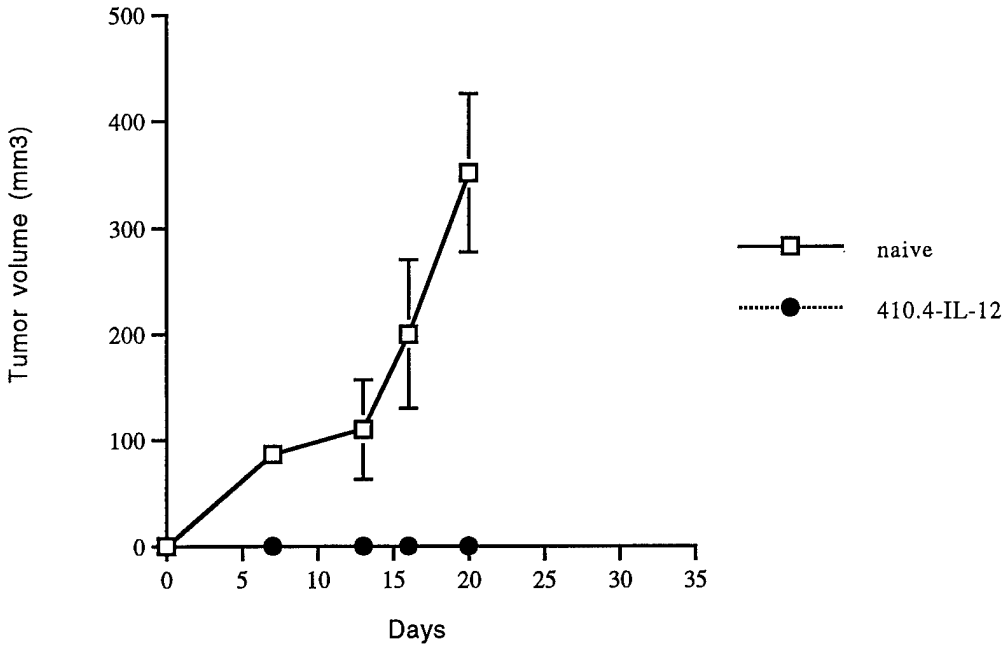


FIGURE 6

Growth of 410.4-IL-12 cells in BALB/c nude mice.

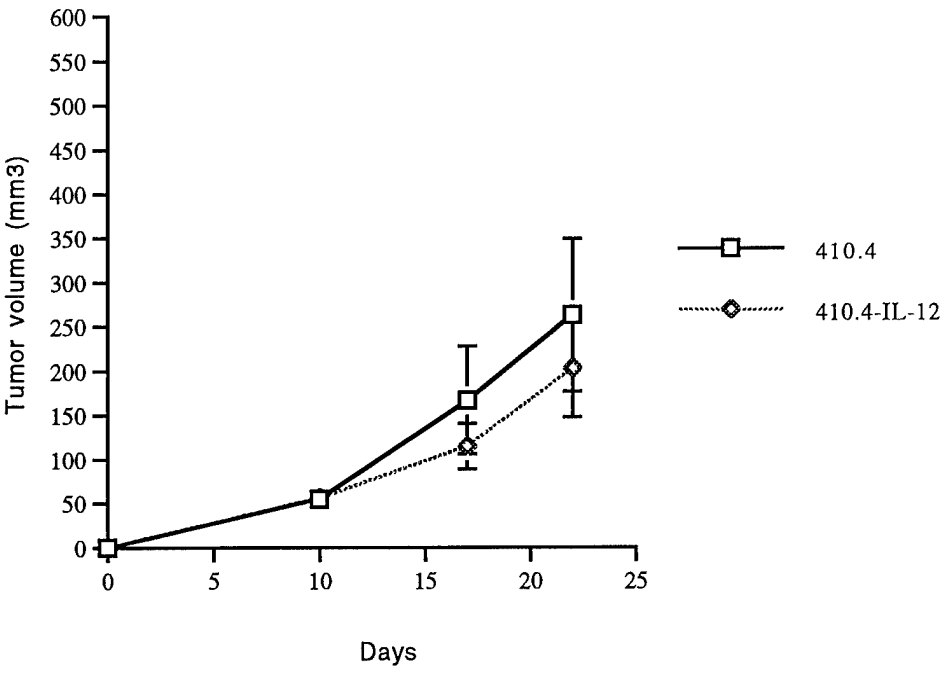


FIGURE 7

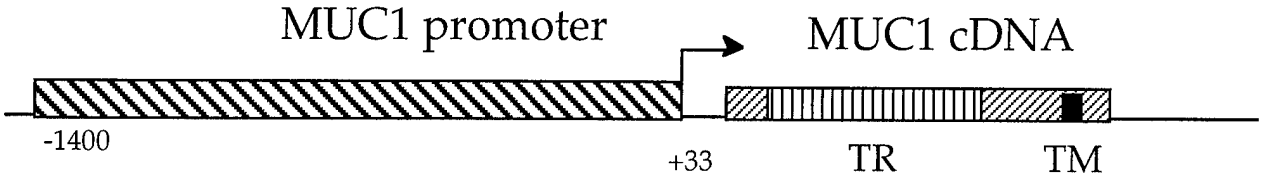
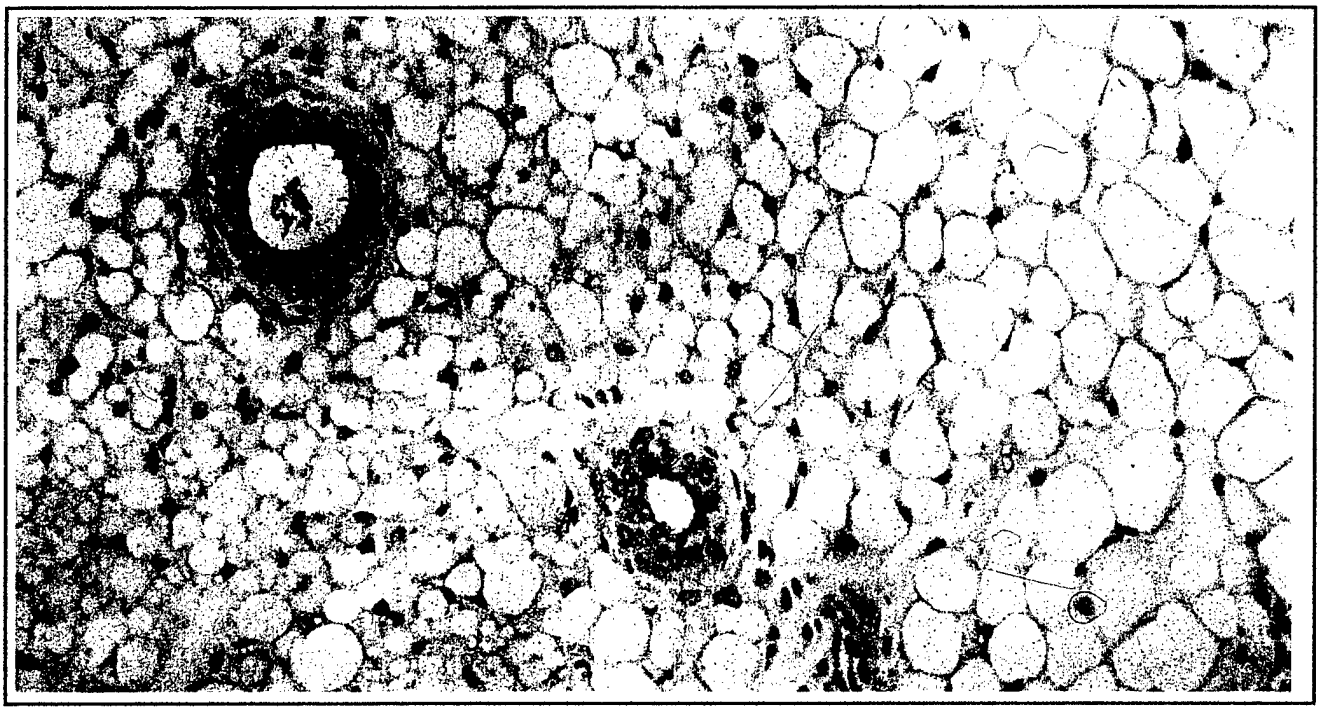


FIGURE 8

A- Expression of MUC1 in normal mouse mammary gland



B- Up-regulation of MUC1 expression in lactating mouse mammary gland.

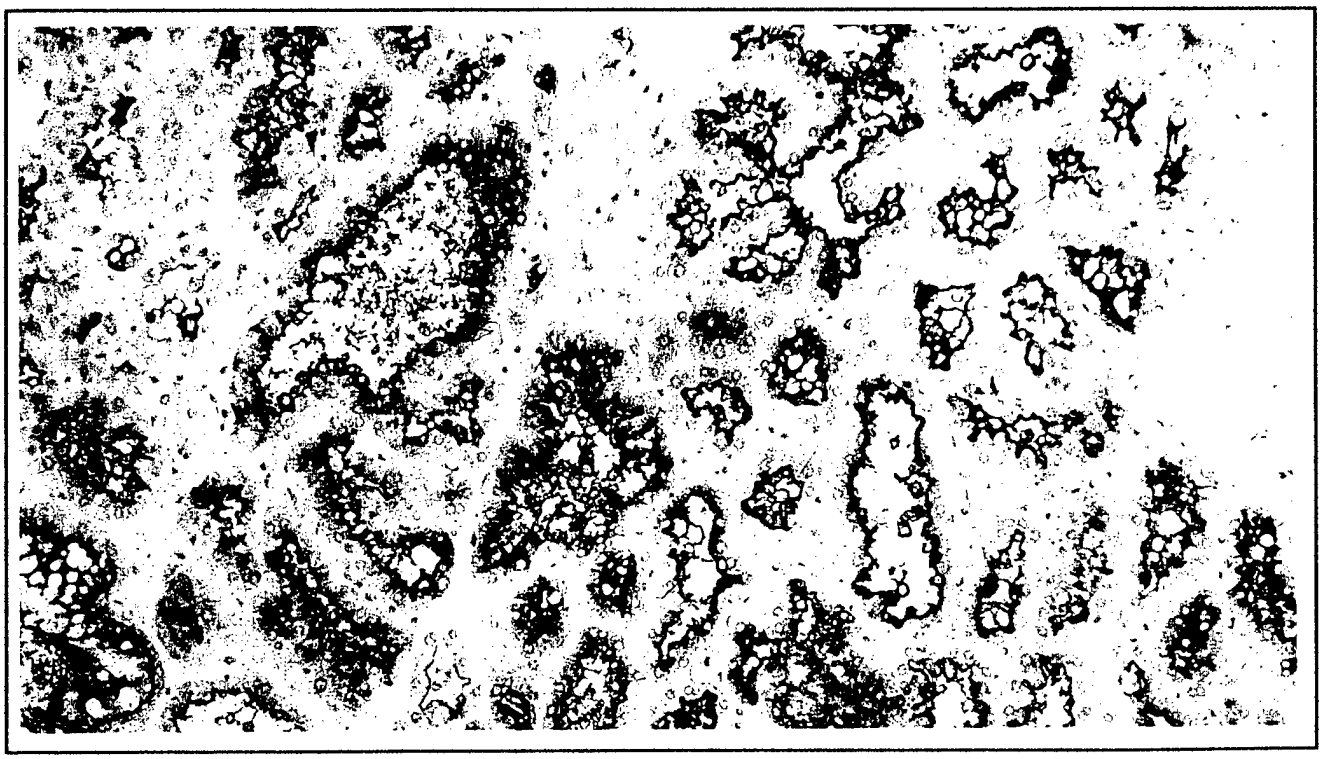
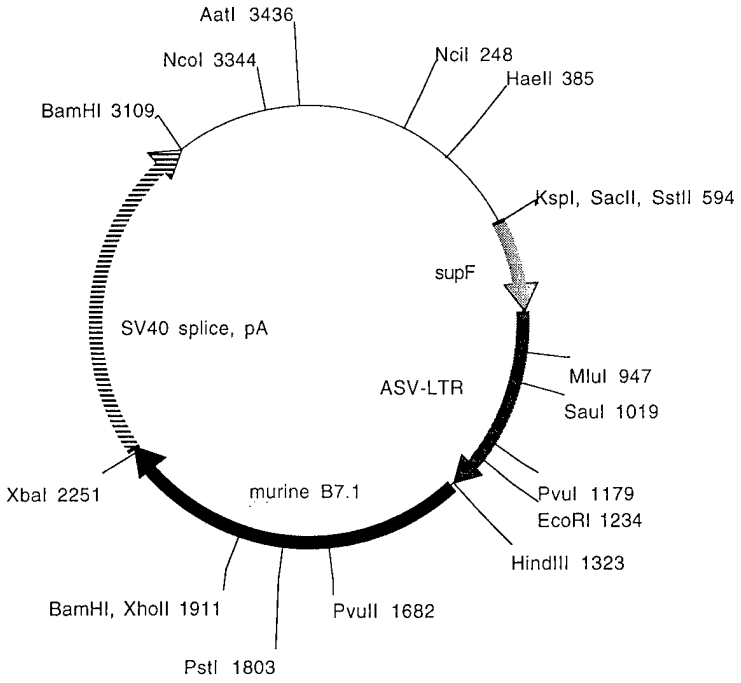


FIGURE 9



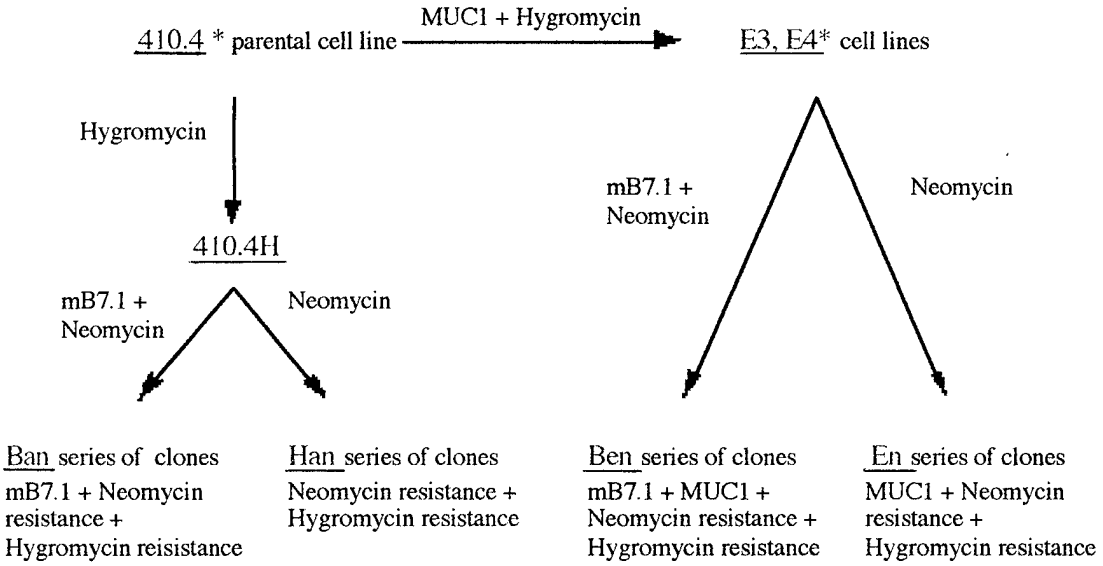
Plasmid:	murB7piLN
Plasmid size	3462bp
Constructed by L. Chen et al., (1992) Cell Vol. 71 No. 7 p1093-1102	

FIGURE 10

Development of 410.4 cell lines expressing mB7.1

Three separate cell lines were selected for transfection: The E3 and E4 cell lines, mouse mammary adenocarcinoma cells which had been previously transfected with the MUC1 gene, and the parental cell line 410.4.These lines were transfected with selectable marker plasmids, and or B7.1 as indicated below.

The derivation of 410.4 cell lines expressing mB7.1 and or selectable markers



The names of cell lines are ~~marked in red~~ underlined. The genes and antibiotic resistance genes transfected to produce the cell lines are positioned next to the arrows of the respective transfections. The cell lines which were already available are marked with an asterix.

Four series of cell lines were produced. All final clones in each series Ban, Han, Ben and En express both Neomycin and Hygromycin resistance genes. The Ban series express mB7.1 along with the resistance markers. The Han series express the resistance markers only. The Ben series express both mB7.1 and MUC1 along with the resistance markers. The En series express MUC1 along with the resistance markers.

TABLE 2

Clones selected for use in further experiments

Name of clone	*Expression*			
	Murine B7.1	MUC1	Hygromycin resistance	Neomycin resistance
410.H			+	
Han1.3			+	+
Ban9	+++		+	+
Ban27*	++		+	+
En3 3.1		++	+	+
Ben4*	++	++	+	+
Ben3 7.1	+++	++	+	+
Ben7	++	++	+	+
Ben84	+	++	+	+

TABLE 3

The tumorigenic properties of B7.1 modified adenocarcinoma cells

Group	Observations	Died	Median survival (days)	Mean survival (days)
1. Ben3 7.1	15	4	(-)	480
2. Ben7	15	8	110	206
3. Ben 84	15	12	75	103
4. En3 3.1	10	9	89	104

Ben3 7.1 = high mB7.1; MUC1 (+)
Ben7 = intermediate mB7.1; MUC1 (+)
Ben84 = low mB7.1; MUC1 (+)
EN3 3.1 = mB7.1 (-); MUC1 (+)

The MUC1 transgenic mice received a s.c injection of 5 X 10^6 of the relevant genetically modified cells.

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APPENDIX

ELSEVIER

A Microsatellite within the MUC1 Locus at 1q21 is Altered in the Neoplastic Cells of Breast Cancer Patients

Margaret R. Waltz, Steven M. Pandelidis, Wendy Pratt, Diana Barnes, Dallas M. Swallow, Sandra J. Gendler, and Edward P. Cohen

ABSTRACT: *Paired DNA samples from the neoplastic and nonneoplastic cells of 118 patients with the sporadic, nonfamilial form of breast cancer were analyzed for evidence of genetic alteration at a polymorphic microsatellite mapped to intron 6 within the MUC1 gene at 1q21. Two other microsatellite loci, D1S104 and APO-A2, which also map to 1q21, were analyzed as well. The frequency of alteration at the microsatellite within the MUC1 locus was significantly higher than D1S104 or APO-A2 ($P < 0.001$). Analysis by Southern blotting of the VNTR region of the MUC1 gene revealed an amplification of one allele in 34 of 54 informative cases (63%). There was no significant association between these alterations and alterations of the microsatellite within the same locus, suggesting independent mechanisms were responsible for the genetic changes. Microsatellite loci D17S579 at 17q21, the site of the BRCA-1 gene, and D18S34 at 18q21-qter, the deleted in colorectal cancer locus, were also analyzed by PCR. Alterations at D17S579 and D18S34 were detected in 18.8% and 6.2% of patients, respectively ($P < 0.001$, and $P < 0.1$ relative to the frequency of alteration at D1S104 or APO-A2). A previously described polymorphism of hMSH2 was altered in 16.4% of cases.* © *Elsevier Science Inc., 1997*

INTRODUCTION

An accumulation of genetic changes within an individual somatic cell is responsible for conversion to the malignant phenotype [1–4]. As the malignant cell population increases, genetic instability often results in the appearance of neoplastic cells with varying abnormal properties, such as the capacity for metastasis, and resistance to drugs commonly used in cancer treatment [5–8].

Various genetic loci have been identified that are frequently altered in neoplastic cells. Certain changes are associated with the malignant phenotype. The BRCA-1 gene at 17q21, and the VNTR region of the MUC1 gene at 1q21 are notable examples [9–11]. A high frequency of alterations in microsatellite sequences [12–17] in genomic DNA from the tumor can be a first indication of the presence of significant genetic change in neoplastic cells, and may lend insight into the genetic mechanisms involved in mediating the alterations [15].

Pratt et al. [18], recently described the polymorphism of a microsatellite (CA-repeat) sequence within intron 6 of the MUC1 gene. The gene includes a polymorphic VNTR region within exon 2 that is frequently altered in breast cancer cells [11]. Figure 1 illustrates the location of these features of the MUC1 gene. Whether the microsatellite is altered in breast cancer cells and whether such alterations are associated with alterations of the VNTR region has not been determined.

To investigate these questions, we analyzed the microsatellite and VNTR region of the MUC1 gene in paired neoplastic and nonneoplastic cells of 118 patients with the sporadic form of breast cancer. In addition, four other loci were analyzed for microsatellite instability. Two of these have been associated with tumor suppressor genes, *D17S579* at 17q21 [9] and *D18S34* at 18q21-qter [19], whereas the other two, *APO-A2* and *D1S104*, are located near the MUC1 gene, at 1q21.

MATERIALS AND METHODS

Human Tissue Samples

Fresh solid tumor tissues, taken during the usual course of the patients' treatment, were dissected free of fat and stored in liquid nitrogen. Peripheral blood buffy coat cells obtained at the time of surgery were used as the source of

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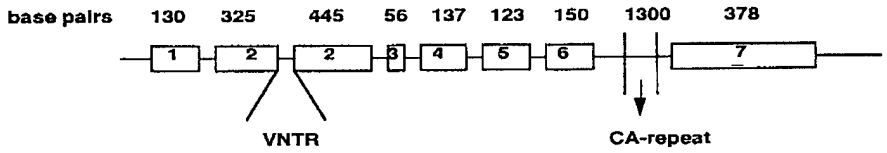

Figure 1 Diagram of the MUC1 gene. The variable number tandem repeat region (VNTR) is located within exon 2 of the gene. The CA repeat is located within intron 6 of the gene.

DNA from nonneoplastic cells from the same individuals. Confirmation of the malignant nature of the tumor was determined in stained paraffin-embedded sections, prepared according to conventional techniques.

Preparation of High–Molecular-weight DNAs From Paired Neoplastic and Nonneoplastic Cells of the Same Individuals With Breast Cancer

Frozen tumor tissues (approximately 0.3 gms) were disaggregated in a Braun (Melsuugen, Germany) Mikro-disembrator II, and the DNA was isolated from the cells in an Applied Biosystems (Foster City, CA) 340A DNA extractor. Nonneoplastic cells from the patients peripheral blood were lysed with water before addition to the extractor. After dialysis, the DNA concentrations were measured spectrophotometrically. Before the various analyses were performed, aliquots of undigested DNA from the paired samples were subjected to electrophoresis through 0.7% agarose gels. Degraded specimens were not included.

Analysis of Microsatellite DNAs From Paired Neoplastic and Nonneoplastic Specimens of the Same Patients

Analyses of each of five microsatellite loci were performed by PCR, using oligonucleotide primers that flanked the region of interest. The loci investigated and the primer sequences are presented in Table 1. Fifty-microliter reaction mixtures consisted of 300 ng of DNA 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1% Triton X-100), 2 mM $MgCl_2$, 1.25 mM of each dNTP, 50 pMol of each primer, 3 uCi α-^{32}P-dCTP, and 0.5 units Taq polymerase (Promega, Madison, WI). The samples were overlaid with oil, placed in a thermocycler (Perkin-Elmer, Norwalk, CT) and subjected to 27 cycles of 94°C, 45 seconds; 55°C, 45 seconds; 72°C, 45 seconds. That the PCR products were of the correct size was confirmed by agarose gel electrophoresis. The PCR products were separated in 8% polyacrylamide denaturing gels and then exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C for 1 to 3 days.

The autoradiographs were compared for differences in the microsatellites from the neoplastic and nonneoplastic cells of the same patients. Changes in the size of a band, deletion or addition of a band in the DNA from the tumor, compared to DNA from the nonneoplastic cells, were considered as evidence of alterations.

Analysis of the VNTR Region of the MUC1 Gene at 1q21 in the Neoplastic and Nonneoplastic Cells of Breast Cancer Patients

Southern blotting was used to detect possible alterations in the polymorphic VNTR region of the MUC1 gene in the neoplastic cells of the breast cancer patients. Approximately 10 μg of DNA from the neoplastic or nonneoplastic cells of the same patients was digested to completion with Hinf1 (Gibco BRL, Gaithersburg, MD), and fractionated in 0.7% agarose gels. HindIII digested lambda DNA (Gibco BRL, Gaithersburg, MD) was used as a molecular-weight marker. After electrophoresis, the agarose gels were denatured in alkali and transferred to nylon membranes (Biodyne, Pall, Glen Cove, NY). After transfer, the membranes were incubated for 1 hour at 80°C under vacuum, and then hybridized with a probe homologous to the VNTR region at exon 2 of the MUC1 gene [20]. The probe was labeled to highly specific activity with [a-^{32}P]-dCTP by the random priming method [21]. Hybridization was performed at 42°C in the presence of nonspecific DNA (herring testis, Promega, Madison, WI) in a buffer containing 5X SSC (1X SSC in 150 mM NaCl, 15 mM trisodium citrate, (pH 7.0), and 50% formamide). After incubation, the filters were

Table 1 Sequence of primers used for analysis of microsatellites

Microsatellite	Primer Sequence	Reference
D17S579	5′-AGT CCT GTA GAC AAA ACC TG 5′-CAG TTT CAT ACC AAG TTC CTA	[15]
D18S34	5′-CAG AAA ATT CTC TCT GGC TA 5′-CTC ATG TTC CTG GCA AGA AT	[17]
APO-A2	5′-GGT CTG GAA GTA CTG AGA AA 5′-GAT TCA CTG CYG TGG ACC CA	[10]
D1S104	5′-ATC CTG CCC TTA TGG AGT GC 5′-CCC ACT CCT CTG TCA TTG TA	[23]
Ms within MUC1	5′-AGG AGA GAG TTT AGT TTT CTT GCT CC 5′-TTC TTG GCT CTA ATC AGC CC	[26]

Table 2 Summary of microsatellite alterations

	MUC1	D17S579	D18S34	D1S104	APOA2
No. of cases	104	101	94	74	70
No. of information cases	100 (96.1%)	90 (89.1%)	81 (87.2%)	65 (87.8%)	70 (100%)
Total no. of changes	24 (23.3%)	17 (18.8%)	5 (6.2%)	3 (4.7%)	2 (2.8%)
LOH	5	9	3	0	0
Allelic imbalance	7	4	1	0	0
Size change	4	4	1	2	1
Additional bands	8	0	0	1	1

washed at 55°C and exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C for 1 to 3 days.

Analysis by SSCP of the hMSH2 Locus in the Paired Neoplastic and NonNeoplastic Specimens of the Breast Cancer Patients

Analysis of the conserved region of hMSH2, a replication error repair gene [2], was performed according to the method described by Orita et al. [22], using oligonucleotide primers specific for codons 668-736 of the hMSH2 locus. The primers were:

PF:5′-CGC GAT TAA TCA TCA GTG-3′
PR: 5′-GGA CAG AGA CAT ACA TTT CTA T-3′.

The reaction mixture consisted of 200 ng genomic DNA, 5 ml of 10X reaction buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 50 pMol of each primer, 25 nMol of each nucleotide, 10 mCi [a-^{32}P]-dCTP, and 2.5 Units Taq polymerase (Promega, Madison, WI) in a total volume of 50 µl. The reaction mixtures were cycled 27 times, at 94°C, 45 seconds; 57°C, 45 seconds; and 72°C, 45 se onds for each cycle. Afterward, 6 µl of the mixture was heated to 95°C for 3 min before it was analyzed in a neutral 7% polyacrylamide gel containing 10% glycerol. The gels were subjected to electrophoresis at room temperature for 6 hours at 50 W. They were dried and exposed to XAR-5 film (Kodak, Rochester, NY) at −70°C for 1 to 3 days. Sequencing of the PCR products was performed using a double strand DNA Cycle Sequencing System (Gibco/BRL, Gaithersburg, MD), according to the directions of the supplier.

RESULTS

A Microsatellite at Intron 6 Within the MUC1 Gene at 1q21 is Altered in the Neoplastic Cells of Breast Cancer Patients

The microsatellite within the MUC1 gene was analyzed in the paired neoplastic and nonneoplastic specimens from 100 informative patients with the sporadic form of breast cancer. For comparison, two other microsatellite loci mapping to 1q21, and two loci at 17q21 and 18q21, which are associated with tumor suppressor genes, were also analyzed.

Table 2 indicates the changes in microsatellites observed and a breakdown of the changes seen at each locus. The numbers of Losses of Heterozygosity (LOH), size changes, and additional bands present are indicated. "Allelic imbalance" has been used to describe those microsatellite alterations that appear as an increase or decrease in the band intensity of one allele relative to the other allele [8]. The frequency of alteration of the microsatellite at intron 6 within the MUC1 gene at 1q21 was significantly ($P < 0.001$) higher than that of *APO-A2* and *D1S104*, the other two microsatellites at the same locus. *D17S579* and *D18S34* were also altered at a high frequency relative to *APO-A2* and *D1S104*, 18.8% and 6.2%, respectively. Figure 2B and C presents the types of changes observed at microsatellite loci. The majority (57%) of alterations were

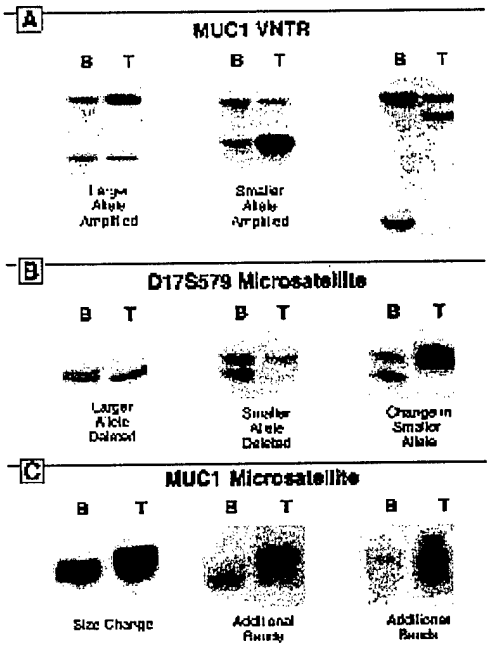

Figure 2 Representative examples of alterations of the VNTR of MUC1 and microsatellite loci. "B" denotes DNA extracted from blood, and "T" denotes DNA extracted from tumor.

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LOH, 23% were size changes of an allele, and 20% were indicated by the presence of additional bands.

The VNTR Region Within the MUC1 Gene at 1q21 was Altered in the Neoplastic Cells of the Breast Cancer Patients

An analysis of the polymorphic VNTR within the coding region of the MUC1 gene revealed a high frequency of alteration in the neoplastic cells of the patients. The analysis was performed by Southern blotting. Representative alterations are presented in Figure 2A. The gene was altered in 34 of 54 informative patients. There was LOH in 3 instances (5.2%), amplification of one of the two alleles in 32 cases (56%), and one translocation (1.8%). Amplification of either allele was considered as "allelic imbalance" in Table 2.

Of the three cases of LOH at the VNTR of the MUC1 gene, none showed LOH of the microsatellite in intron 6 of the gene. This was unexpected, as losses of heterozygosity tend to reflect the deletion of large segments of genetic material [26]. If such a loss had occurred at 1q21, both the VNTR and the microsatellite should show LOH. However, an alternate explanation for the LOH at the VNTR is that there has been homologous recombination of the MUC-1 gene in the tandem repeat region, resulting in two alleles of the same size. If this were the case, the 1q21 locus may be present, though the VNTR of the MUC1 gene is no longer heterozygous. This would explain how the three individuals with LOH at the VNTR may also be informative for the three microsatellite markers at 1q21.

There was no significant association between an alteration of the VNTR region and an alteration of the microsatellite at intron 6 within the MUC1 gene in the neoplastic cells of the same patient ($P > 1.0$). The likelihood that patients whose neoplastic cells revealed both an alteration at the VNTR region and alteration of the microsatellite within intron 6 was not greater than random.

Alterations of the hMSH2 Gene were Not Detected in the Neoplastic Cells of the Breast Cancer Patients

The hMSH2 gene encodes a DNA repair protein that binds to base pair mismatches generated during DNA replication [2]. An analysis of the gene for hMSH2 was carried out by SSCP, using primers for codons 668-736 and flanking sequences (corresponding to bps 2072 to 2208) of the hMSH2 locus [2]. This portion of the gene is conserved across species and is thought to encode the DNA binding region of the protein. Sixteen of 97 cases examined (16.4%) revealed an alteration of the hMSH2 gene. The same alteration was present in both the neoplastic and nonneoplastic cells of the same patient. DNA sequencing was carried out to characterize the alteration in greater detail. It revealed a G to T transition at the −6 splice acceptor site. Because the alteration was found in DNA from both normal and tumor cells of the same individuals, it was likely that the variation was a germline, rather than somatic change in sequence. (These data are not presented.)

There was no significant association between a change in the hMSH2 gene and a change in any of the microsatellite loci investigated. DNA from the neoplastic cells of one patient revealed instability of microsatellite DNA at two of the five loci. The hMSH2 gene was not altered in the neoplastic or nonneoplastic cells of this patient.

DISCUSSION

Genetic instability of malignant cells is indicated by widespread alterations in microsatellite DNAs. Because the alterations are not random, they are taken as indications of genetic changes that are invovled in the malignant phenotype. Here, we present the results of a comparative analysis of five microsatellite loci and of the VNTR region of the MUC1 gene in malignant and nonmalignant cells of 118 breast cancer patients. The investigation was an extension of prior studies in which it was found that the MUC1 gene was frequently altered in the neoplastic, but not the nonneoplastic cells of patients with primary breast carcinomas [19].

Both the VNTR region of the MUC1 gene and a microsatellite within the same gene were altered in a high proportion of the patients. However, the likelihood that changes in the VNTR region were accompanied by changes in the microsatellite within the gene was not greater than random. This finding may not be unexpected, as microsatellite and VNTR loci, though both frequent sites of genetic alteration, may be subject to different types of mutation. Indeed, the data were consistent with the presence of different genetic mechanisms responsible for alteration of the VNTR region and a change in the microsatellite. Because the MUC1 gene includes tandemly repeated DNA, it is possible that alteration of the VNTR region was mediated by homologous recombination, whereas alterations in microsatellite DNA may arise from slippage during the replication of simple repetitive sequences, followed by failure of the cell to repair the damage. In support of this hypothesis is a previous finding that VNTR loci are not affected by the replication error phenotype [15]. Why the CA repeat within the MUC1 gene was altered at a higher frequency than two other microsatellites at 1q21 was not determined. Conceivably, a change in the microsatellite conferred an undefined growth advantage to the malignant cells.

The Replication Error Repair (RER^+) phenotype has been found in HNPCC colon cancers as well as in other sporadic tumors associated with HNPCC syndrome [1,3, 23]. In our investigation, microsatellite alterations were occasionally detected, though most were losses of heterozygosity. None of the specimens revealed the ladder-like expansions seen in the microsatellite instability of colorectal cancers [1,3]. Similar changes have been noted previously in studies of patients with the sporadic form of breast cancer [23].

Fishel et al. [2] and Leach et al. [3] reported that approximately 60% of patients with colon cancer with the RER^+ phenotype had mutations of the hMSH2 gene. We attempted to detect analogous changes at the hMSH2 locus in the specimens we examined. A T to C substitution at the −6 position of the splice acceptor site was present in 16 of 97 cases. However, because it was found in both the neoplastic as well as nonneoplastic cells of the same individuals, it was likely to have been a ploymorphism. Leach et al. [3] found the same mutation in 2 of 20 tumor-free

individuals. No significant association between this alteration at the hMSH2 locus and alterations of the microsatellite loci was detected.

We conclude that alterations in microsatellites in sporadic breast cancer are likely to result from a different mechanism from that responsible for microsatellite instability in HNPCC. In addition, we confirm the high frequency of alteration in the region of the BRCA1 locus and in the VNTR at exon 2 of the MUC1 gene in sporadic breast cancer. The high frequency of alteration of the CA repeat within the gene was an unexpected finding. Its possible involvement in progression of the tumor remains to be determined.

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