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13. ABSTRACT *(Maximum 200*

The purpose of this grant is to examine interactions between stroma and epithelium in mammary oncogenesis in transgenic mice that expressing Shope growth factor (SGF). Different lines of SGF transgenic mice express this EGF-like cytokine using inducible (metallothionein, MT) and constitutive (RSV-LTR) promoters. We have fulfilled and surpassed stated goals for year 01. The first project year proposed producing sufficient SGF-transgenic mice from RSV-SGF and MT-SGF lines for analysis of mammary development and tumorigenesis, including measuring SGF expression and expression of genes associated with mammary cancer in humans and animals. Availability of sufficient mice has permitted us to begin these histologic and molecular analyses. We have, further, made functional anti-SGF antiserum. Breeding between RSV- and MT-SGF mice, and p53– mice, has begun, to address experimentally the strong association of p53 mutations with human breast cancers, and to test whether this association holds for experimental mammary carcinogenesis in this system. Thus, we have initiated new studies that are progressing well, and progress in pursuing specific aims is on or ahead of schedule. We expect this project to be completed as scheduled.

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Introduction

Nature of the problem/project

In our original application, we proposed to study mammary gland (MG) tumor development in transgenic mice that express Shope growth factor (SGF). SGF is an epidermal growth factor (EGF)-like growth factor that elicits mammary tumors in virgin transgenic mice. Because we had found that SGF is produced principally in the stromal cells of the mammary gland (and other organs), and that the proliferative and differentiative responsiveness to SGF was principally by the epithelial cells of the mammary gland, we proposed to use this system to study the mechanisms of stromal and epithelial cell interaction to produce preneoplasias and tumors in SGF transgenic mice.

Background of Previous Work by ourselves and others

This background section will discuss (i) the roles of EGF-like growth factors (ELF) in cell activation and growth; (ii) participation of ELF in oncogenesis, particularly mammary and breast oncogenesis; (iii) what is known about interactions between stromal and epithelial cells in oncogenesis; and (iv) mammary oncogenesis in SGF transgenic mice. The first two subsections of this background reflect work performed by our and other laboratories. The final subsection describes work done on this system in our laboratory.

(i). the roles of EGF-like growth factors in cell activation, growth and oncogenesis

There is a family of EGF-like growth factors(ELF). ELF stimulate responsive cells to proliferate and, sometimes, to differentiate (1). Therefore, ELF play roles in cell proliferation and differentiation activities such as oncogenesis, wound healing and organ maturation.

SGF is a glycoprotein related to EGF (2). It is encoded by malignant fibroma virus (MV), which produces malignant tumors of fibroblasts. Epithelial proliferation overlies fibrosarcomas in MV-infected animals, and is felt to represent the influence of SGF production by MV-infected cells. (3). When the SGF gene is deleted, MV's virulence is attenuated. Epithelial proliferation and tumor spread are diminished. Instead of dying uniformly, most animals survive (4).

To understand how SGF acts as a growth factor, free of other viral genes we produced transgenic mice that express SGF. In this setting SGF induces mammary differentiation, proliferative preneoplastic lesions, or invasive adenocarcinomas, depending on the promoter construct used and the animal's age when SGF expression begins. In this application, we propose to study how stromal and epithelial SGF secretion and responsiveness affects target cells and leads to neoplasia.

The family of ELF includes EGF, transforming growth factor-α (TGFα), amphiregulin, cripto, three poxviral products (SGF, vaccinia growth factor (VGF) and myxoma growth factor (MGF)) and the potential HER2/neu ligands gp30 and p175. These all differ in primary structure, but generally share a constrained tertiary structure characterized by 3 overlapping disulfide bonds (5). Of these growth factors, EGF and TGFα are the best understood. EGF and TGFα are 53 and 50 amino acids (aa) respectively, and are produced by cleavage of larger precursors (6). The TGFα precursor may be glycosylated and anchored at the cell membrane, but its post-translational modifications are lost when the secreted form is cleaved from its membrane bound precursor (7). Thus, secreted forms of EGF and TGFα are not glycosylated. The poxviral growth factors and gp30 are larger and are all glycosylated (8). SGF is encoded as an 80 aa polypeptide, then cleaved and glycosylated to 12-16 kDa (1,9,10).

The effects of most of this family on cells depend on interactions with EGF receptor (EGFR). EGF, VGF, SGF and TGFα all bind the extracytoplasmic region of EGFR at different sites (11-14). Human EGFR is a 170 kDa transmembrane glycoprotein that resembles the products of viral oncogene, v-*erb^b* and the cellular protooncogene HER2/neu (15). GF-receptor interaction initiates a cascade of events that leads to cell division. When ligand binds EGFR, the receptor oligomerizes and its affinity for ligand increases (16). Cytoplasmic EGFR tyrosine kinase activity is activated, and adjacent receptor molecules trans-phosphorylate each others' cytoplasmic domains (17).

Activated EGFR mobilizes a series of intracellular responses. Many enzymes associate with the cytoplasmic domain of EGFR via SH2 (src-homology-2) domains that bind activated EGFR phosphotyrosine, e.g., PI3 kinase, GTPase activating protein and phospholipase $C\gamma_1$ ($PLC\gamma_1$, 18-20). These enzymes are substrates for EGFR kinase activity. They are activated on phosphorylation by EGFR, and carry the message of ELF-induced cellular stimulation to the activation apparatus beyond. This leads to activation of $PLC\gamma_1$, followed by hydrolysis of phosphatidyl inositol phosphates into inositol phosphates, which increase intracellular Ca (21,22). Ras protein is also activated via intermediate proteins, Grb2 and Sos1 (23-25). Ras activation is associated with phosphorylation/activation of raf1 kinase, leading to activation of MAP kinases (26,27), and then of jun-fos AP1 transcription factor to increase gene transcription and initiate cell division (28,29).

(ii) participation of ELF in oncogenesis, particularly mammary and breast oncogenesis

The EGF group of cytokines is important in both organogenesis and oncogenesis. They are needed for normal development and differentiation of many organs, including kidney, GI tract, lung and breast. EGF or TGFα stimulate normal mammary ductal growth, even in the absence of steroids, and are vital to mammary differentiation (30-32). We have found that SGF may induce mammary tumors or differentiation in transgenic mice, depending on the timing of its induction. A similar observation has been made for gp30 (33).

Tumor development is also linked to signal transduction via EGFR. EGF, TGFα and EGFR appear to be important for tumorigenesis in several organs via an autocrine loop: tumors make EGF and/or TGFα, plus high levels of EGFR. Tumor cells make EGFR and an EGFR ligand grow more slowly when the ligand is removed with anti-GF antibody (34). Anti-sense RNA that blocks EGFR expression reportedly suppresses transformation (35).

EGF alone does not elicit phenotypic transformation, but it may do so in concert with other agents (36). The v-*erb^b* protein may transform cells precisely because it lacks ligand binding sites. This deficiency allows v-*erb^b* protein to be constitutively activated in the absence of ligand (37). In addition, cells with high concentrations of EGFR may become phenotypically transformed in response to low concentrations of EGF (38).

The roles of EGFR and HER2/neu in breast cancer have been studied extensively (39). Several investigators have reported that EGFR+ tumors make and/or respond to members of the EGF family (40,41). In addition, EGFR expression is associated with aggressive behavior and poor prognosis. Tumors that produce EGFR (EGFR+) are of higher grade and stage than EGFR- tumors, and express less estrogen receptor. EGFR+ tumors are also more highly proliferative and aneuploid (40). They tend to recur and kill patients more than EGFR- tumors (42).

Detectable EGFR may be the most accurate predictor of survival (43). *C-erb^b* (HER2/neu) protein is an EGFR-like receptor, whose expression has also been associated with poor prognosis in breast and other tumors (44).

A better understanding of the roles of ELF in oncogenesis has come from the study of transgenic animals expressing ELF. TGFα induces mammary hyperplasia, and in some cases, differentiation (alveolarization). Aged virgin TGFα-mice mainly showed atypical hyperplasias, while aged multiparous mice may develop secretory tumors (32,45,46). MG tumors are reported in transgenic mice expressing c-*myc*, c-*erbB2/neu*, *int-1*, *int-3* and Ha-*ras*, and in F1 hybrid mice transgenic for both *wnt-1* and *TGFα* (47,48).

The most common mammary preneoplasias are hyperplastic alveolar nodules (HAN) and ductal hyperplasia (DH). These are usually induced by mouse mammary tumor virus (MMTV), chemical carcinogens, hormonal stimulation or radiation (49-52). HAN and DH cells are immortal populations. That is, they can be serially transplanted indefinitely. On transplantation, these preneoplasias show hyperplastic growth patterns and are at high risk for neoplastic transformation (53,54). Studies proposed here will help to define the progression of preneoplasias into tumors and the effects of SGF expressed as a transgene.

We proposed to study transgenic mice expressing SGF, an EGF-like growth factor, as a model of mammary oncogenesis and differentiation. We had produced transgenic mice in which SGF expression was controlled by the metallothionein (MT) promoter or Rous sarcoma virus LTR (RSV-LTR, 55,56). RSV-LTR is a strong, constitutively active regulator. MT is substantially inducible by heavy metal (Zn).

Virgin RSV-SGF transgenic mice showed marked preneoplastic MG ductal proliferation by 6 mo. By 8 mo., 1/3 had developed adenocarcinoma. Virgin MT-SGF mice induced to express SGF at 2 mo. of age, showed MG differentiation without atypia. By *in situ* hybridization analysis, SGF was mainly expressed in mammary stroma, although tumors and preneoplastic proliferations were all epithelial. These observations suggested that since mouse mammary neoplasias and preneoplasias are typically readily cultured and are transplantable *in vivo,* SGF-mice might represent a model system to examine stromal-epithelial interactions in GF-related oncogenesis.

(iii) what is known about interactions between stromal and epithelial cells in oncogenesis

A peculiar strength of this model is the opportunity it provides to study epithelial responsiveness to growth stimulation by stromal cells. Much evidence implicates interactions between breast stroma and epithelium in the growth of malignant tumors. This interaction involves secretion of and responses to insulin-like growth factors-I and -II (IGF-I, IGF-II). IGF-I and -II have different cell membrane receptors, though responses to IGF-II may be mediated through IGF-I receptor (57-59). Breast cancer cell lines usually respond to both cytokines but do not produce IGF-I. IGF-I is, instead, elaborated by mammary stroma adjacent to the tumor. Thus, IGF-I is a paracrine growth factor for breast tumor cells (60-62). IGF-II is also produced by breast stroma, but is also made by some tumor cell lines. IGF-II, then, acts in both paracrine and autocrine fashions (63).

The interaction between SGF expressed as a transgene and target mammary epithelium resembles these reported data on the IGF's. SGF transgenic mice develop mammary tumors. The growth factor is recognized by EGFR. SGF is expressed in both epithelium and stroma, mainly in the stroma. Therefore, by developing mammary epithelial cell lines from transgenic and normal mice, and then transplanting them into the opposite recipients, we had proposed an experimental model that will allow the study and dissection of mechanisms of stromal-epithelial interactions that promote and sustain tumor and mammary gland growth.

Human breast cancer is a complex disease or group of diseases, involving a variety of independent risk factors such as parity, family history, etc. (64). Understanding human breast tumor development thus requires a number of model systems. The proposed studies of SGF-related mammary carcinogenesis complement other systems of breast carcinogenesis. For example, SGF elicits preneoplasias and invasive tumors in young virgin mice. Multiple parity starting at an early age decreases risk for human breast cancer but increases breast tumors in TGFα transgenic mice (32). SGF transgenic mice should thus provide additional insight into mechanisms of development and progression of breast tumors, and supplement other experimental models of mammary carcinogenesis.

(iv) mammary preneoplasia and neoplasia in SGF transgenic mice

a. Constructs used to produce transgenic mice

SGF constructs were made by cloning the SGF gene into mMT-1 and pRSVcat as expression vectors (65,66). (Insert orientation was confirmed by DNA sequencing.) These plasmids use the MT promoter and Rous sarcoma virus long terminal repeat (RSV-LTR) respectively as regulators of gene expression. The molecular strategies that were used to make these constructs are described in detail in the appended reprint (67), and are not recapitulated here.

Transgenic mice were made by microinjection of promoter-SGF constructs into (C57Bl/6 x DBA/2)F1 [hereafter, BDF1] embryos using standard techniques. Animals were screened for carriage of SGF transgene by assaying tail DNA. Two founder mice (#8, #9) carried SGF, and both had 3-5 copies of the gene. These mice were backcrossed to normal BDF1's. Offspring were examined for transgene carriage, and lines established from positive animals by sibling mating. We identified 3 founder mice carrying RSV-SGF and established lines from them in the same way. All progeny are now >15 generations beyond the founder mice.

Thus, 5 lines carry the SGF transgene, 2 with SGF an inducible gene controlled by the MT promoter, and 3 with SGF expressed constitutively under the control of RSV-LTR. The next section describes our clinical and pathologic observations in these mice. These findings form the basis of this application.

b. Histologic, clinical and *in vitro* observations on these transgenic mice.

Histologic findings in mammary glands of virgin transgenic mice are summarized in Table 1 for MT-SGF mice in which SGF expression was induced after sexual maturity, and in Table 2 for the RSV-SGF mice, expressing SGF constitutively. The histology on which these summaries are based is illustrated in ref. #67. RSV-SGF mice are the basis of this application; so this discussion will focus on the preneoplastic and malignant proliferations found in those animals. Other observations are described and illustrated in the accompanying preprint (67). Unless otherwise stated, all observations are made on histologic sections taken near the nipples of the abdominal mammary glands.

Table 1. MAMMARY HISTOLOGY IN VIRGIN MT-SGF TRANSGENIC MICE EXPRESSING SGF FOR 2 MONTHS

Construct	GF Expression	MG Histology		
		Ducts	Lobules	Other
pMTSGF	Uninduced/2 mo.	Normal	None	Normal
pMTSGF	Uninduced/4 mo.	Normal	None	Normal
pMTSGF	Induced at 2 mo. for 2 months Observed at 4 mo. of age	Mild hyperplasia; Abundant protein-rich secretions in ducts, with +++ periductal fibroplasia	Development of lobules with protein-rich secretion	Normal

The findings from MT-SGF transgenic mice can be summarized as follows:

•SGF expression elicits differentiation (alveolarization) and protein secretion in virgin mice when expression is begun at the age of sexual maturity for two months.

By contrast, RSV-SGF transgenic mice develop clear preneoplasia by 6 months of age and invasive secretory carcinomas by 8 months of age in 1/3 of RSV-SGF mice examined.

Table 2. MAMMARY HISTOLOGY IN VIRGIN RSV-SGF TRANSGENIC MICE

Construct	GF Expression	Breast Histology		
		Ducts	Lobules	Other
pRSGF	Constitutively expressed Observed at 2 months of age	Mild hyperplasia & atypia in ducts and ductules	None	Normal
pRSGF	Constitutively expressed Observed at 6 months of age	Highly abnormal. Marked hyperplasia extending through duct walls, into surrounding fat	None	Normal
pRSGF	Constitutively expressed Observed at 8 months of age	Highly abnormal. 1/3 of mice show invasive secretory adenocarcinoma	None	Normal

SGF has effects on other organs as well. These effects, which are not the subject of the current application, are described and illustrated in reference #67, which is appended.

Production of and responsiveness to SGF by stroma and epithelium, and consequent cellular growth *in vitro* and *in vivo*, are important aspects of this application. We studied explanted fibroblasts from SGF transgenic mice *in vitro*. Skin fibroblasts from adult SGF mice transformed spontaneously in culture within 4 weeks: they lost contact inhibition, formed foci in monolayer culture and established colonies in soft agar. Control BDF1 fibroblasts invariably die within 6 weeks. Thus SGF in culture acts as a potent transforming agent.

Thus RSVSGF transgenic mice developed ductal hyperplasias, followed by occasional invasive carcinomas by 8 months of age. The oncogenic potential of SGF as it is produced by fibroblasts, are underscored by the rapid transformation of SGF transgenic fibroblasts *in vitro.*

c. Growth factor transgene expression

SGF transcription in the mammary gland was ascertained by RNA dot and Northern blot analyses, and *in situ* hybridization. SGF expression was studied in MTSGF mice ± Zn for 2 mo., and in RSVSGF mice. MT-SGF and RSV-SGF MG, but not control MG, made mRNA that hybridized with SGF probe (See ref. #67 for *in situ* hybridization data and Northern analysis.)

Cellular patterns of transgene expression were studied by in situ hybridization (ISH) using SGF DNA as a probe. SGF DNA incorporating biotinylated dUTP (Boehringer-Mannheim) was hybridized to MG tissue sections from MTSGF, RSVSGF and normal BDF1 mice. This was followed by avidin, then biotin-alkaline phosphatase, according to established protocols (68). SGF transcript was detected in epithelial and stromal cells of many organs in both transgenic lines, but is expressed most strongly in vascular endothelium and other connective tissue cells, and MG epithelium.

SGF is expressed throughout the body in SGF-transgenic mice, in both stromal and epithelial cells. In the mammary gland SGF is mainly expressed by stromal cells.

(d) Production and characterization of recombinant SGF

We proposed to produce biologically active recombinant SGF in order to elicit antiserum against the biologically active form of SGF. Other investigators have reported little success in making active SGF, using either prokaryotic expression (2), or chemical synthesis (14). To date, antibodies that can bind to SGF have not been reported. Antibodies vs. SGF peptides (A. Opgenorth, personal communication) or translation products made in E. coli do not recognize native SGF glycoprotein (2).

SGF and MGF in lysates of SF9 cells infected with MGF-baculovirus (a), wild type baculovirus (b), or SGF-baculovirus (c). Lysates of infected cells were electrophoresed without reducing agents in SDS-PAGE. Proteins were visualized by staining with Coomaissie brilliant blue. SGF (c) and MGF (a) glycoproteins are indicated by the arrows and were 12-16 kDa. They were detected only in GF-AcMNPV, and were absent in cells infected with wt AcMNPV

We produced recombinant glycoprotein SGF (rSGF) using baculovirus (AcMNPV, 69). Sf9 cells infected with wild type (wt) baculovirus, which matches published descriptions for SGF (2).

We have been able to recover approximately 15 mg growth factor/liter Sf9 cells by electroeluting from SDS-PAGE gels like the one shown here. The rSGF prepared from Sf9 cell lysates by electroelution from SDS-PAGE stimulated target cell proliferation comparably to EGF (Fig. 1) (70).

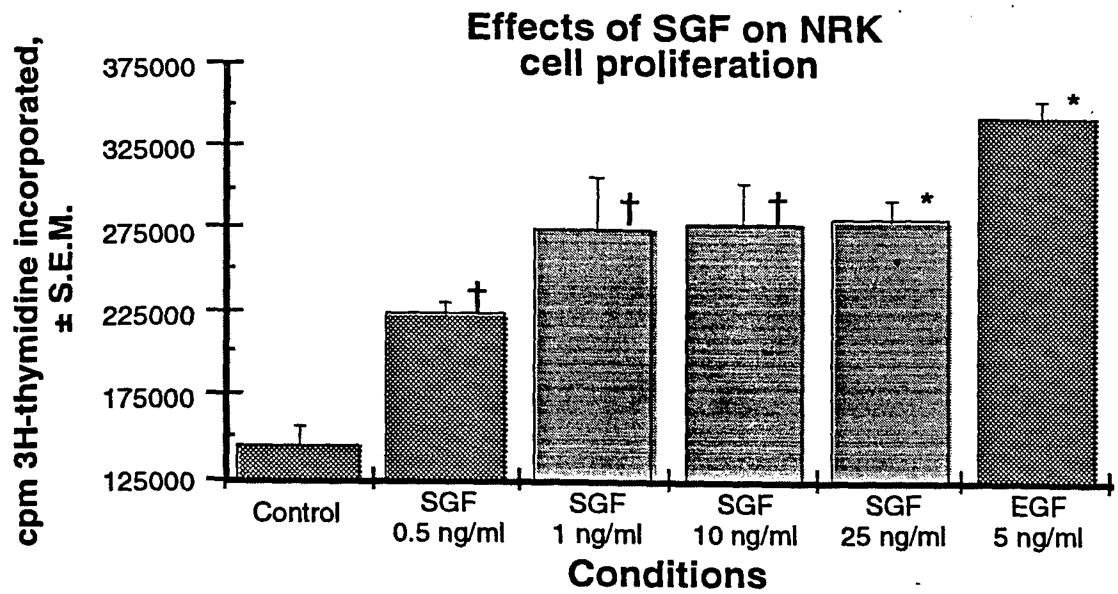

Figure 1 (above). NRK proliferation stimulated by purified SGF or EGF. NRK cells were serum-starved overnight, and cultured for 4 days with SGF or EGF as shown. After 3 days 3H-thymidine was added. Cells were harvested one day later and incorporated radionucleotide counted. *, $P<0.01$; †, $P<0.05$, both compared to Control.

For all experiments described here, we will use recombinant SGF prepared by electroelution from SDS-PAGE of lysates of Sf9 cells infected with SGF-containing baculovirus.

Therefore, SGF produced from baculovirus yielded a glycoprotein similar in size to SGF from SFV-infected mammalian cells. This recombinant SGF has ≈75% of EGF's stimulatory activity. A chemically synthesized SGF peptide is reportedly 10% as active as EGF (14).

SGF is mainly expressed by the glandular stroma in mammary glands of transgenic mice. In this setting, its expression elicits mammary preneoplasia, leading to invasive carcinomas. We proposed in this grant to study tumor development using SGF transgenic mice and biologically active recombinant SGF as tools. This system may help to elucidate cellular, pathologic, and molecular mechanisms involved in mammary oncogenesis.

Purpose of the Present Work

•It is the purpose of the work performed in this grant to study mammary oncogenesis in SGF-transgenic mice as it involves both production of growth factor and responsiveness to it. The studies performed in pursuit of this project are intended to determine the extent to which this requirement may be met by interactions between growth factor-producing stroma and -responsive epithelium.

Methods of Approach

We proposed to use transgenic mice that express SGF as a transgene to study mammary oncogenesis. Cellular, biochemical and molecular parameters of oncogenesis in this system are to be defined, particularly as they relate to epithelial-stromal interactions. Therefore, we proposed to:

1. *Define the natural history of mammary oncogenesis in SGF transgenic mice*

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias

Thus, we proposed to use SGF-transgenic mice to study interactions between mammary stromal and epithelial cells and mechanisms of growth factor-related oncogenesis. The experimental approaches proposed, the methods to be applied, and the time frames in which these studies were proposed to be completed are as follows:

1. Define natural history of mammary oncogenesis in SGF transgenic mice (months 1-24)

(a) Transgenic mice will be mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

(b) Expression of recognized genetic markers associated with breast oncogenesis will be quantitated in RNA from mammary tissue.

2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines (months 12-30)

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation (months 12-36)

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and in situ hybridization will be used to localize SGF production and site of action within the mammary gland

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias (months 24-48)

(a) The ability of cells from transgenic mice to sustain their own growth will be measured by transplanting them into normal mice. The phenotypes of resultant proliferations will be studied by in situ hybridization and immunohistochemistry.

(b) Similarly, the ability of SGF-producing stroma to regulate oncogenesis will be determined by implanting normal cells into transgenic mice and assessing the outcome by in situ hybridization and immunohistochemistry.

Body

The work done in the past year will be described and illustrated with reference to the several tasks proposed in the original application. These will be recapitulated above. Experimental approaches that have to date been described at length in the original application and will not be repeated here, except to note changes that we have made in the original experimental plan. These changes have been made for one of several reasons: (1) to accommodate new data from our own laboratory; (2) to resolve experimental issues raised by new data; or (3) to reflect important contributions to this field described in recently reports from other laboratories.

1. Define natural history of mammary oncogenesis in SGF transgenic mice (months 1-24)

Our studies in the first year concentrated on generating the mice necessary for these studies. This was done by breeding the several lines of SGF-transgenic mice, testing them for transgene carriage, and grouping them for sacrifice at the time intervals specified in the original proposal.

(a) Transgenic mice were mated and left unmated to determine the natural history of SGF effects on the mammary gland following induction of SGF expression, from birth, in pregnancy and at different stages of development.

For this purpose, we bred transgenic mice from two different MT-SGF lineages and two different RSV-SGF lineages to provide sufficient numbers to begin our proposed systematic examination of the natural history of SGF-induced mammary gland epithelial proliferation. The proposed studies include the following:

•breeding and testing the SGF transgenic mice for transgene carriage. In the course of these studies, we decided that it was most advantageous to use only mice that were homozygous for SGF transgene carriage. Examination of heterozygotes would potentially complicate the analyses as we found that these mice occasionally express the transgene at lower levels that did homozygotes. Thus, substantial additional breeding was necessary to accommodate this need.

Nonetheless we have succeeded in breeding SGF transgenic mice to produce homozygous animals capable of expressing SGF.

•accumulation of sufficient numbers of transgenic mice to sacrifice the prescribed numbers of animals at the stated intervals (2 mo., 6 mo., etc.) and following the prescribed treatment regimens (Zn^{2+} treatment or control treatment for MT-SGF mice; pregnancy x0, x1, etc.). During the past year, we accumulated sufficient mice both to allow breeding to continue apace, and to begin to sacrifice them in accordance with the proposed protocols.

Thus, we produced control (nontransgenic) mice that have been sacrificed following 0 or $\geq$ 2 pregnancies. Mammary glands and other organs from these animals were saved for histologic examination, RNA extraction, etc., as proposed.

Furthermore we produced sufficient 2 month old virgin homozygous MT-SGF mice not fed Zn^{2+}, which have been sacrificed, again as previously proposed.

Along these lines, we have examined many mammary gland and other organ histologies from RSV-SGF, MT-SGF and normal mice. We have found that a significant percentage of SGF transgenic mice, mostly of the RSV-SGF lineage but occasionally of the MT-SGF lineages, show mammary gland proliferation and differentiation, even when they are virgins. The degree of mammary gland proliferation is highly advanced in some cases. In other cases, mammary differentiation includes milk production and extensive differentiation reminiscent of lactation.

(b) Expression of recognized genetic markers associated with breast oncogenesis will be quantitated in RNA from mammary tissue.

We have made RNA preparations from mammary glands from many of the proposed groups of animals, and have accumulated the appropriate molecular probes with which to analyze these mice. The probes used are cDNA probes for c-myc, β-casein, whey acidic protein (WAP), retinoblastoma protein (Rb), p53, int-3, and Ha-ras, and oligonucleotide probes of α-lactalbumin and gelsolin. Although not all of the animals proposed in the application have yet been studied by these techniques, most have, and the results are summarized below.

Table 3

Genotype(number)	History	c-myc	β-cas	WAP	Rb	p53	α-lac	gelsolin	int-3	Ha-ras
					Gene tested					
Control										
2 month (5)	virgin	–	–	–	–	–	–	–	+	–
6 month (2)	virgin	–	–	–	–	–	–	–	+	–
6 month (2)	pregnant	+	+	–	–	–	–	–	+	–
12 month (1)	virgin	–	–	–	–	–	–	–	–	–
12 month (1)	pregnant	+	+	–	–	–	–	–	–	–
Experimental										
MT-SGF (-Zn)										
6 month (5)	virgin	+	–	–	–	–	–	–	+	–
MT-SGF (+Zn)										
6 month (4)	virgin	+	+	+	–	–	–	–	+	–
RSV-SGF										
6 month (9)	virgin	+	–	–	–	–	–	–	+	–
RSV-SGF										
6 month (2)	pregnant	+	–	+	–	–	–	–	–	–

The table shown above is remarkable for the following observations:

(1) Expression of c-myc in whole tissue homogenates is highly unusual, yet appears to be a hallmark of SGF transgene expression. Myc is also expressed at detectable levels in control pregnant mouse mammary glands.

(2) Even more striking is the association of SGF production with expression of differentiation-associated proteins, β-casein and whey acidic protein (WAP).

The lack of detection of α-lactalbumin is of indeterminate significance, since in our hands the sensitivity of oligonucleotide probes is less than of the cDNA probes used for most of the other cellular genes. (The cDNA probe for α-lac was requested but not provided to us.)

In addition to the above studies, we have initiated an additional breeding program to reflect recently described findings in mammary oncogenesis. These findings relate to the likely role(s) of abnormal p53 in the pathogenesis of breast cancers in humans (71-75). Therefore, in collaboration with Larry Donehower, Department of Molecular Virology, Baylor College of Medicine, we began breeding our SGF transgenic mice with p53-knockout mice that he had developed.

We began this breeding program shortly after the onset of this grant project. We received a pair of p53 -/+ mice, provided to us by Dr. Donehower. Our first goal with these animals was to produce a stock of p53 -/+ mice with which to breed the SGF mice. This was accomplished. We then identified both p53-/- and p53-/+ mice, and have crossed these animals with SGF mice of both the RSV-SGF and MT-SGF lineages.

The goal of establishing SGF+p53-/- mice has proven difficult. We have produced many p53 -/+, SGF+ mice of both sexes, and their phenotypes appear to be indistinguishable from normal mice. Most of these mice are heterozygous for RSV-SGF, perhaps explaining the lack of a more SGF-like mammary gland phenotype. We have generated a number of p53-/- mice, ♀ and ♂, which have died at 6-9 months of age. Most of these deaths have been from lymphomas. One mouse developed a widely metastatic osteosarcoma.

In trying to produce SGF+p53-/- mice, we have succeeded in producing several such males. The oldest of these are now 5-8 months old, and are overwhelmingly male. We have only documented one SGF+p53-/- female. The reason for this sex preponderance, which is not seen in p53-/+SGF+ mice, is not yet clear, but appears to represent a selective disadvantage of p53-/-SGF+ females either *in utero* or within the first and second days of extrauterine life.

Furthermore, although we have found that the p53-/- mice die spontaneously at 6-9 months of age, the SGF+p53-/- mice (all males so far) that have died have done so at 5-6 months of life. (There is one 8 month old male of this genotype, however.) For both groups of mice, malignant lymphomas have been the causes of death. The reason that SGF+p53-/- mice appear to develop or die from lymphomas earlier is not yet clear, but is under investigation.

Once our lines of these combination transgenic-knockout mice are developed, we will begin a systematic analysis of their mammary glands and other organs. Initially, the analysis of these animals will parallel analysis of the straight SGF transgenic animals described above. Subsequent analyses will depend upon the nature of the findings in our early studies.

<u>2. Produce cell lines from preneoplasias and tumors from SGF mice, and characterize the growth characteristics of these cell lines (months 12-30)</u>

(a) Cell lines will be established from ductal hyperplasias and tumors that arise in transgenic mice

(b) These cell lines will be studied for ligand binding by EGF receptor using Scatchard analysis

Work on establishment of these cell lines is in progress. Two of our laboratory staff were sent to Dr. Medina's laboratory (Baylor College of Medicine) to learn the necessary procedures. As a result, we have successfully established stromal cell lines. These have been well enough established that aliquots have been frozen to preserve them for future analysis. These cells continue to be passaged. Progress has been somewhat slower in producing mammary epithelial cell lines, as several attempts have not yet yielded the expected results. We are currently continuing these studies.

<u>3. Define production of SGF in transgenic mice and study its induction of mammary neoplasias and differentiation (months 12-36)</u>

(a) Antibody vs. SGF will be produced

(b) This anti-SGF antibody, in conjunction with cDNA probes, will be used to measure SGF production in the cultured transgenic fibroblasts and epithelial cells

(c) Immunohistochemistry and *in situ* hybridization will be used to localize SGF production and site of action within the mammary gland

We are working on this specific aim. (Specifically, Aims #3(a) and (c).) The first step in Aim (3(a) was to produce anti-SGF antiserum We have used SGF from our baculovirus system, and have immunized rabbits with 10-25 μg protein intravenously in saline every 3 weeks, bleeding one week after immunization. By Western blot, we have detected antibody Therefore, we have developed antisera with antibody activity against SGF. Continued immunization and testing is being performed to develop sufficiently high anti-SGF activity for the second and third subaims of this specific aim.

In situ hybridization studies have been applied to the analysis of SGF production in transgenic mammary glands and other tissues. We have found, as was previously suggested, that the expression of SGF in the mammary gland is principally a function of stromal cells. We are currently applying these approaches to our stromal cell lines.

Epithelia of a number of organs do support SGF expression, however. We have found that epithelium of kidney, liver and gastrointestinal tract express SGF. The relative proportions of stromal: epithelial expression vary from organ to organ, however. The identification of c-myc, β-casein and WAP transcripts in virgin mammary glands from SGF transgenic mice has provided stimulus for us to expand our *in situ* hybridization studies to these genes as well.

4. Assess stromal and epithelial interaction in GF production and responsiveness in the generation of mammary tumors and preneoplasias (months 24-48)

(a) The ability of cells from transgenic mice to sustain their own growth will be measured by transplanting them into normal mice. The phenotypes of resultant proliferations will be studied by in situ hybridization and immunohistochemistry.

(b) Similarly, the ability of SGF-producing stroma to regulate oncogenesis will be determined by implanting normal cells into transgenic mice and assessing the outcome by in situ hybridization and immunohistochemistry.

As indicated in the task outline, projected work on this aim is not expected to begin until the third year of this grant. We have not yet begun to work on the studies proposed in this aim.

This grant focuses on the effects of SGF on the mammary gland. In the course of the proposed studies, we expect to define the natural history of murine mammary tumor development as a function of SGF stimulation, and to categorize these effects according to current concepts of mammary oncogenesis. Growth factor production and responsiveness by the mammary stroma and epithelial cells will be examined to understand how these different cell types interact to yield preneoplastic and neoplastic proliferations.

Conclusions

The goals of the first years of this project included the generation of a transgenic mouse colony of sufficient numbers to permit the analyses proposed in the second and third years of this project, and to supply the animals needed to complete the second through fourth years of this proposed research. We have completed this expansion of our transgenic mouse colony. We have further expanded our research to accommodate experimental study of the pathogenetic role that may accompany the clinically established correlation of mutant p53 with aggressive human breast cancer.

The goals of the second year have been to apply the expanded transgenic mouse colony to our studies proposed, and much of this work has been accomplished. We have performed extensive gene expression studies using MT-SGF and RSV-SGF mice. We are now analyzing these mice by *in situ* hybridization to localize the transgene expression in the mammary glands of these mice. We have, furthermore, expanded the scope of our work to include the analysis of mice produced by crossing p53 knockout mice with our SGF transgenic mice.

We have been successful in producing anti-SGF antibody in rabbits and are currently increasing anti-SGF antibody production in the expectation that considerable quantities will be needed to pursue our studies.

While these two years of work do not yet allow us to draw definitive conclusions as to the mechanisms of stromal-epithelial interactions in the development and spread of mammary cancer in transgenic mice, as we had anticipated in our proposal the work produced in the first two years of this four year project has provided insight into SGF-induced changes in gene expression and has laid the foundations for the subsequent experiments. It is these subsequent studies that will produce conclusions that will bear directly on this issue.

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