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Distinctive Patterns of Hyperplasia in Transgenic Mice with Mouse Mammary Tumor Virus Transforming Growth Factor-α

Characterization of Mammary Gland and Skin Proliferations

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Eight lines of transgenic mice expressing a mouse mammary tumor virus (MMTV) human transforming growth factor-α (TGFα) fusion gene were established. Three lines with distinctive phenotypes are presented. All have proliferative changes of the mammary gland. One line has sebaceous gland hyperplasia of the skin. Five histologic patterns of mammary gland hyperplasia based on two of these lines were identified: cystic hyperplasia, solid hyperplasia, dysplasia, adenoma, and adenocarcinoma. Human TGFα mRNA and protein were produced in all patterns but appeared reduced in solid hyperplasia, dysplasia, and adenocarcinoma. TGFα immunoreactivity in the mammary tissue, cystic fluid, and serum did not show significant differences; hyperplasia developed in 65% of multiparous mice and 45% of virgin mice by 12 months of age. Adenocarcinoma developed in 40% of multiparous mice and 30% of virgin mice by 16 months of age. These transgenic lines may provide useful models of mammary and sebaceous gland hyperplasia analogous to human disease. (Am J Pathol 1992, 140:1131–1146)

Carcinogenesis is recognized as a multistep process.[1,2] Although genetic damage is considered an integral component of carcinogenesis, not all carcinogenic agents are genotoxic.[3] The mode of action of nongenotoxic agents involves proliferation as an important ingredient.[4] Cellular proliferation plays a critical role[5,6] in the action of agents classified as tumor promoters. These agents may act by increasing the proliferation of initiated cells resulting in the propagation of genetic errors.[2,5]

Transforming growth factor-α (TGFα) is a mitogenic polypeptide.[7,8] The mature peptide consists of 50 amino acids derived from a 160 amino-acid precursor. Pro-TGFα is membrane anchored and the mature peptide is produced by proteolytic cleavage.[9] TGFα shares approximately 35% homology with epidermal growth factor (EGF) and acts through binding with the EGF receptor.[10] Although TGFα was originally found in transformed cells,[11] it has been identified in a number of cell types, including a wide range of normal cells[12,13] and embryonic tissues.[14,15] TGFα is a known mitogen for a variety of different cell types.[13]

We have developed transgenic mice-expressing human TGFα under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer region.[16] Eight lines have been established. Six lines have been examined and three with distinctive phenotypes have been studied in detail and form the basis of this article. We present here a classification of the mammary gland pathology in the females of two of these lines. The mammary glands of the female mice in the 29 and 254 lines are

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characterized by proliferations of the epithelium which can be classified into five distinctive patterns. Carcinoma develop in both multiparous and virgin mice in both of these lines. TGFα immunoreactivity does not vary significantly among the different histologic types, but TGFα mRNA expression and protein production appear to be decreased in the more cellular hyperplastic regions. In contrast, the mammary glands of the females of the 64 line are characterized by marked cystic and solid hyperplasia. Adenomas do occur but no dysplasia or carcinoma has yet been detected. Line 254 has both skin and mammary gland abnormalities. There is marked sebaceous gland hyperplasia in the skin as well as the development of hyperplasia and adenocarcinoma in the mammary glands of this line.

Materials and Methods

Development of Transgenic Mice

The construction of the MMTV-TGFα transgenic mice has been described previously.[16] In brief, transgenic mice were generated by injecting one-cell embryos with human TGFα under the control of the long terminal repeat of the mouse mammary tumor virus. Splicing events were provided by including exons 2 and 3 of the rabbit β-globulin gene in the MMTV-TGFα vector. Ten founder mice were produced and eight lines established. Six of the lines have been studied extensively. Three lines will be described in detail in this article.

Histologic Methods

Mammary tissue was obtained by dissection at sacrifice. For wholemount preparation, the skin containing the mammary fat pads was fixed in 10% buffered formalin for at least 24 hours. After fixation, the mammary glands were dissected from the skin and processed using a modification of the method of Medina.[17] Briefly, they were treated with acetone and stained with Harris' hematoxylin. After decolorization in acid alcohol and tap water, they were treated with 0.12% ammonia water, stored in 95% ethanol, and examined under a dissecting microscope. Tissue for light microscopic examination was immediately fixed in 10% buffered formalin and embedded in paraffin using routine methods. The tissue was sectioned at 5 μm and stained with H&E.

Transmission Electron Microscopic Examination

Specimens of mammary gland were fixed in 3% glutaraldehyde and postfixed in osmium tetroxide. The tissue was dehydrated, en bloc stained with uranyl acetate and embedded in Spurr (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with uranyl acetate and lead citrate and viewed with an Hitatchi H-60 microscope (Hitachi Inst. Incorp, Santa Clara, CA).

In Situ *Hybridization*

Tissues were obtained at the time of sacrifice and fixed in 4% paraformaldehyde. After fixation and dehydration in graded alcohol, the tissue was embedded in paraffin and cut at 6 μm. Pathologic assessment was made by one of us (SAH) from sections stained with H&E obtained from the same block as those used for *in situ* hybridization and immunocytochemical analysis. The human TGFα probe was a cDNA fragment obtained from G. Bell (University of Chicago) inserted into the transcription vector pGEM7Z (Promega, Madison, WI). Sense and anti-sense RNA strands were synthesized and labeled with ^{35}S by *in vitro* transcription in the presence of 500 μM GTP, CTP, ATP, and 12 μmol/l of ^{35}S-labeled UTP, 40 units RNasin, 2 mmol/l spermidine, 100 μmol/l dithiothreitol, 20 mmol/l NaCl, 40 mmol/l TRIS (pH 7.5), 6 mmol/l $MgCl_2$, and SP6 or T7 RNA polymerase as previously described[12]. Hybridization was carried out at 55 C overnight. Posthybridization washes consisted of 30 minutes at room temperature in 20 μg/ml RNase A followed by 2 hours in 0.1× standard sodium citrate (SSC) at 55 C. Slides were exposed for 14 to 21 days.

Immunohistochemical Staining Procedure

Tissues were obtained at sacrifice and fixed immediately in 4% paraformaldehyde. After fixation and dehydration in graded alcohol, the tissue was embedded in paraffin and cut at 6 μm. The avidin-biotin method (Vectastain Elite, Vector Laboratories, Burlingame, CA) was used to determine tissue TGFα immunoreactivity. Nonspecific background was reduced by staining the tissue sections in 10% normal rabbit serum in phosphate buffered saline (PBS) for 15 minutes before the addition of the primary antibody. Sheep anti-recombinant human TGFα antibody (made in collaboration with East Acres Biologicals, Southbridge, MA) diluted 1:1000 to 1:2000 in 10% normal rabbit serum in PBS was used as primary antibody. The primary antibody was applied overnight followed by biotinylated rabbit anti-sheep IgG for 90 minutes at room temperature. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol. Avidin-biotin peroxidase complex was added for 30 minutes at room temperature. Hematoxylin was used as counterstain. Negative controls included absorption of the primary antisera with an excess of recombinant human

TGFα (Berlex Biosciences Inc., Alameda, CA) bound to nitrocellulose and the use of normal sheep serum in place of sheep anti-TGFα serum.

Sample Preparation

Blood, tissue, and cystic fluid were obtained under anesthesia at the time of sacrifice. Blood was collected by cardiac puncture. Cystic fluid was collected by aspiration and immediately stored at −70 C until assayed. Mammary tissue samples were snapfrozen in liquid nitrogen and stored at −70 C. Tissue (0.1–0.5 g) was thawed in 2 ml of homogenate buffer (50 mmol/l NaCl, 25 mmol/l TRIS-HCL [pH 8.0] containing 0.02% (w/v) sodium azide) containing protease inhibitors (1mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin) and homogenized. Two 100 μl aliquots were taken for DNA[18] measurement. The remaining tissue homogenate was solubilized by the addition of 2 ml of buffer containing 1% (w/v) NP-40 and 1% (w/v) sodium deoxycholate and rocked for 1 hour at 4 C. Detergent-insoluble material was removed by centrifugation at 10,000*g* for 30 minutes at 4 C. Any fat was aspirated and the resulting supernatant was collected and stored at −20 C until assayed by TGFα radioimmunoassay (RIA).

Radioimmunoassay for Human TGFα

Recombinant human TGFα (Berlex Biosciences Inc., Alameda, CA) was used both as a standard and as a ^{125}I-labeled tracer. Briefly, 100 μl of recombinant TGFα standard or 100 μl of sample diluted in RIA buffer (62.5 mmol/l Na_2HPO_4, 12.7 mmol/l EDTA, 3 mmol/l NaN_3 [pH 7.4]) containing 0.1% Triton X-100 were mixed with 100 μl of sheep anti-TGFα diluted 1:20,000 in 1% normal sheep serum in RIA buffer containing 0.1% Triton X-100 and incubated for 3 days at 4 C. One hundred μl of ^{125}I-labeled[19] human TGFα (2000 cpm) diluted in RIA buffer plus 0.1% Triton X-100 was added and incubated for 24 hours at 4 C. The bound ligand was precipitated by the addition of 100 μl of donkey anti-sheep serum (Biomedical Technologies, Stoughton, MA) diluted 1:8 in RIA buffer. After a 4-hour incubation at 4 C, 1.5 ml of RIA buffer containing 2.5% bovine serum albumin (BSA) and 2% polyethylene glycol (PEG) 8000 was added and immediately spun at 3000 rpm for 30 minutes. Bound and free label were separated by pouring off the supernatant. The pellets (bound label) were counted for 5 minutes in a Isomedic 4/600 HE IC gamma counter. Results were calculated using a 4-parameter log-logit transformation on the Isodata program (ICN Biomedicals Inc. Costa Mesa, CA). The sensitivity of the assay as measured by 10% displacement of ^{125}I-labeled trace was 4 pg TGFα per tube. The assay was highly specific for human TGFα with essentially no displacement of ^{125}I-labeled trace by rat, mouse, or human EGF up to 1 μg/ml. Minimal crossreactivity with rat TGFα was observed with only 16% displacement of ^{125}I-labeled trace at 1 μg/ml. Intra- and interassay coefficients of variation were 6.9% and 5.7%, respectively.

Results

To date, ten founders with the transgene have been established. All of the founders have gross evidence of mammary gland abnormalities. Eight of the founders have been able to transmit the transgene to their progeny and all of the lines established have histologically proven proliferations of the mammary gland. Four of the lines have histologically confirmed adenocarcinoma of the mammary gland. All of the lines have been able to conceive and deliver normal young. Three of the lines (29, 64, and 254) have been studied in detail and form the basis of this report.

Histologic Characterization of Line 29

Light Microscopic Examination

Abnormal histology was found only in the mammary glands of female transgenic mice. The histologic appearance of the mammary gland tissue in virgin transgenic mice was similar to their female virgin nontransgenic littermates at 29, 70, and 83 days. However, at 90 days of age, the transgenic mice began to show more alveolar development (Figure 1A) compared with nontransgenic virgin littermates (Figure 1B). Alveolar proliferation persisted until the mice were more than 1 year old (Figure 1C) but not to the same degree as seen in the younger mice. Atrophy was present in virgin mice older than 1 year but scattered hyperplastic nodules could still be seen. These hyperplastic foci were not present in age-matched nontransgenic virgin littermates (Figure 1D).

Pregnancy is a natural state of hyperplasia for the mammary gland. However, the appearance of the gland in the pregnant transgenic mice was different than in the nontransgenic pregnant littermates. Cysts were more prominent and the secretion was more inspissated[16] (data not shown). These changes persisted postpartum. None of the MMTV-TGFα transgenic mice can nurse their pups.

Many multiparous transgenic mice developed numerous lumps of varied size and shape in the mammary glands beginning at 4 months of age. These masses were most prominent in the thoracic region and the in-

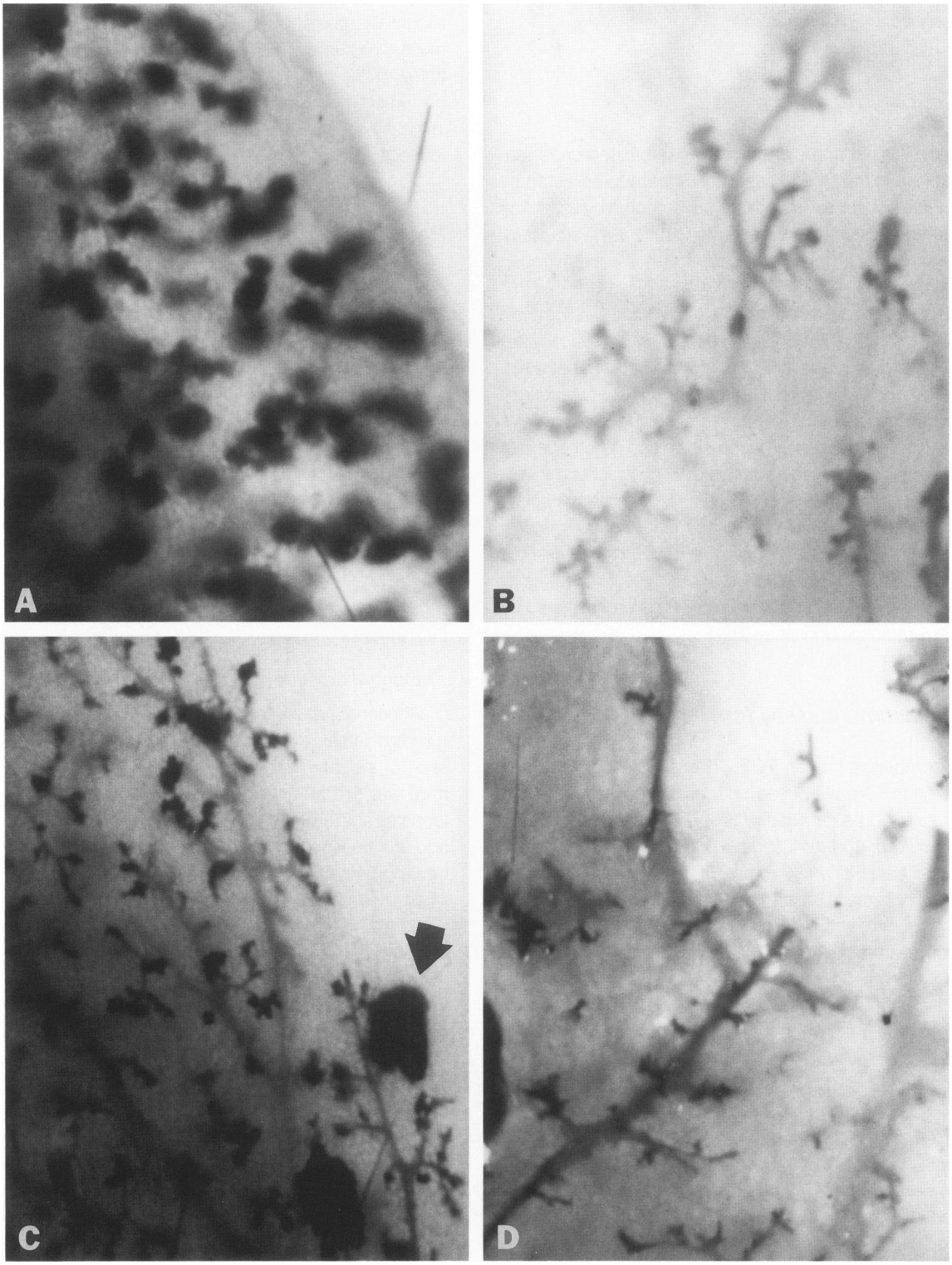

Figure 1. *Wholemount preparation of 90-day-old virgin transgenic female mouse* **(A)** *shows well-developed and proliferative alveolae compared with nontransgenic* **(B)** *female littermates. Wholemount preparation of 476-day-old virgin transgenic female mouse* **(C)** *demonstrates retention of hyperplastic alveolae with scattered focal hyperplastic nodules (arrow) compared with marked atropy seen in nontransgenic littermate* **(D)** *(Hematoxylin, ×30).*

guinal area but occurred in all glands in the mammary chain. They were fluctuant, mobile, and contained clear, milky or sanguinous fluid. These masses measured from 1 mm to 2 cm in diameter. Histologic examination showed large cystic spaces lined by cuboidal epithelium (Figure 2A) with no atypia. Abundant secretion was present in the lumen. This type of hyperplasia is called cystic hyperplasia.

Another type of hyperplasia seen in these MMTV-TGFα transgenic mice is termed solid hyperplasia (Figure 2B). The cysts were smaller than in cystic hyperplasia and there was more stromal proliferation between the cysts. The nuclei of the epithelial cells were hyperchromatic but there was abundant cytoplasm and no alteration of the nuclear/cytoplasmic ratio.

Dysplasia is seen when the epithelial cells form solid sheets and there are rare cysts present (Figure 2C). It is not known, however, if the biological behavior of these lesions is premalignant. The term is used purely in a descriptive sense and will require further study to determine if there is a biological link to neoplasia. Dysplastic foci were often found around areas of frank adenocarcinoma but could be found in the absence of carcinoma. Histologic patterns of dysplasia included closely packed epithelial cells and hyperchromatic nuclei with rare nucleoli and mitotic figures. However, nuclear and architectural atypia were insufficient for a diagnosis of carcinoma.

Ten transgenic mice in line 29 developed lumps that were solid, circumscribed, mobile, and nonfluctuant. Histologic examination showed adenocarcinoma (Figure 2D) consisting of glands or sheets of malignant cells with abundant mitoses. Most of the adenocarcinomas were poorly differentiated but all contained some areas of glandular differentiation. The majority of the carcinomas were well circumscribed and noninvasive but in several animals invasion of surrounding soft tissue and muscle was seen. No distant metastases were detected. Multiple carcinomas developed in five of the mice. All but one of these tumors occurred in the thoracic mammary glands.

One multiparous transgenic mouse in line 29 developed a mass grossly similar to the adenocarcinomas. However, histologic examination showed that the tumor was benign (Figure 2E). Glands were more widely spaced and the stroma was markedly increased. Because of the histologic similarity to human fibroadenomas, this tumor has been called an adenoma, but the biological potential is, as yet, undetermined. Atypia of the epithelium was not seen and mitotic figures were rare.

Of the 24 mice in line 29 examined in detail histologically, all showed either cystic hyperplasia or solid hyperplasia or a combination of both. Those mice that developed dysplasia or adenocarcinoma also had evidence of cystic hyperplasia and/or solid hyperplasia in the same mammary gland and in other mammary glands. In a minority of mice, the other mammary glands did not appear grossly to be cystic, but on histologic examination, all mammary glands demonstrated either cystic and/or solid hyperplasia.

In Situ *Hybridization*

In situ hybridization using a ^{35}S-labeled human TGFα antisense riboprobe showed high levels of TGFα mRNA expression in the epithelial cells lining the cysts (Figure 3A) in cystic hyperplasia. As controls, serial sections were hybridized with a ^{35}S-labeled sense TGFα probe (Figure 3B). In solid hyperplasia, grains were fewer and more scattered, compared with cystic hyperplasia (Figure 3C). TGFα mRNA expression was also decreased in areas of dysplasia (data not shown). *In situ* hybridization for TGFα showed heterogeneous gene expression in adenocarcinomas (Figure 3D). Some areas of the tumor did not have any gene expression whereas other areas had scattered grains. Expression was decreased and more heterogeneous in adenocarcinomas compared with cystic hyperplasia.

Immunocytochemical Analysis

Immunocytochemical analysis for TGFα protein showed strong reactivity in the epithelial cells lining cysts with lower intensity in the stromal cells in cystic hyperplasia (Figure 4A). Staining was especially intense in the epithelium lining large cysts. Reduced staining of TGFα protein was seen in solid hyperplasia compared with cystic hyperplasia (Figure 4B). Immunoreactivity for TGFα within these areas showed faint, scattered positivity. A similar heterogeneous pattern for TGFα was seen in dysplasia and adenocarcinoma (Figure 4C).

Histologic Characterization of Line 64

Light microscopic examination of the mammary glands of eleven female mice of line 64 showed that all animals developed cystic hyperplasia similar to that seen in line 29. Many of these mice, however, also had solid hyperplasia with crowded glands and smaller cystic spaces. One female mouse developed an adenoma in line 64. No adenocarcinomas have been seen in this line. However, follow-up has been shorter in line 64 than in line 29.

Transmission Electron Microscopic Examination

To determine the origin of the hyperplastic cells, transmission electron microscopic examination was done on

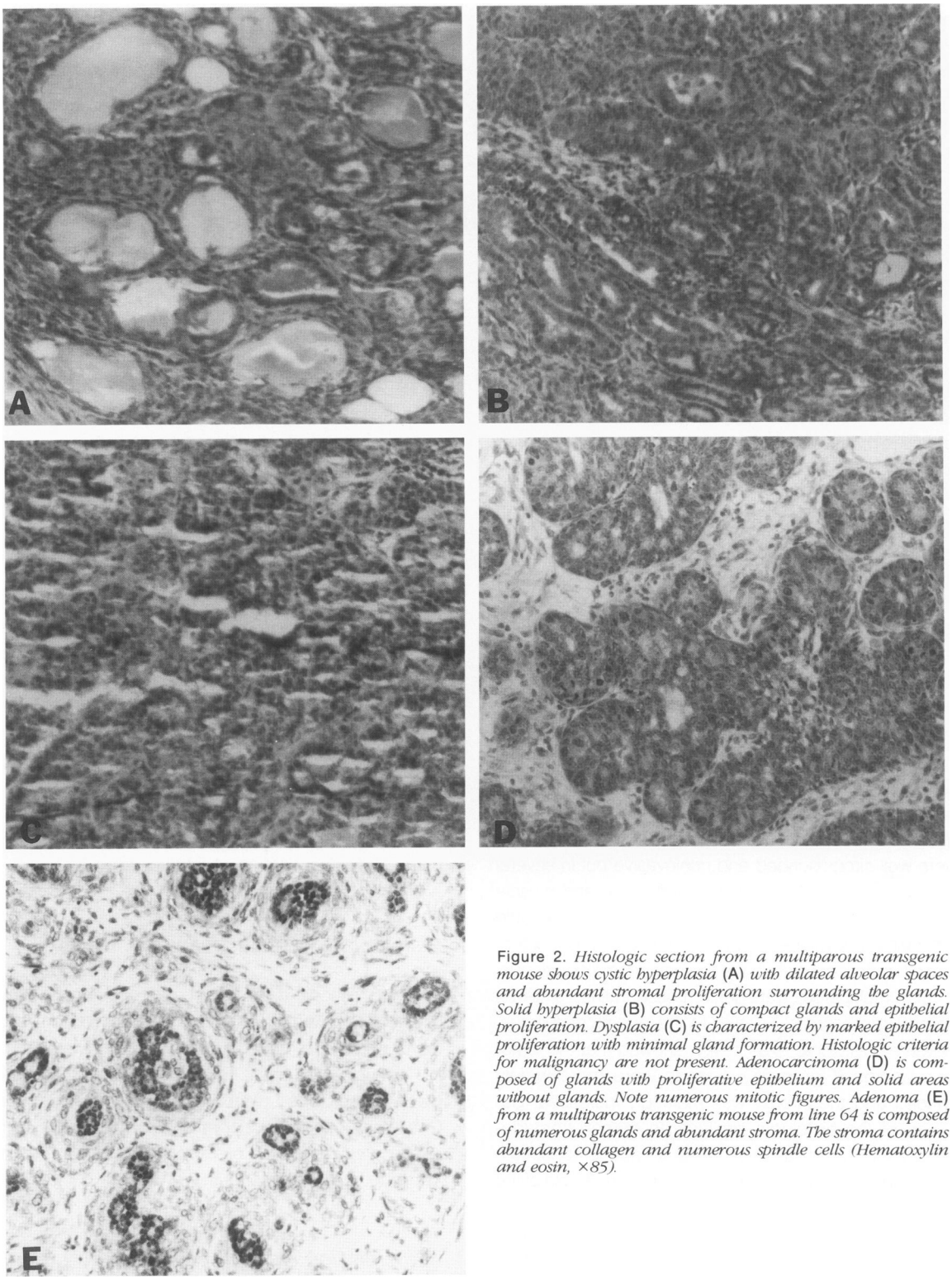

Figure 2. *Histologic section from a multiparous transgenic mouse shows cystic hyperplasia* (A) *with dilated alveolar spaces and abundant stromal proliferation surrounding the glands. Solid hyperplasia* (B) *consists of compact glands and epithelial proliferation. Dysplasia* (C) *is characterized by marked epithelial proliferation with minimal gland formation. Histologic criteria for malignancy are not present. Adenocarcinoma* (D) *is composed of glands with proliferative epithelium and solid areas without glands. Note numerous mitotic figures. Adenoma* (E) *from a multiparous transgenic mouse from line 64 is composed of numerous glands and abundant stroma. The stroma contains abundant collagen and numerous spindle cells (Hematoxylin and eosin, ×85).*

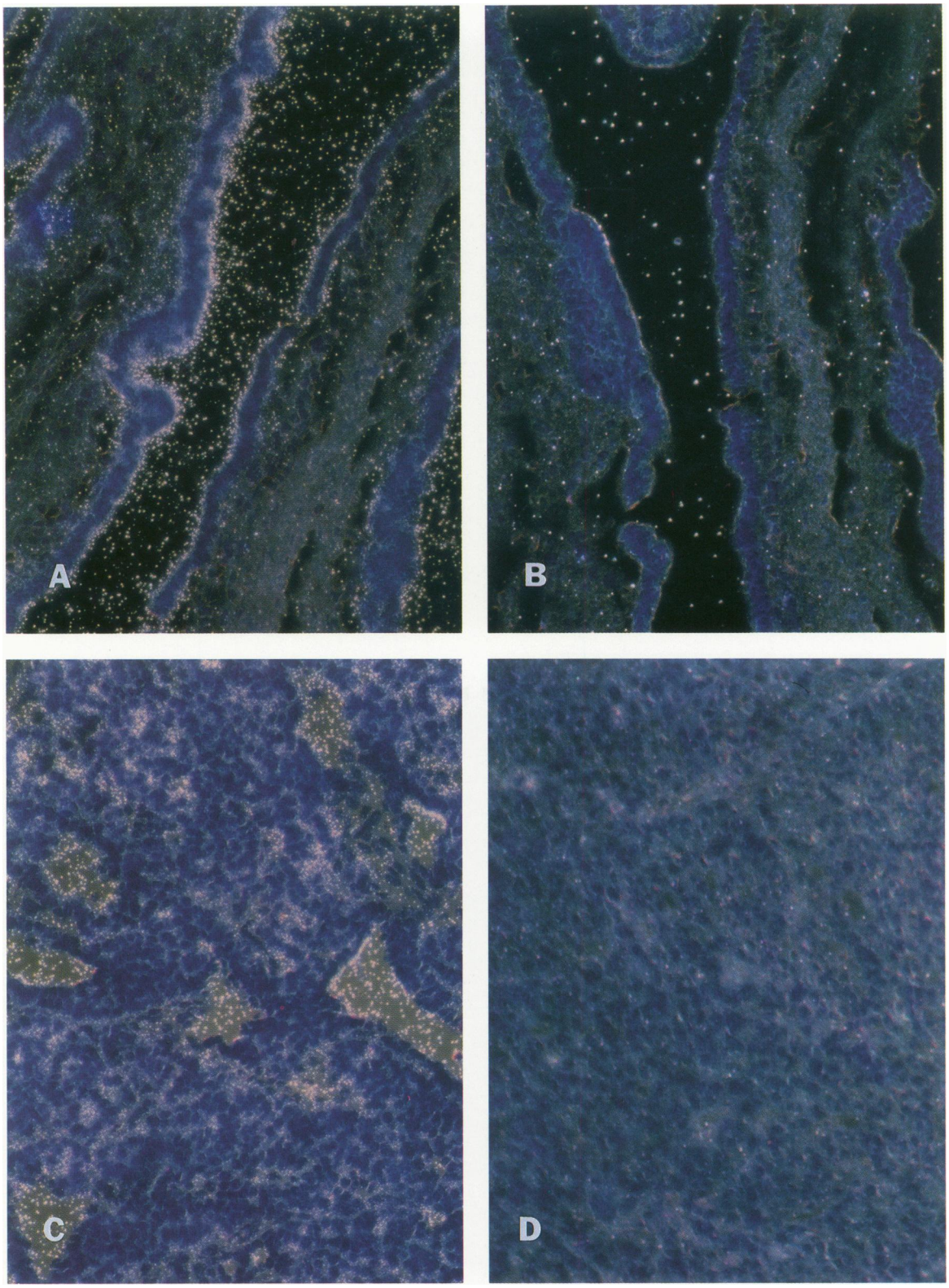

Figure 3. *Darkfield illumination of* in situ *hybridization of cystic hyperplasia* **(A)** *with ^{35}S-labeled anti-sense TGFα riboprobe shows highest expression in epithelial cells lining cysts.* In situ *hybridization with sense probe* **(B)** *shows background staining.* In situ *hybridization of solid hyperplasia* **(C)** *with ^{35}S-labeled anti-sense TGFα riboprobe shows heterogeneous expression.* In situ *hybridization of adenocarcinoma* **(D)** *with ^{35}S-labeled anti-sense TGFα riboprobe is also heterogeneous (×110).*

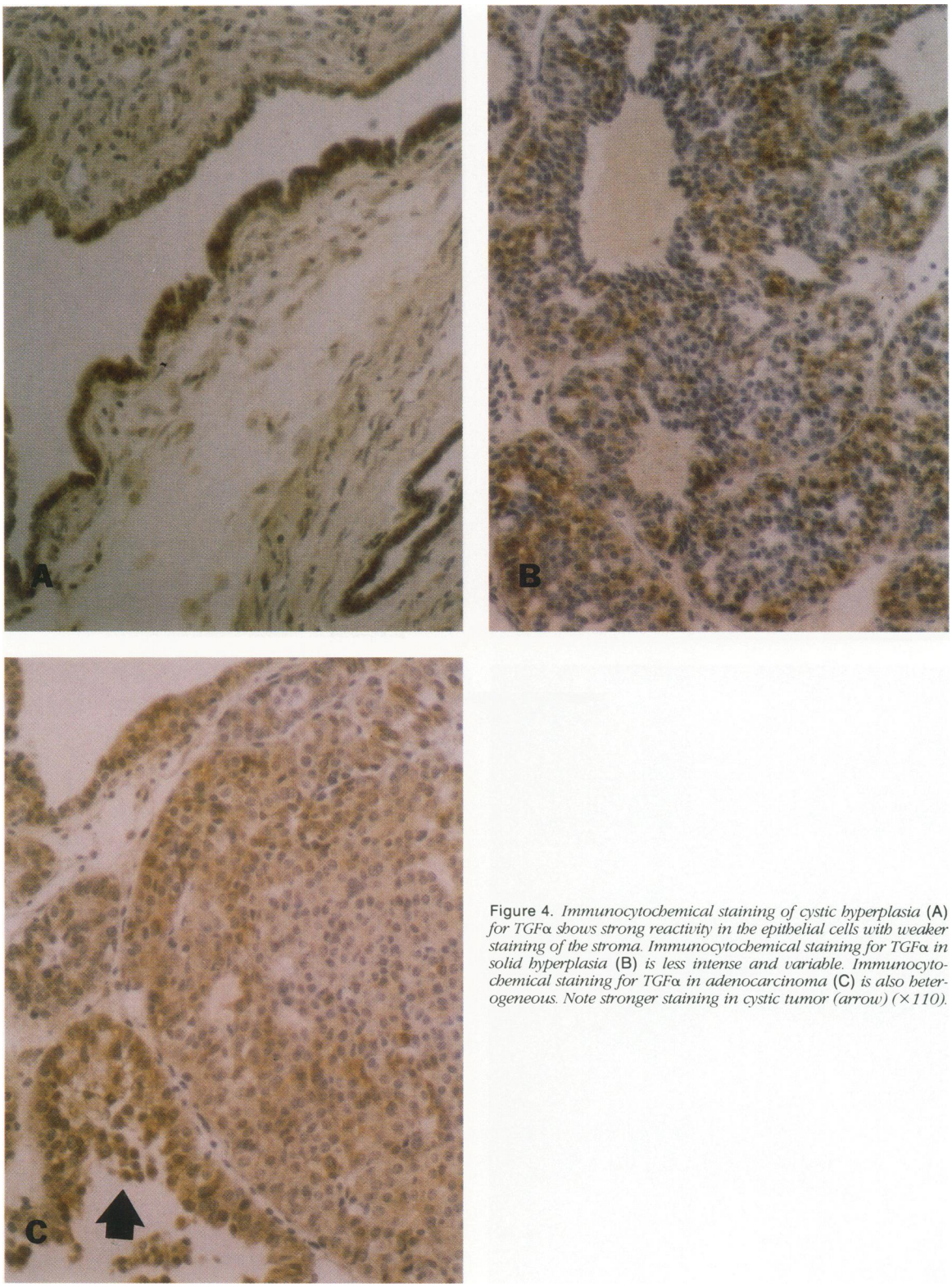

Figure 4. *Immunocytochemical staining of cystic hyperplasia* (A) *for TGFα shows strong reactivity in the epithelial cells with weaker staining of the stroma. Immunocytochemical staining for TGFα in solid hyperplasia* (B) *is less intense and variable. Immunocytochemical staining for TGFα in adenocarcinoma* (C) *is also heterogeneous. Note stronger staining in cystic tumor (arrow) (×110).*

nonmalignant mammary tissue from seven multiparous transgenic mice from lines 29 and 64. Ultrastructural examination showed abundant glandular formation with scattered fibroblastic and chronic inflammatory cells in the stroma (Figure 5). All of the normal cellular components of the mammary gland were involved in the hyperplasia with maintenance of normal glandular configuration. There was no alteration in the types of cells found in

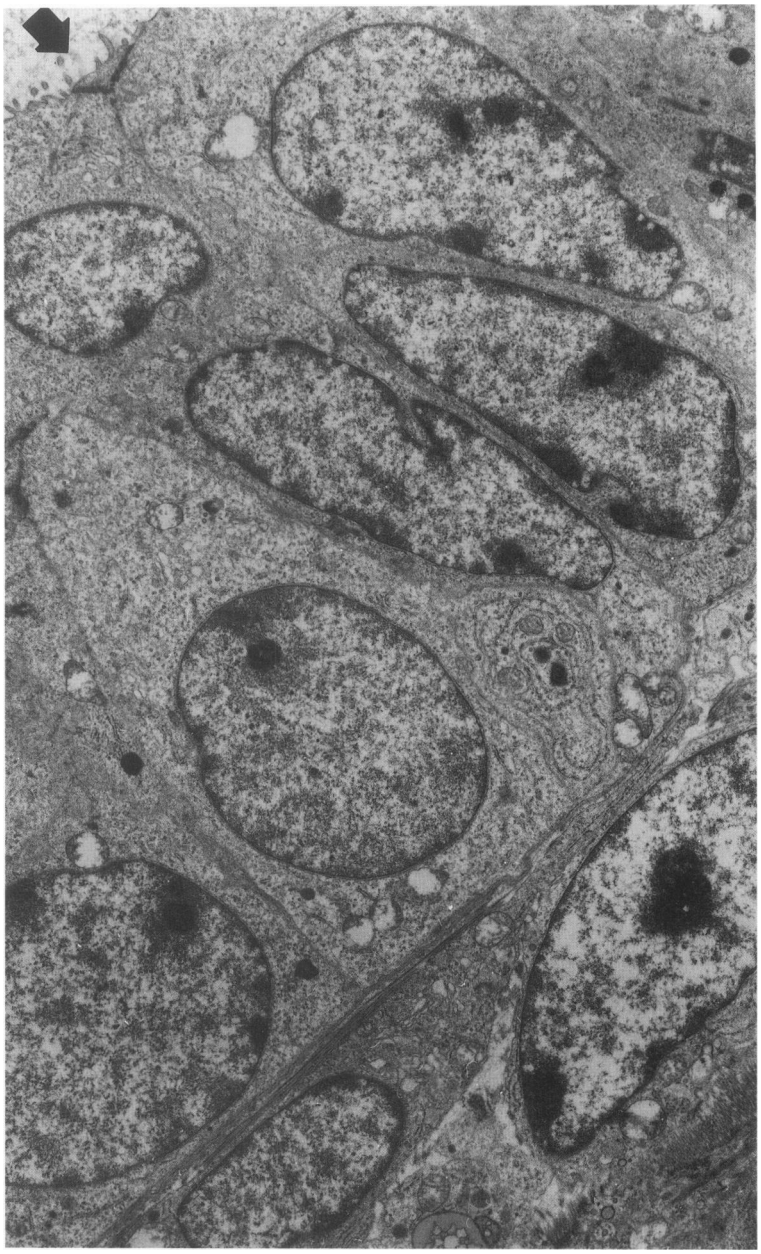

Figure 5. *Electron micrograph of cystic hyperplasia in a multiparous transgenic mouse. Gland (arrow) is composed of epithelial cells joined by intercellular junctions and subtended by a basal lamina. Spindle cells in the stroma are fibroblasts (×7,000).*

the hyperplastic process compared with normal mouse mammary gland.

Radioimmunoassay

TGFα protein production was determined in the cystic fluid, serum, and tissues of transgenic mice using a sensitive and specific immunoassay for human TGFα. The results for lines 29, 64, and 254 are shown in Figures 6 and 7. The cystic fluid showed consistently high levels of TGFα in both multiparous and virgin animals (Figure 6A–D). Cyst fluid levels were higher than serum in all the lines studied. There was little difference in the amount of TGFα in the different histological types of hyperplasias (Figure 7A,B). However, the atrophic lobules of the old virgin mice had lower mean levels than the hyperplastic or carcinomatous tissue.

Natural History of Lines 29 and 64

By 1 year of age, 65% of multiparous transgenic mice in line 29 developed mammary hyperplasia (Figure 8). Virgin transgenic mice also developed mammary hyperplasia but it occurred in fewer animals and at a later age

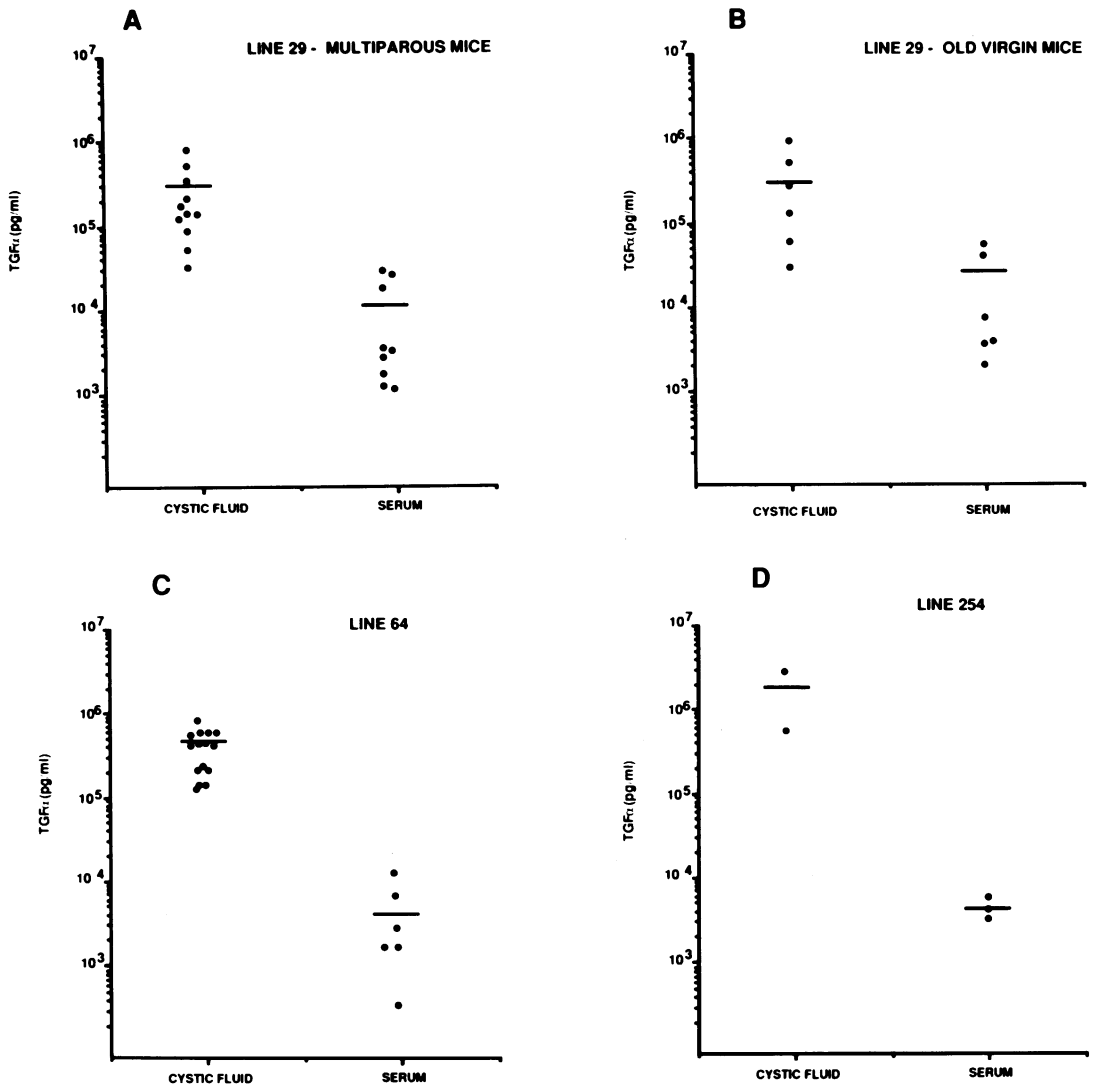

Figure 6. *Radioimmunoassay for TGFα polypeptide production in the cystic fluid and serum of female mice in lines 29* (A, B), *64* (C), *and 254* (D), *(bar = mean).*

(Figure 8). Mammary hyperplasia occurred earlier in line 64 and a larger percentage of animals were involved than in line 29.

Carcinomas were first detected at 6 months of age in line 29 and by 13 months, 40% of multiparous transgenic mice in this line developed adenocarcinomas (Figure 9). Adenocarcinomas occurred in 30% of virgin transgenic mice in line 29 but appeared at an older age (Figure 9). No metastases have been found in any of the sacrificed mice. No primary carcinomas of any other organs have been seen in these animals.

Line 254

Line 254 is a more recently studied line that has histologic changes in the mammary gland that are similar to line 29. Cystic and solid hyperplasia, dysplasia, and adenocarcinoma, similar to line 29, have been documented in seven mice in this line by light microscopic examination. Cyst and serum levels of TGFα have been comparable with the other lines (Figure 6D). The natural history of these mice has not been provided because of the smaller numbers and shorter follow-up compared with lines 29 and 64.

This line is notable for its distinctive skin changes in male and female mice. The mice in this line developed multiple, raised, white plaques and nodules on the abdominal skin. Incision of the nodules revealed white material resembling sebum. Microscopic examination showed sebaceous gland hyperplasia (Figure 10) in the skin. There was marked proliferation of the sebaceous glands in the dermis. No dysplasia or sebaceous cell carcinoma was seen, however. *In situ* hybridization for TGFα mRNA expression showed high expression in the areas of sebaceous hyperplasia (Figure 11A). Immuno-

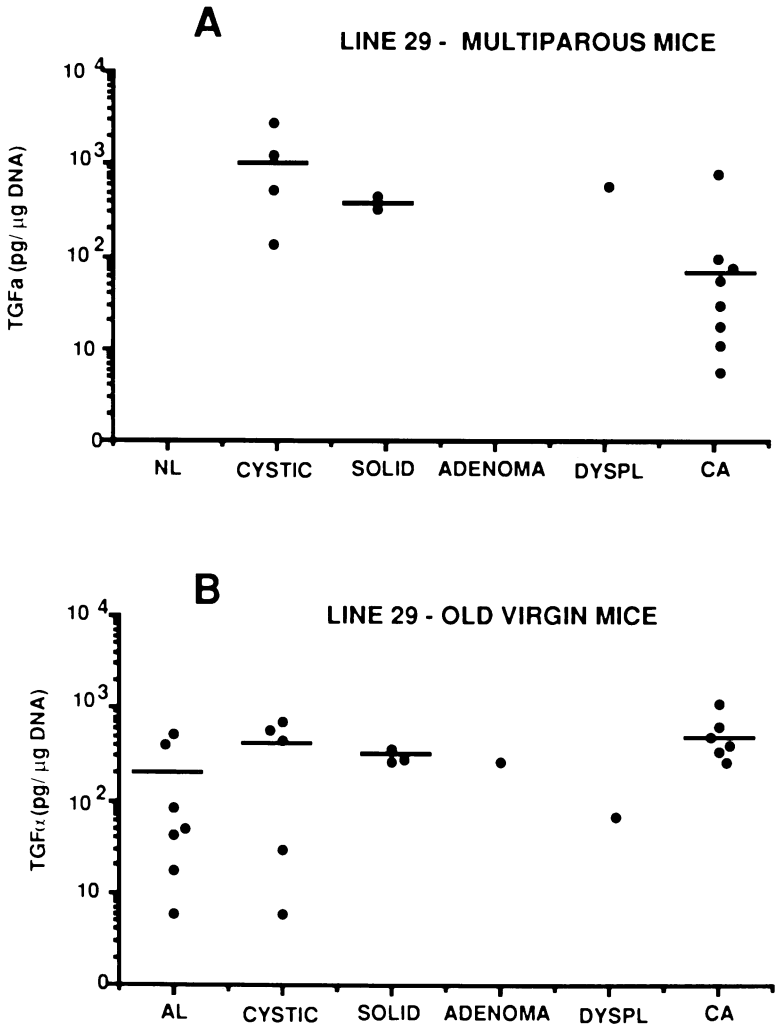

Figure 7. *Radioimmunoassay for TGFα polypeptide production in normal (NL), atropic (AL), cystic hyperplasia (cystic), solid hyperplasia (solid), adenoma, dysplastic (dyspl), or carcinoma (ca) tissues of multiparous* (A) *and virgin female* (B) *mice in line 29 (bar = mean).*

cytochemical staining for TGFα protein showed immunoreactivity in the hyperplastic sebaceous glands, especially in the cells in the periphery of the glands (Figure 11B). Expression of TGFα mRNA by Northern blot analysis was markedly elevated in the skin lesions of the mice of this line (data not shown).

Discussion

The development of carcinoma is a multiple-step process that includes several events, which lead to a malignant tumor with the potential to invade and metastasize. Although hyperplasia plays an important part in this process, its exact role is not entirely clear. Many early studies used irritation and inflammation as mechanisms to elicit hyperplasia.[20] Experimental models of hyperplasia using polypeptide growth factors have not been previously available.

Transgenic mice have provided important *in vivo* models for studying the biological consequences of overproduction of targeted genes (for review, [21–25]). Several human genes have been introduced into mice using the MMTV promoter/enhancer including c-*myc*,[26] H-*ras*,[27,28] int-1,[29] int-2,[30] and c-*neu*.[31,32] Carcinomas have occurred in many of these transgenic mice. The phenotype of these carcinomas may be predictive of their genotype.[33] Not all the transgenes are associated with proliferation of the mammary gland and none of the transgenic mice have had cystic hyperplasia as seen in the MMTV-TGFα transgenic mice described here. TGFα transgenic mice have been developed using the metallothionein promoter.[34,35] These mice have developed epithelial hyperplasia of several organs including pancreas, mammary gland and liver. Cystic hyperplasia of the mammary gland has not been described in these animals.

The TGFα transgenic lines presented in this paper offer several unique characteristics as models of hyperplasia. They provide an experimental model of mammary hyperplasia that is a consequence of sustained overproduction of an endogenously produced peptide growth factor. These mice have hyperplastic mammary gland

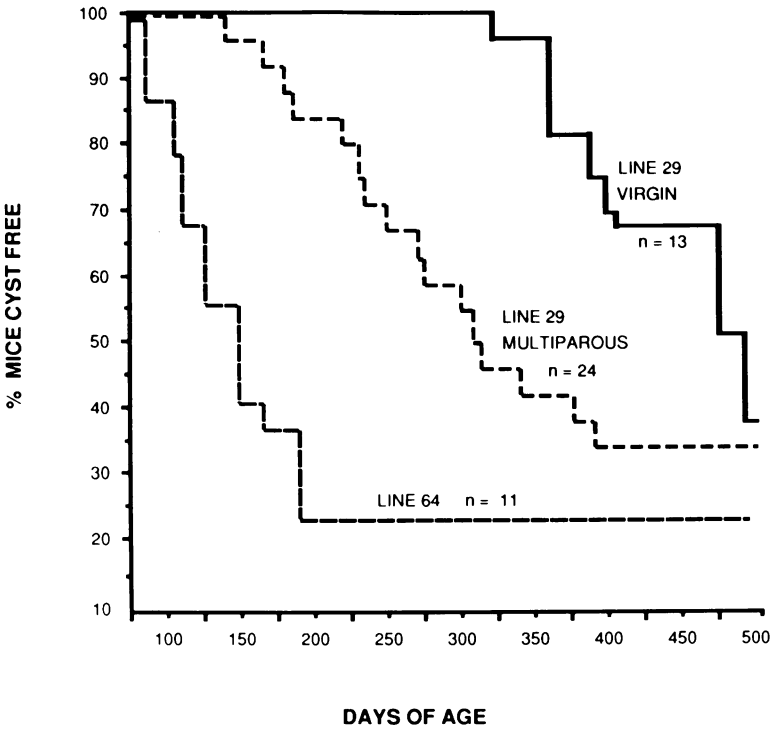

Figure 8. *Incidence of cystic and solid hyperplasia in lines 29 and 64.*

epithelium that may be analogous, although not yet proven, to human mammary epithelial hyperplasia. There is a delay of at least several months before carcinomas appear. This provides a temporal window through which one may view the effects of tumor-accelerating agents such as carcinogens or hormones or conversely protective agents such as the retinoids. The tumor incidence is not 100% in this model, which is advantageous when one introduces initiators and/or tumor promoters. Lastly, the MMTV-TGFα construct used in this model is selective for the mammary gland in female mice of at least two lines. This eliminates the development of tumors in other organs that could complicate the model system.

The delayed interval from expression of the transgene (approximately 5 weeks; Dempsey and Coffey, unpublished observation) to tumor formation, as well as the ran-

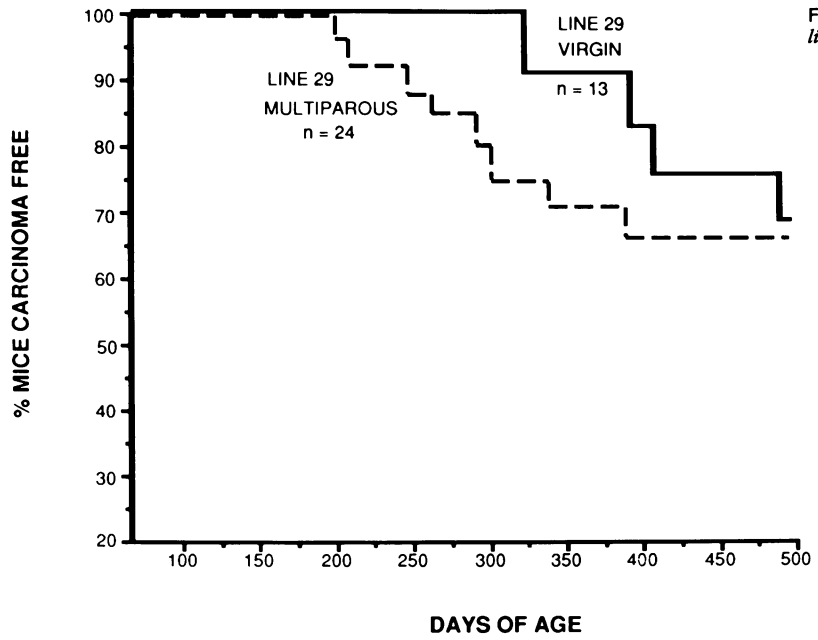

Figure 9. *Incidence of adenocarcinoma in line 29.*

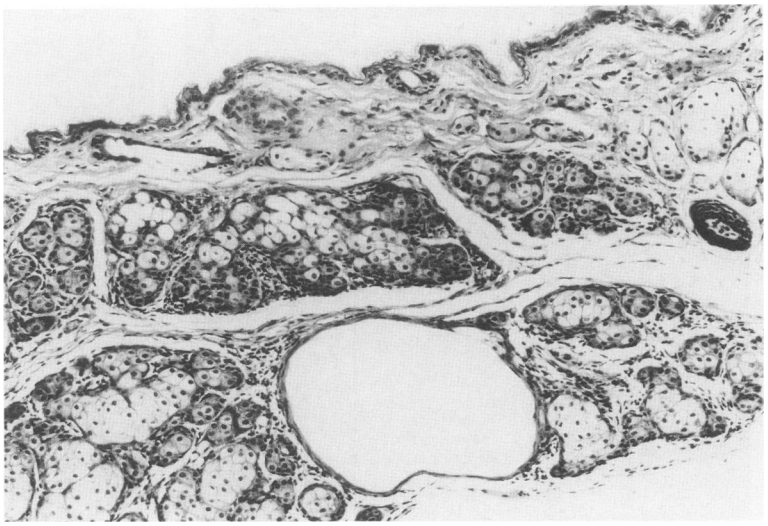

Figure 10. *Sebaceous gland hyperplasia is characterized by marked proliferation of the sebaceous glands in the dermis of a female mouse in line 254, (×85).*

dom and scattered foci of malignant transformation indicate that the development of mammary neoplasia is a stochastic process and that additional events are required. We have previously observed that EGF receptor mRNA expression is upregulated in the tumor and peritumoral tissue of these MMTV-TGFα transgenic mice.[16] Others have suggested that neoplastic transformation may occur if a critical threshold level of expression of ligand and receptor is achieved.[36] Additional events that are presently under study include p53 and ras mutations.

The role of TGFα in the development of neoplasia is unclear. TGFα is a known mitogen[37] and may function as a tumor promoter by enhancing proliferation of mammary epithelial cells. EGF is also known to be a mitogen and has been implicated as a possible promoter of mammary tumor development.[38,39] Although the exact mechanism of action for tumor promoters is unknown, clonal expansion of an initiated population of cells is considered a major mode of action.[2,5] This results in a premalignant lesion and provides an enlarged population of initiated cells for further promotion and/or progression. Alternatively, a tumor promoter, through its mitogenic action, may expand the number of cells upon which an initiator may act. Administration of the classic tumor promoter, 12-0-tetradecanoylphorbol 13-acetate (TPA), to cultured human keratinocytes results in a 20-fold increase in TGFα mRNA and protein[40] suggesting that some effects of TPA may be mediated by enhanced production of TGFα. This finding is consistent with the hypothesis that TGFα may act to promote mammary tumor formation.

TGFα mRNA expression and protein production appear to be decreased in solid lesions compared with cystic hyperplasia. Within the same lesion, the more dysplastic lesions appear to have reduced TGFα production. We speculate that TGFα participates early in the malignant process, perhaps through a tumor promoting effect, and is not necessary to maintain the transformed phenotype. Studies are underway to test this hypothesis more directly by the administration of an initiating dose of dimethylbenzanthracene to MMTV-TGFα transgenic virgin mice and their age-matched nontransgenic littermates.

We have studied three lines in detail in this article. All three lines have similarities but there are important differences between the lines. Two of the lines exhibit phenotypic changes only in the mammary gland whereas the third line has both skin and mammary gland abnormalities. Two of the lines with mammary epithelial hyperplasia have developed carcinoma whereas the third line has not, despite a high incidence of hyperplasia in this line. The reasons for these differences are not entirely clear. Currently, we are exploring different insertion sites or possible secondary events involving p53 and/or *ras* mutations as possible explanations.

Line 254 provides a unique *in vivo* model of sebaceous gland hyperplasia. TGFα mRNA expression and protein production are concentrated in the periphery of the sebaceous gland. This is the proliferative compartment in which the EGF receptor resides.[41] It is tempting to speculate that sebaceous gland hyperplasia results from enhanced proliferation, which is mediated by local overproduction of TGFα acting through its cognate receptor.

An unexplained finding in these lines is the lack of phenotypic changes in organs in addition to the mammary gland and skin despite high circulating levels of TGFα. Although an explanation for this finding is not clear from the data presented, local presentation of TGFα within the cell in which it is produced may contribute to the tissue-restricted effects that we observe. As discussed earlier, TGFα is produced as a transmembrane protein that is proteolytically cleaved to its secreted form. Membrane-fixed forms of TGFα retain biological activ-

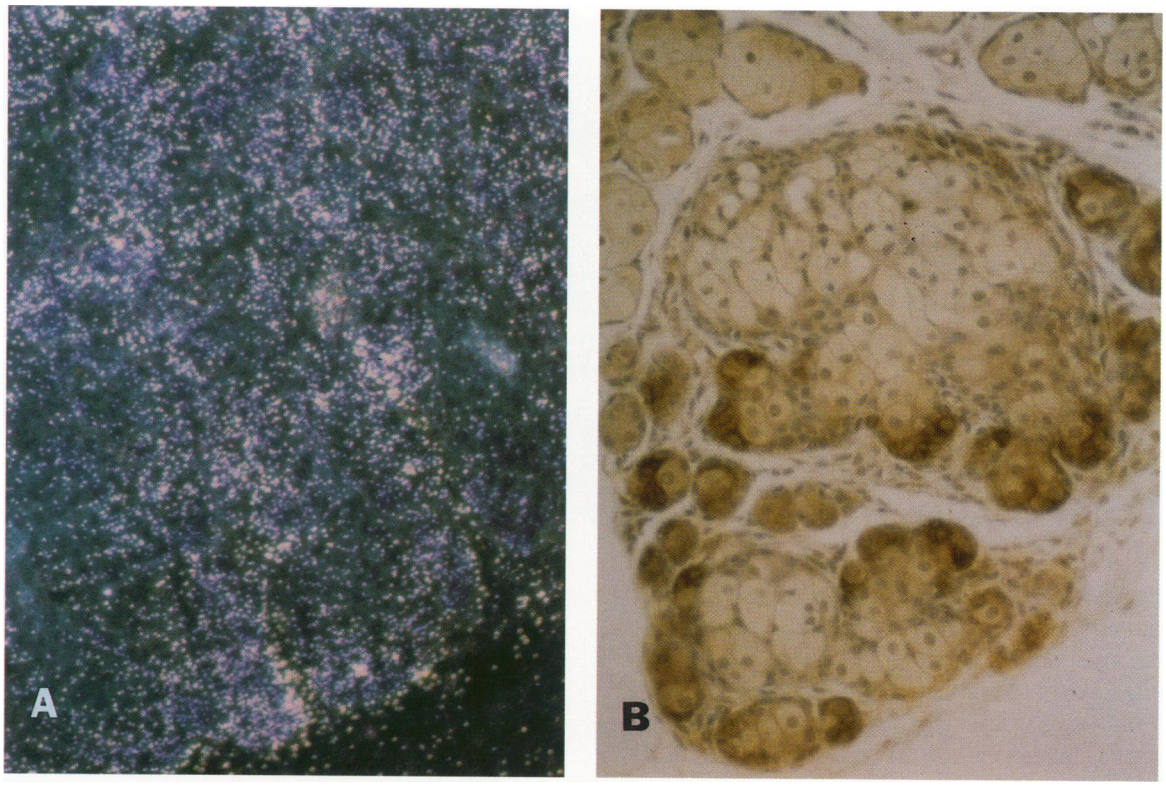

Figure 11. *Darkfield illumination of* in situ *hybridization of sebaceous gland hyperplasia* **(A)** *with* ^{35}*S-labeled anti-sense TGFα riboprobe shows highest expression in the sebaceous cells. Immunocytochemical staining for TGFα* **(B)** *is most intense in the sebaceous cells in the periphery of the glands but is also strongly positive in the cells comprising the rest of the gland (×110).*

ity.[33,42,43] NRK cells transfected with a mutant membrane-fixed TGFα retroviral construct exhibit a transformed phenotype.[44] In addition, Ju and coworkers[45] have recently observed that retroviral mediated introduction of TGFα into NIH 3T3 cells resulted in a transformed phenotype; however, administration of TGFα to parent 3T3 cells stimulated growth but did not induce transformation. Addition of TGFα to 3T3 cells expressing the transferred gene actually suppressed growth and focal transformation. We speculate that the sizeable amounts of TGFα produced within the mammary epithelium and sebaceous glands of these transgenic mice overwhelm the cellular machinery for the normal processing of pro-TGFα and may result in large amounts of membrane bound TGFα which, in turn, contributes to the distinctive phenotypes that we observe.

In conclusion, we have presented a transgenic mouse model of mammary gland and sebaceous gland hyperplasia which is characterized by high expression of human TGFα mRNA in the mammary gland of three lines and in the skin of one line. All of the lines have high circulating levels of human TGFα. A high percentage of mice develop hyperplasia of the mammary gland and a significant number subsequently develop adenocarcinoma. Although the histology resembles a continuity of premalignant to malignant change, similar to the situation in the human breast, the definitive biological potential of these patterns is, as yet, unknown. Nevertheless, these MMTV-TGFα transgenic mice that overproduce an endogenously produced peptide growth factor may provide useful *in vivo* models for the study of mammary carcinogenesis.

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