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13. ABSTRACT (Maximum 200) Transforming growth factor-alpha (TGF α) is an oncogenic protein that induces a transformed phenotype when overexpressed. Its receptor, Epidermal Growth Factor Receptor (EGFR), is a protooncogene that is overexpressed in breast carcinomas. This suggests that these two genes may be important contributors to breast tumorigenesis. Therefore, we examined TGF α expression in normal and transformed epithelium. We also examined TGF α and EGFR protein in human pre-invasive pathologies. Breast cancer cell lines expressed primarily the 4.6 kb TGF α transcript; no splice-variants were identified by Northern analysis. Western blot analysis showed that breast cancer cell lines expressed primarily the transmembrane form of TGF α , while freshly isolated breast tissue, tumors, epithelial organoids and early passage epithelial cultures expressed primarily the soluble form of TGF α . Due to the heterogeneity of patient samples it is difficult to determine what effect tissue culture has on normal TGF α protein synthesis and processing. TGF α protein expression in human archival tissue was constant during progression from benign to invasive disease. EGFR expression, however, was increased during the early hyperplastic phase of tumor progression, suggesting that EGFR may play a role in this pre-invasive pathology. In vitro treatment of isolated epithelial organoids with EGF and TGF α showed that TGF α was uniquely mitogenic. (200 words)				
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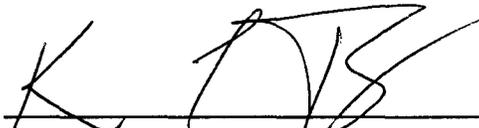
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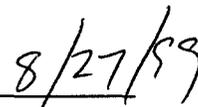
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INTRODUCTION

The etiology of breast cancer can be defined in terms of the genetic changes which predispose breast epithelium to disregulated growth. These genetic changes may be inherited or the result of environmental insults. Regardless of the mechanism, these genetic changes result in the increased expression of oncogenes and the loss of expression of tumor suppressor genes, leading to uncontrolled proliferation (1-3). TGF α is frequently expressed at high levels in invasive breast cancer (4-6). The protein is a dominant oncogene, i.e. when overexpressed in breast epithelium, it produces a fully transformed cell (5-9). It is, however, also expressed at high levels in normal breast epithelium, and its synthesis is driven by estrogen (10-15). The overall goal of this project is to determine the function of TGF- α in both normal and cancerous epithelium. To do this we examined the cell specific expression of TGF- α and its receptor, Epidermal Growth Factor Receptor (EGFR) in breast cancer cell lines, fresh human tissue and formalin fixed human archival tissue.

Results

1. Prepare riboprobes for in situ hybridization for in situ hybridization: subcloning of cDNAs, sequencing constructs. (Month 0-6).

This goal is completed and presented in the 1996-97 Annual Report.

Conclusion: 925 basepairs of the TGF α gene has been subcloned into the PGEM 3Z plasmid and is in the 5' to 3' orientation relative to the T7 promoter.

2. Identify TGF α splice variants in breast cancer cell lines. (Month 6-9).

This goal is completed and presented in the 1997-98 Annual Report.

Conclusion: Northern blots of isolated RNA on breast cancer cell lines were performed, and probed with the radiolabeled TGF α oligo-probe. All cell lines expressed the expected 4.6 kb transcript as quoted in the literature (4, 6, 7). No unusual transcripts which would indicate splice variants were detected.

3. Identify TGF α protein sizes in breast cell lines: Western blot. (Month 6-9).

This goal is completed and presented in the 1996-97 Annual Report.

Conclusion: Protein was isolated from: Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A cell lines and all primarily express the transmembrane form.

4. Repeat 2 and 3 in normal breast cells or organoids. (Month 9-12).

This goal was partially completed and presented in the 1997-98 Annual Report.

Conclusion: TGF α mRNA from normal epithelium is too low to detect by Northern blot analysis, therefore, we examined TGF α mRNA expression in normal epithelium by RT-PCR. Normal epithelium (organoids) from reduction mammoplasties and mastectomies

were collected; RNA was isolated and examined for TGF α message. Epithelium from two of the three reduction specimens and two of the three mastectomy specimens expressed TGF α mRNA (Figure 1). In addition, we analyzed message expression in fibroblasts isolated from both normal and tumorous breast tissue. Both the normal and tumor fibroblasts expressed detectable levels of TGF α mRNA (Figure 1). Due to the heterogeneity of expression from patient samples we did not detect a discernable pattern of TGF α expression.

Unlike cell lines that express the transmembrane form of TGF α (see #3 above) by western blot analysis, patient samples (whole tissue, isolated organoids, and early passage culture) primarily express the soluble form of the protein. These data suggest that fresh tissue may be a more appropriate model to study TGF α protein regulation in breast tissue (Figure 2).

5. Develop splice-specific probes. (Month 9-12).

This goal is completed and presented in the 1997-98 Annual Report.

Conclusion: Northern blot analysis of seven breast cancer cell lines, fresh breast tissue samples, and organoids using a radiolabeled TGF α oligo-probe does not indicate any potential splice variants.

6. Examine normal and proliferative breast tissue for cell-specific expression of TGF α . (Month 12-24).

This goal is completed and presented in both the 1997-98 Annual Report and attached manuscript.

Conclusion: Analysis of TGF α and EGFR protein expression by IHC in human archival breast tissue reveals: 1. TGF α protein expression is unaltered in the luminal cells as the

epithelium progresses through the histopathological subtypes. 2. TGF α is upregulated in endothelium adjacent to breast lesions. 3. EGFR protein expression increases in the luminal cells during the proliferative phase of breast tumorigenesis. This suggests EGFR is an important early event in the progression towards invasive disease.

7. Examine tissue as above for proliferation, estrogen receptor expression, and apoptosis.

(Month 18-24).

Partially completed and reported in 1997-98.

Conclusion: Functional expression of ER (i.e. ER/PR positive) correlated with expression levels of TGF α . IHC using the mib-1 monoclonal antibody to determine proliferation has been performed on all cases and are currently being analyzed. Data is currently being analyzed by principal investigator's thesis advisor and will be incorporated into manuscript. Histological analysis of the human archival breast tissue does not reveal an appreciable amount of apoptotic bodies.

8. Prepare Riboprobes for PKC. (see Conclusions).

9. Examine tissues as above for alterations in PKC isozyme expression (see Conclusions).

Reportable Outcomes

The principal investigator will be defending his thesis this summer to earn a Ph.D. in Pathobiology and Molecular Medicine from the University of Cincinnati, College of Medicine, Cincinnati, OH 45267-0529.

Furthermore, the principal investigator has accepted a post-doctoral position in the Department of Molecular Genetics at the University of California at Irvine.

Abstracts:

Taylor KL, Yassin R, Miller MA, Gear R, Heffelfinger SC. Altered Expression of Transforming Growth Factor-alpha and Epidermal Growth Factor Receptor protein expression during the progression from normal breast epithelium to tumor formation. Presented at American Association for Cancer Research Annual Meeting, Philadelphia, PA, 1999.

Taylor KL, Gear R, Heffelfinger SC. Inhibition of Angiogenic Potential of the Breast Cancer Cell Line MCF-7 by Tamoxifen. FASEB Annual Meeting, Washington, D.C., 1999.

Publications:

Taylor KL, Yassin R, Miller MA, Gear R, Heffelfinger SC. Altered Expression of Transforming Growth Factor-alpha and Epidermal Growth Factor Receptor protein expression during the progression from normal breast epithelium to tumor formation. Manuscript in Preparation.

Heffelfinger SC, Gear RB, Taylor KL, Miller MA, Schneider, LaDow K, Warshawsky D. DMBA-Induced Mammary Pathologies are Angiogenic In Vitro and In Vivo. Lab Investigation, submitted.

Taylor KL, Gear RL, Miller MA, Heffelfinger SC. Increased Angiogenic Capacity of Normal Breast Epithelium Isolated from Women with Invasive Breast Cancer.

Manuscript in preparation.

Taylor KL, Gear RL, Miller MA, Heffelfinger SC. Estrogen Regulation of the Angiogenic Potential of MCF-7 Cells. Manuscript in preparation.

Conclusions:

We have subcloned 925 basepairs of the TGF α gene into the PGEM 3Z plasmid and have made a ^{32}P labeled probe. Northern blots hybridized with the radiolabeled TGF α probe identified the 4.8 kb mRNA transcript as the primary TGF α message in seven breast cancer cell lines: Hs-578 Bst, SKBR3, MCF-7, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A. No splice variants were detected, despite extensive exposure on phosphoimaging plates. Northern blots on 8 patient samples (fresh tissue or organoids) have been performed. Using the radiolabeled TGF α probe no mRNA was detected. To detect TGF α mRNA RT-PCR was developed and showed that breast epithelium and fibroblasts expresses TGF α (Figure 1). Due to the heterogenous expression among patient samples no trend in expression was identified between epithelium isolated from reduction mammoplasties and mastectomies or from normal and tumor fibroblasts.

We examined TGF α and EGFR cell-specific protein expression in paraffin embedded formalin fixed tissues from 90 patients by immunohistochemistry. Protein expression was determined in the luminal epithelial cells and myoepithelial cells of ducts and lobules, microvasculature, stromal cells and lymphocytes. Expression of these proteins in each histological subtype was compared with one another as breast tissue progressed towards invasive disease. Our data indicate that TGF α expression does not increase in the luminal epithelial cells of the ducts or lobules as the epithelium progresses through the histopathological subtypes. In the microvasculature adjacent to tumors TGF α is upregulated but the results were not statistically significant. However, EGFR expression increases in the luminal epithelial cells during the proliferative phase of breast

tumorigenesis ($p = 0.006$). These data are presented in the 1997-98 Annual Report and the manuscript attached.

Previously, protein was isolated from seven cancer cell lines: Hs-578 Bst, SKBR3, MCF-7, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A and Westerns performed. The results indicate that the cell lines express primarily the transmembrane form (approximately 22 kD) of TGF α . Western blots performed on patient samples (fresh tissue, organoids, or early passage culture) express primarily the soluble form (6 kD) of TGF α . Protein isolated from a tumor specimen also expresses primarily the soluble form of TGF α . One explanation for these data is that the difference in protein expression in the cell lines is due to a tissue culture artefact. Therefore, fresh tissue may be a more appropriate model to study alterations in TGF α expression during breast tumorigenesis.

In the 1997-98 Annual Report it was mentioned that my thesis committee felt that the role of TGF α obtained from breast cell lines did not reflect the role of TGF α in the early proliferative process. This taken together with the data showing EGFR is upregulated in the proliferative phase of breast disease they felt that I needed to develop functional assays that would examine the role of TGF α /EGFR in breast proliferation. Therefore, I developed tissue culture techniques to isolate, culture, and characterize normal breast epithelium (presented in 1997-98 Annual Report). With this, we then addressed the following questions regarding TGF α processing in the breast: 1. determine if TGF α processing is different between the in vivo and in vitro situation by examining TGF α from fresh tissue vs. primary and early passage culture from the same patient. 2. determine the role stromal cells play in TGF α protein processing. 3. determine if EGFR

expression is altered with conversion from a relatively quiescent organoid to a rapidly proliferating monolayer culture. 4. investigate the proliferative effect of TGF α and EGF on isolated organoids by IHC using bromo-deoxyuridine (BrDU) incorporation.

Western blot of five organoids and three early passage culture shows that all five organoids expressed the soluble TGF α form and one of the early passage cultures expressed the soluble form of the protein (Figure 2a.). These results were consistent with our previous data showing patient samples expressed primarily the soluble form. To determine if TGF α protein processing differed between the in vitro and in vivo situation protein was isolated from fresh tissue, organoids, and early passage culture from the same patient. The soluble form was present in fresh tissue samples from two patients but expression subsequently decreased with culture (Figure 3). However, expression was lost in the organoids and early passage culture in one patient while it persisted, albeit at a lower level, in the other patient (Figure 3.). Given the fact that TGF α protein expression was highly variable among patient samples, the role of fibroblast co-culture on protein processing was not determined.

Since EGFR expression is upregulated during the proliferative phase of tumor progression we examined EGFR expression in the transition from quiescent organoids to monolayer culture. All five organoids and the three early passage cultures expressed EGFR and its expression level did not appear to be altered in vitro (Figure 4). This suggests that EGFR expression does not change from a resting in vivo situation to a rapidly proliferating in vitro situation. Our IHC data of human archival tissue showed relatively constant EGFR expression in the myoepithelium of ducts and lobule and an increase in the luminal cells in early progression. The fact that the early passage cultures contain both

myoepithelial and luminal cells may mask a change in expression between the two cell types as determined by Western.

To determine the proliferative effects of TGF α and EGF on isolated organoids, organoids were isolated and put into medium containing 100 ng/ml of TGF α , EGF or no growth factor. BrDU incorporation showed that there were significantly more cells taking up BrDU in the TGF α treated organoids compared to EGF treated organoids (Figure 5). These data suggest that TGF α acts as a mitogen for normal epithelium, whereas EGF does not.

Our findings show that breast cancer cell lines primarily express a 4.6 kb TGF α mRNA transcript; no splice variants are identified. Analysis of TGF α protein show that breast cancer cell lines express primarily the transmembrane protein while human tissue express primarily the soluble form of TGF α . It was initially believed that this difference in expression was due to a tissue culture artefact. However, human breast epithelium in early passage culture also expressed primarily the soluble form, although the not all samples expressed TGF α . The heterogeneity of expression in human tissue samples makes it difficult to determine why expression is different in cell lines. Possibly, the expression of the transmembrane form is a characteristic of immortalized cell lines. IHC for both TGF α and EGFR in human archival tissue reveals that TGF α remains constant with tumor progression. EGFR, however, is upregulated during the proliferative phase of tumor progression, but is then down-regulated in the later stages. This finding suggests that EGFR may help drive epithelial proliferation early in progression, but other factors are needed to continue progression.

As noted in previous reports, the direction of my graduate research was dictated by my thesis committee. Although fruitfull to some extent, the work on TGF α will only result in a single manuscript (enclosed). To fulfill the requirements for the degree, my committee steered me toward other interesting questions regarding pre-invasive breast disease. These data are summarized in the manuscripts listed under "reportable outcomes". All but one of these manuscripts are "in preparation" and will be submitted for publication within the next year.

MATERIALS & METHODS

Tumor cell culture: Breast tumor cell lines Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A were obtained from ATCC and cultured according to their protocols. Cells were passaged weekly using trypsin-EDTA.

Organoid isolation: Breast epithelium (organoids) was dissected from both mastectomy and reduction mammoplasty specimens, minced, and enzymatically digested with collagenase as previously described (16). Briefly, tissue is obtained within 1 hour of surgery, minced with opposing scalpels to 1 mm³ pieces, and enzymatically digested for 12 to 18 hours at 37 °C in a rotary shaker (60 rpm) with 150 U/ml Collagenase Type I (Gibco), 100 U/ml Hyaluronidase (Gibco) in CDM3 media. CDM3 media consists of DMEM/f12 (1:1), 2.6 ng/ml selenium, 100 ng/ml EGF, 0.1 µg/ml fibronectin (all from Collaborative Research, Bedford, MA), 3 µg/ml insulin, 25 µg/ml transferrin, 10⁻¹⁰ M estradiol, 10⁻⁶ M hydrocortisone, 10⁻⁸ M cAMP, 10⁻⁸ M triiodothyronine, 10⁻⁴ M ethanolamine, 10⁻⁴ M phosphoethanolamine, 0.1% BSA, 10 µg/ml ascorbic acid, 20 µg/ml fetuin (all from Sigma, St. Louis, MO), and 1X trace element mix (Biofluids, Rockville, MD). Following digestion, epithelial organoids and fibroblasts were separated from vascular fragments and from one another by sedimentation.

Northern Blot Analysis. Total cellular RNA was isolated using RNazol (CINNA/BIOTEX) and electrophoresed on formaldehyde-denatured agarose gels. RNA was transferred onto Nytran for hybridization. Prehybridization of the filters was carried out for 4-18 hours at 37°C in 50% formamide, 5x SSC, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1 mg/ml salmon sperm DNA, 20 mM sodium phosphate (pH 6.8), and 0.1% SDS. For hybridization the appropriate 5'-end labeled oligonucleotide or ³²P-labelled

riboprobe was added and incubated at 37° overnight. The filters were washed in 2x SSC and 1% SDS twice at room temperature, 2x SSC and 1% SDS twice at 37°C, and 0.2x SSC and 1% SDS for 15 minutes at 37°C.

Western blot: Cells or tissues are homogenized in 20mM Tris-HCl, pH 7.4, 2mM EDTA, 0.5mM EGTA for protein analysis (Bradford assay) and then electrophoresed under reducing conditions on 10% acrylamide SDS-PAGE (Laemmli) and transferred to PVDF membranes (Millipore) by the Towbin method. Membranes from each well were cut for replicate analysis, blocked in 5% non-fat dry milk, 0.5M NaCl, 10 mM Tris-HCl, pH 7.5. Primary antibodies (5-10 µg/ml) were incubated in blocking solution at RT overnight. The membranes were washed and incubated one hour at RT with biotinylated anti-mouse/rabbit Ig (1:100, Sigma), washed, and incubated another hour with Streptavidin-alkaline phosphatase (1:200, Sigma) in 5% non-fat milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5. The reaction was developed with BCIP/NBT substrate. Samples were calibrated for equal loading by using antibodies against actin.

BrDU incorporation: Organoids were isolated and plated in 35 mm dishes in CDM3 media containing 100 ng/ml of TGF α , EGF or no growth factor. Cells were cultured for 24 hours in the indicated media, the last 6 hours of which mg/ml of BrDU was added. Organoids were collected, pelleted, and fixed in 10 % formalin before being processed for immunohistochemistry. Proliferating cells were identified by those that were positive for BrDU incorporation. 200 nuclei were counted in the three most proliferative organoids in each group.

RT-PCR: Total cellular RNA was extracted from tissue using Tri-Reagent (Molecular Research Center, Cincinnati, OH). RNA was reverse transcribed using random

oligonucleotides (cDNA Cycle Kit, Invitrogen, Carlsbad, CA). PCR primers were derived from the TGF α sequence and are: 5' CGC CCG CCC GTA AAA TGG TC: 3' GCC AAA CTC CTC CTC TGG GC. These primers detect all known TGF α splice variants. The PCR reaction mixture utilized reagents from Life Technologies (Gaithersburg, MD) except as noted: 5 μ l 10x buffer, 2 μ l 50mM MgCl₂, 1 μ l 10nM dNTP (Invitrogen), 50 pmoles of primers, 1 μ g template DNA, 0.5 μ l 0.1M DTT, and 1 Unit Taq polymerase. Each reaction began with a 15 minute denaturation at 96°C and 5 minute 72°C, followed by 5 minute annealing 60°C and 90 second extension at 72°C. Following this 35 cycles of a 60 second denaturation (95°C), a 60 second annealing (60°C), and a 2 minute extension (72°C). A final 13 minute extension at 72°C was also included. PCR products were verified on a 5 % acrylamide gel. The predicted TGF α fragment was 519 basepairs and actin fragment was 610 bp.

Immunohistochemistry: 4 μ m tissue sections were deparaffinized and stained using the Ventana 320 automated immunostainer (Ventana Medical Systems, Tucson, AZ). The TGF α antibody Clone 31G7 (Zymed, San Francisco, CA) was used at a dilution of 1:10. The EGFR antibody Clone 213-4.4 (Oncogene Research Products, Cambridge, MA) was used at a dilution of 1:25. The monoclonal antibodies to BrDU (clone Ab3) is used at a concentration of 1:20. The monoclonal antibodies were pretreated with trypsin on the immunostaining system. Each reaction utilized a horse-radish peroxidase-conjugated secondary. Slides were then counterstained with hemotoxylin.

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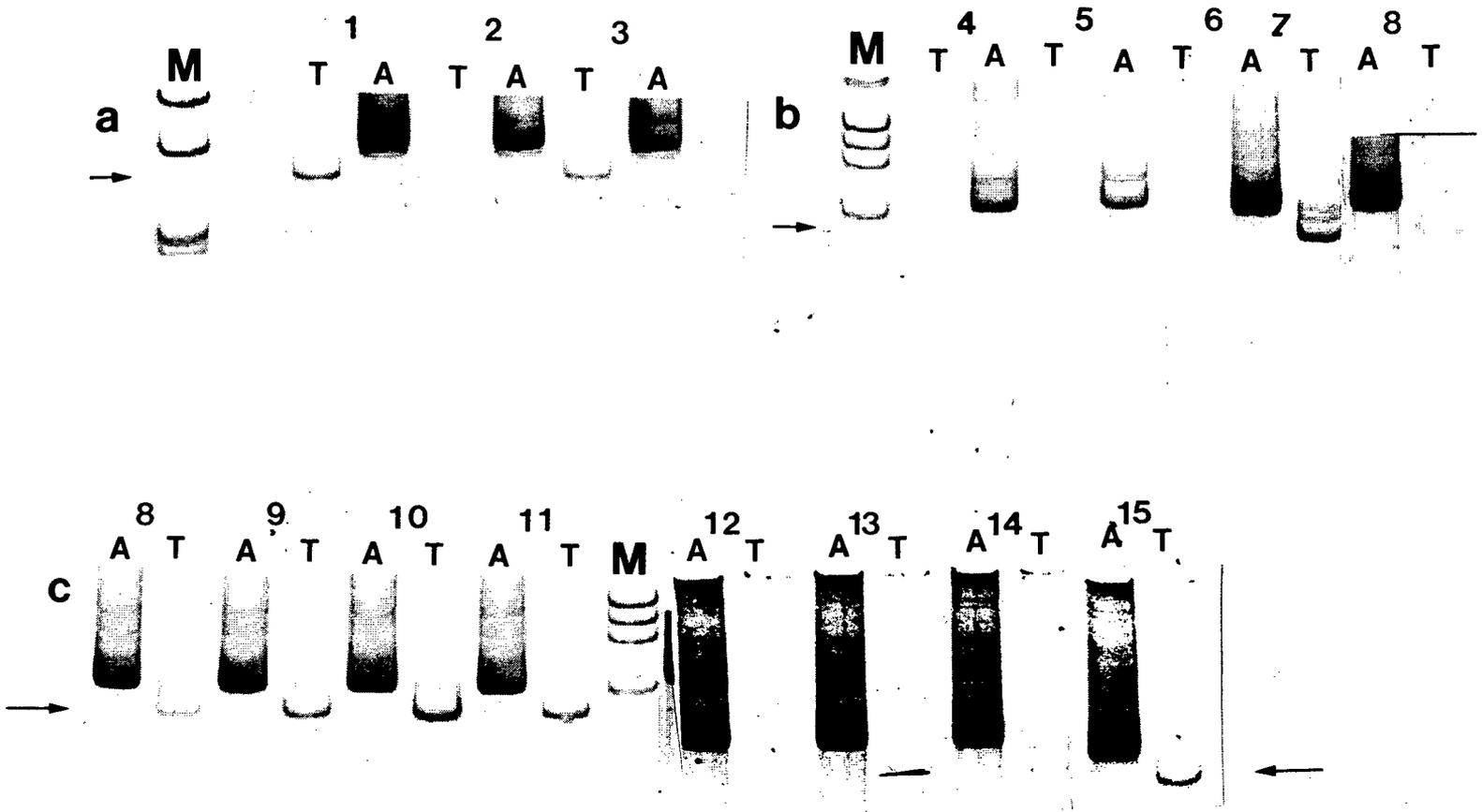


Figure 1. PCR products run on a 5 % acrylamide gel. TGF α fragment is 519 basepairs (lanes marked T) and actin fragment is 610 basepairs (lanes marked A). (a) organoids from three reduction mammoplasties (patients 1-3), (b) organoids from 3 mastectomies (patients 4,5,7), and one early passage culture (patient 6), (c) cultured normal fibroblasts (patients 8, 9, 15) and tumor fibroblasts (patients 10-14).

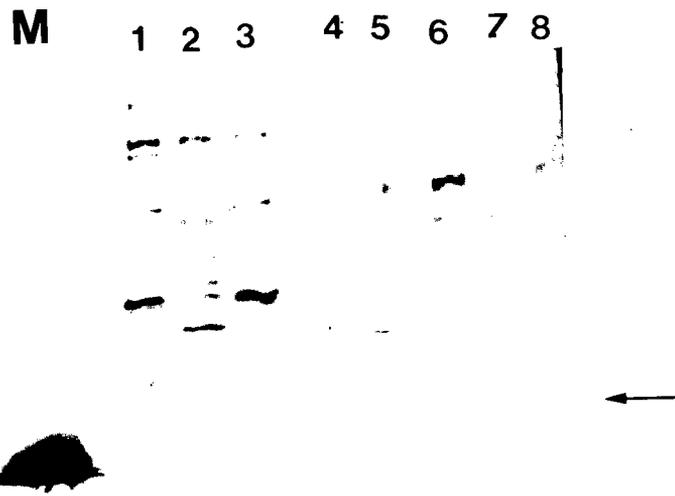


Figure 2: western blot of TGF α protein from 3 early passage cultures (patients 1-3) and 5 organoids (patients 4-8). Patients 2 and 4-8 express the soluble form of TGF α . All higher molecular weight forms seen are well above the size for any known TGF α protein.

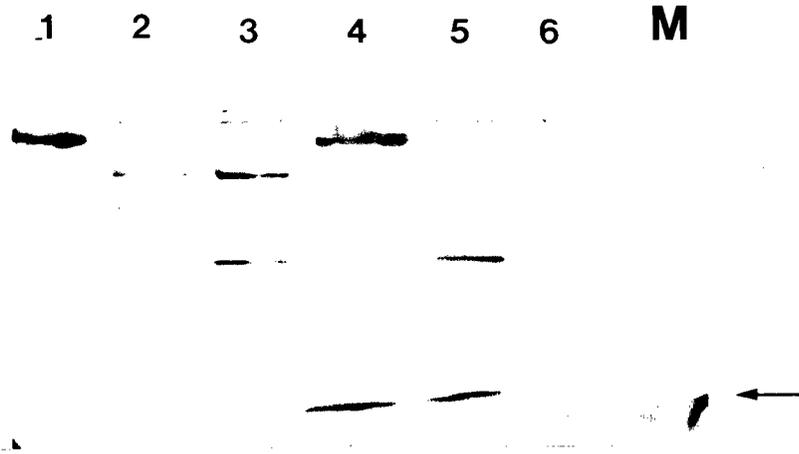


Figure 3: western blot for TGF α protein from 2 patients (lanes 1-3 and 4-6). Fresh tissues (1, 4), organoids (2, 5) and early passage cultures (3, 6). TGF α protein expression is highest in fresh tissue and decreased with culturing. Again high molecular weight forms are greater in size than any known TGF α product.

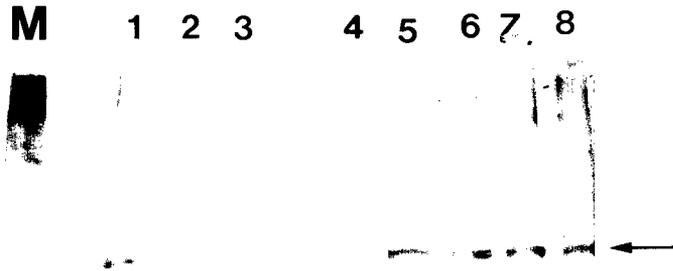


Figure 4: western blot for EGFR on 3 early passage epithelial cultures from 3 patients (lanes 1-3) and isolated organoids from 5 patients (lanes 4 – 8). All express the expected EGFR protein.

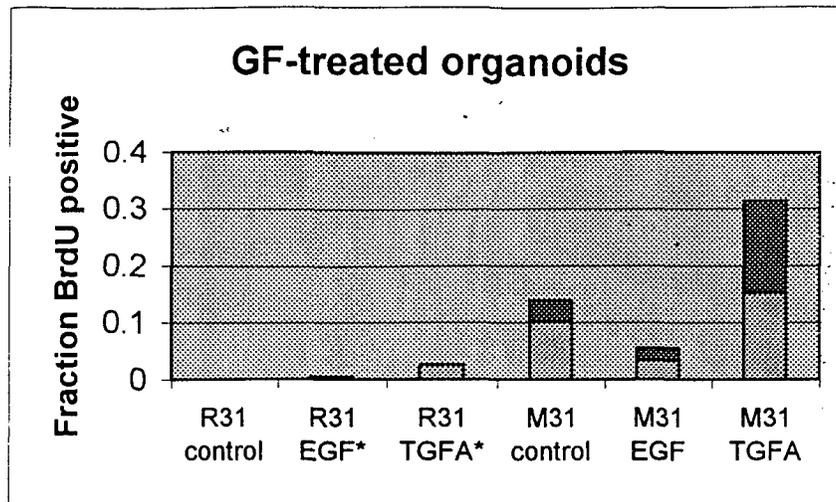


Figure 5: BrDU incorporation in EGF and TGF α treated organoids. * indicates statistically significant versus control ($p < 0.05$).

Altered Expression of Transforming Growth Factor – alpha and Epidermal Growth
Factor Receptor Protein Expression During Progression from Normal Breast to Invasive
Carcinoma

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Key Words: Breast cancer, proliferative breast disease, in situ carcinoma, TGF α , EGFR,
oncogene, autocrine, paracrine.

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Transforming Growth Factor-alpha (TGF α) is an oncogenic growth factor expressed in up to 70% of invasive breast cancers. Its receptor, Epidermal Growth Factor Receptor (EGFR), is a protooncogene overexpressed in a third of breast tumors. To determine if these oncogenic proteins are important in the earliest stages of breast tumorigenesis, we examined TGF α and EGFR expression by immunohistochemistry in normal breast epithelium (89 patients), proliferative breast disease (54 patients), in situ carcinoma (59 patients), and invasive breast carcinoma (26 patients) from human archival tissue. TGF α immunoreactivity was present in luminal epithelium throughout progression; whereas, expression in the myoepithelium increased coincident with the development of cellular atypia ($P = < 0.05$). The total tissue burden of TGF α increased in carcinoma in situ and invasive disease as a result of the influx of TGF α immunoreactive leukocytes. On the other hand, EGFR, which is normally expressed in the basal region of normal ducts and lobules showed a circumferential cell distribution in luminal cells primarily during the proliferative phase of tumor progression. These findings suggest that alterations in TGF α and EGFR expression may be important in the earliest stages of tumorigenesis.

Introduction:

In a subset of women, invasive breast cancer is preceded by histopathologic lesions such as usual hyperplasia (UH), atypical hyperplasia (AH), and carcinoma in situ (CIS).

Although it is not yet known what proportion of these lesions are direct precursors of invasive disease, their presence clearly increases the risk for subsequent development of invasive disease from two to ten fold, depending on the pathologic features identified (1).

As with invasive breast cancer, the genetic/epigenetic mechanisms that regulate the formation of these pre-invasive pathologies are poorly understood. However, epidemiologic studies show that breast cancer formation is a hormone dependent process, although the mechanisms through which hormones effect this process are not clear. One mechanism through which hormones may mediate breast tumorigenesis is via the interaction of hormone-dependent growth factors with their receptors (2, 3).

Transforming Growth Factor – alpha (TGF α) is a dominant oncogene expressed in 40-70% of breast cancers (3). The TGF α gene contains an estrogen response element in the 5' flanking region (4). When expressed in NIH3T3 cells, TGF α induces a transformed phenotype (5). Furthermore, TGF α is expressed in normal breast epithelium, as well as UH, AH, and DCIS (6-11).

In rodents TGF α is expressed in normal mammary epithelium, playing a role in breast development and maturation during pregnancy (12). During pregnancy TGF α drives epithelial proliferation. However, during lactation TGF α is downregulated coincident

with upregulation of milk proteins including whey acidic protein. During involution TGF α is once again upregulated coincident with milk protein loss. In transgenic mice over-expressing TGF α and in in vitro mouse mammary epithelial cell cultures, TGF α is able to suppress whey acidic protein production (13). Furthermore two transgenic models over-expressing mammary TGF α , in which TGF α is driven by either the mouse mammary tumor virus (MMTV) or an inducible zinc promoter, exhibit a transformed phenotype in adult mice. In early development these mice exhibit no morphologic changes; however, multiparous mice develop invasive carcinoma (14, 15). Thus, TGF α appears to play a role in breast tumorigenesis.

TGF α is one of several ligands for the receptor tyrosine kinase, epidermal growth factor receptor (EGFR), or c-erb-B1. Other ligands include Epidermal Growth Factor (EGF), Amphiregulin, and Betacellulin (16). Of these proteins TGF α and EGF have the highest affinity for EGFR. In addition, TGF α has an approximately 100 fold greater affinity for EGFR than EGF and is frequently considered a "superagonist" compared to EGF (17). For instance, TGF α is able to stimulate both mitogenesis and wound healing to a greater degree than EGF (16). All of these EGFR ligands are expressed in both breast tumors and breast cell lines (3, 11).

EGFR is overexpressed in up to 35% of breast tumors and, in node negative patients, is associated with a poor prognosis (18). Although TGF α is a hormone-dependent growth factor, there does not appear to be any hormonal regulation of EGFR. Studies show EGFR inversely correlates with estrogen receptor (ER) expression in breast tumor cells

by immunohistochemistry (19). Furthermore, patients overexpressing EGFR have a poor response to the estrogen antagonist, tamoxifen (20). EGFR is amplified in several breast cancer cell lines. In addition, transfection of cell lines with EGFR can induce both a transformed morphology as well as growth in soft-agar (21). Interestingly, ER positive MCF-7 cells transfected with EGFR were unable to maintain EGFR expression in the presence of estrogen (22). Finally, TGF α is expressed in up to 70% of breast tumors, the majority of which also express EGFR (3). Together these studies suggest that TGF α EGFR signaling may be estrogen driven early in progression, but once EGFR is overexpressed, EGFR drives estrogen-independent cellular events.

To date, numerous studies have explored the role TGF α and EGFR play in invasive breast cancer. Because rodent and in vitro studies clearly show that TGF α and EGFR regulate cell proliferation and differentiation during breast maturation, we decided to test the hypothesis that alterations in TGF α and EGFR expression are up-regulated in the earliest stages of tumorigenesis. Our results show that TGF α is produced by multiple cell types in breast tissue, and TGF α and EGFR are independently regulated during breast tumor formation. Therefore, expression of these proteins early in progression may be important in breast tumorigenesis.

Materials and Method:

Tissue characteristics: Paraffin-embedded archival tissues from 92 patients were retrieved from the pathology files at the University of Cincinnati based on a search for specimens that contained either epithelial hyperplasia, atypical hyperplasia, or carcinoma in situ. Specimens included mastectomies, excisional biopsies, and reduction mammoplasties. Two observers (SCH and RY) independently confirmed each diagnosis based on consensus criteria (23). From these samples we identified 89 cases of normal epithelium, 54 cases of UH, 13 cases of AH, 59 cases of DCIS (of which 15% were of the comedo type), and 26 cases of invasive carcinoma. Patients were seen between 1980 and 1995; 34% were premenopausal (ages 32-50) and 65%, postmenopausal (ages 51-81).

Immunohistochemistry: All immunohistochemistry was performed on 4 μ m paraffin-embedded sections using the Ventana ES immunostaining system. Following deparaffinization in xylenes, slides were placed in the instrument, which adds trypsin, the primary and biotinylated anti-mouse secondary antibodies, and avidin-conjugated peroxidase, as dictated by a bar code. Primary antibodies were incubated for 32 minutes at 37° C. The instrument performed all washes. Primary antibodies recognized TGF α (Clone 31G7, 1:10, Zymed, San Francisco, CA) or EGFR (Clone 213-4.4, 1:25, Oncogene Research Products, Cambridge, MA). The slides were counterstained with hematoxylin, by hand. In all cases an irrelevant mouse immunoglobulin was used instead of primary antibody as a negative control. TGF α and EGFR expression was evaluated in luminal and myoepithelial cells from ducts and lobules, adjacent small vascular

endothelium (from vessels within lobules or touching an epithelial basement membrane) versus nearby vessels (within one 40x field), stromal fibroblasts, and infiltrating lymphocytes. Only cytoplasmic TGF α and plasma membrane EGFR were considered positive. Each stain was graded on a subjective intensity scale of 0-4, with 4 indicating the greatest amount of staining intensity.

Statistics: All statistics were performed using Sigma Stat (SPSS, Chicago, IL). Among histological sub-types, means for each patient were compared by non-parametric One Way Analysis of Variance (ANOVA); multiple comparison testing was performed by Dunn's Method.

Results:

Immunohistochemistry for TGF α and EGFR protein expression was performed on human archival breast tissue. Mean TGF α immunoreactivity was 2.47 ± 0.2 and 2.62 ± 0.22 in the ductal and lobular luminal epithelium, respectively, in 90-100% of cases throughout progression. However, within the myoepithelium of ducts and lobules, TGF α staining intensity was 2.6 and 1.8 fold greater, respectively, with the development of atypia ($p < 0.05$) (**Figure 1**). CIS also exhibited approximately a 2.5 fold greater staining at the periphery relative to normal epithelium, which may represent either residual myoepithelium or adjacent fibroblasts and myofibroblasts ($p = < 0.001$). To determine whether this increased myoepithelial immunoreactivity represented increased mean intensities or a greater number of cases with TGF α immunoreactivity, we examined the percent positive cases, as shown in **Table 1**. These data show that there is an increase in both the number of cases with TGF α immunoreactivity and absolute intensity.

Since TGF α expression is not specific to the epithelium, we examined the expression of this growth factor in other cell types. Distal microvessels and stroma showed mean TGF α intensities of 1.23 ± 0.23 and 0.39 ± 0.09 , respectively, which did not change with progression. Adjacent microvessels were more intensely stained than distal vessels, particularly in AH and CIS (**Figure 2**), an increase that was not statistically significant. In addition, leukocyte TGF α immunoreactivity was greater in CIS and invasion ($p < 0.001$)

as seen in **Figure 3**. This was due to the influx of leukocytes commonly seen at this stage of disease progression (**Table 2**).

EGFR immunoreactivity was weak or absent in all cell types during progression, except within the epithelium. While TGF α immunoreactivity showed increases with the onset of atypia, changes in epithelial EGFR were detectable at an earlier stage. **Figure 4** shows mean EGFR staining in the luminal epithelium. In normal ductal and lobular epithelium EGFR staining was basal and appeared to be primarily myoepithelial, although a low level of staining was consistently seen on the basolateral surface of luminal cells (**Figure 5a**). With the onset of usual hyperplasia the luminal cell staining increased 2-3 fold and became completely circumferential at an intensity similar to that seen in the myoepithelium ($P = < 0.005$) (**Figures 4 and 5b**). However, this luminal expression of EGFR was short-lived since there was significantly less staining in CIS and invasive disease. **Table 3** shows that this apparent increase in stain intensity is due to the increased percent of cases expressing EGFR at the proliferative stages. Therefore, alterations in EGFR staining at the stage of UH include an increase in numbers of cases that are EGFR positive and a redistribution of EGFR immunoreactivity in the cell relative to normal epithelium.

Discussion:

Breast cancer is the result of misregulated cell proliferation resulting from accumulated genetic/epigenetic changes. Defining the early changes in oncogene expression during tumorigenesis will potentially lead to better prognostic criteria and intervention strategies for high-risk women who have pre-invasive breast pathologies. To this end, we examined pre-invasive breast pathologies for alterations in TGF α and EGFR, two oncogenes known to be important in breast tumor model systems. Because this receptor/ligand pair affects multiple cell types, we chose immunohistochemistry as the method to examine cell-specific expression of these proteins.

Although expressed in 40 to 70% of breast tumors, TGF α has also been reported in pre-invasive breast pathologies and normal breast tissue (3, 7, 11). Mizukami et al. found an increase in cases positive for TGF α by IHC when CIS and invasive lesions were compared to benign lesions (8). Yet, Parham et al. found similar expression of TGF α by IHC in UH, AH, and CIS (10). In addition, Perroteau et al. examined TGF α by competitive radioimmunoassay and detected TGF α protein in normal, pre-invasive, and invasive lesions with no significant difference in protein concentration among the groups (6). In our study, we examined expression in both the luminal epithelial cell and myoepithelial cell. TGF α , which is normally expressed in luminal epithelial cells, remained unaltered during tumor progression in both ducts and lobules. However, TGF α expression was greater in the myoepithelium with the onset of atypia. In the later stages

of tumorigenesis, myoepithelial cells are frequently absent for reasons which are unclear, so that influences of myoepithelial TGF α must occur early in disease progression.

TGF α immunoreactivity was also slightly increased in the immediately adjacent microvasculature of AH and CIS. This could be due to an increase in either epithelial or endothelial cell production. TGF α is a known angiogenic factor, which is produced by many cell types and can be expressed in the vasculature adjacent to breast carcinomas (24). We have shown that microvascular area increases adjacent to the epithelium of pre-invasive breast disease coincident with disease progression (26). Thus, TGF α may drive formation of vessels to supply these growing lesions.

Finally, the mean immunoreactivity for TGF α in infiltrating leukocytes was greater in the later stages of tumorigenesis. Our analysis shows that this increase reflects the increase in leukocytic infiltration associated with these types of pathology and not an intrinsic difference in the leukocytes themselves within these lesions. Nonetheless, the presence of TGF β in these cells adds to an increase in total TGF α expression and may play an important role in tissue proliferation and angiogenesis.

While numerous studies have examined EGFR over-expression or amplification in invasive breast disease, only a few studies have examined expression of EGFR in pre-invasive pathologies (26-30). To our knowledge, none of these studies have examined alterations in expression within the specific cell types that occur over the progression from normal epithelium to invasive disease. In our study, EGFR expression in luminal

epithelium increased during the proliferative phase of breast tumorigenesis in the ducts. Whether this is a redistribution of this cell surface receptor over the entire cell surface or a true increase in cell-specific expression is not clear. A detailed examination of normal epithelium showed that intense EGFR staining appeared to be restricted to the myoepithelium, with very low level staining identified on the basolateral surface of luminal cells. Our observation that EGFR is mostly expressed in the myoepithelium and not luminal epithelium fits immunolocalization studies examining EGFR expression in luminal and myoepithelial cells separated by flow cytometry from primary isolates (31). Since the luminal epithelial cells express TGF α , upregulation of EGFR early on in the process of tumorigenesis may activate an autocrine loop and further drive luminal cell proliferation. These data are consistent with another study that examined EGFR overexpression in epithelial hyperplasia in high-risk women and found a statistically significant increase in EGFR expression in both UH and AH when compared to normal epithelium (26). It would be useful to determine if the expression of EGFR is of any prognostic significance.

In conclusion, TGF α and EGFR are oncogenes that may play a role in breast tumorigenesis in a subset of women. Our study examined the cellular distribution of these two proteins in progressive stages from normal epithelium to invasive disease. We found several key alterations in protein expression. First, TGF α immunoreactivity is intense and ubiquitous in the luminal epithelium. It increases in the myoepithelium with the acquisition of atypia. Finally, and most importantly, EGFR is expressed in luminal epithelial cells during usual hyperplasia. Expression or redistribution of EGFR in the

luminal epithelium may implement an autocrine loop with TGF α and drive this cell type to proliferate.

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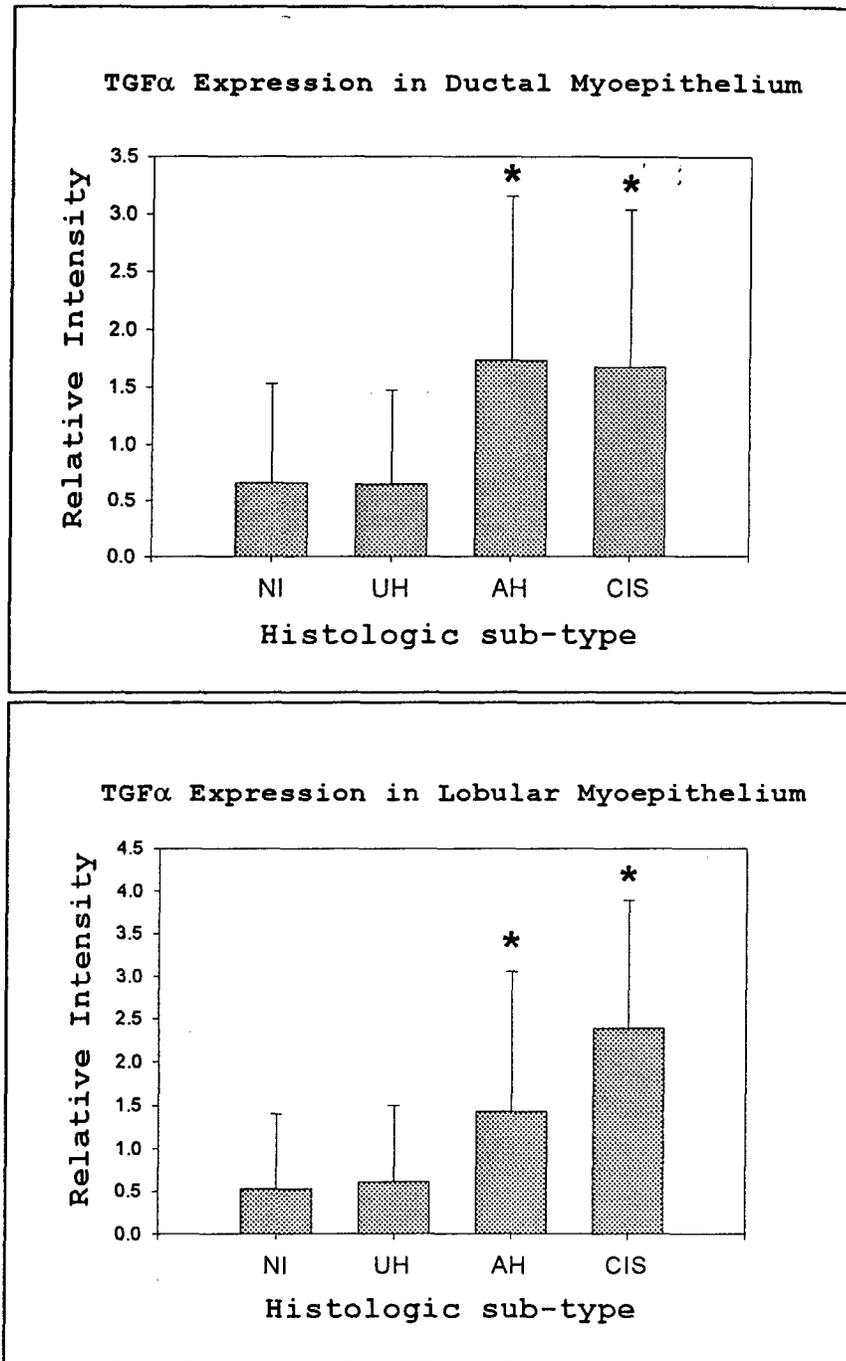


Figure 1. Shown are the mean and standard deviation for TGF α immunoreactivity in both ductal and lobular myoepithelium. The * indicates mean values that are statistically significant relative to normal tissue ($p < 0.05$).

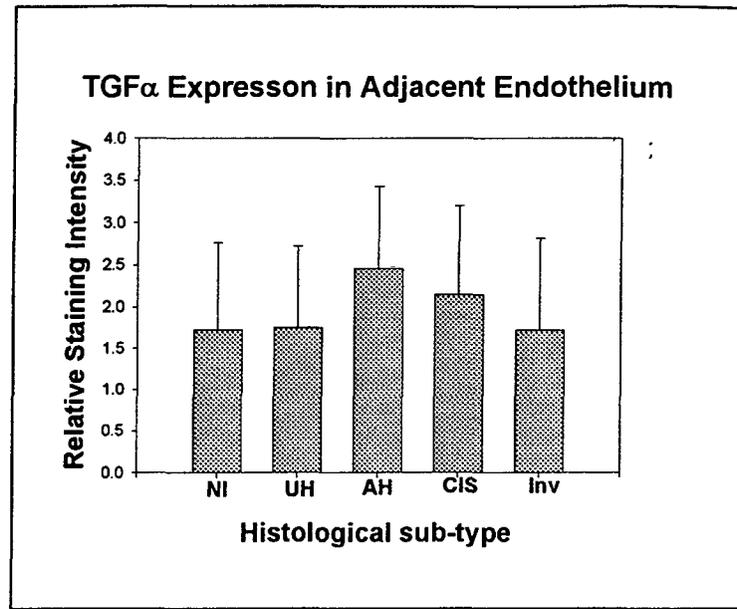


Figure 2 shows mean staining intensity for TGF α \pm the standard deviation in microvascular endothelial cells adjacent to the epithelial basement membrane.

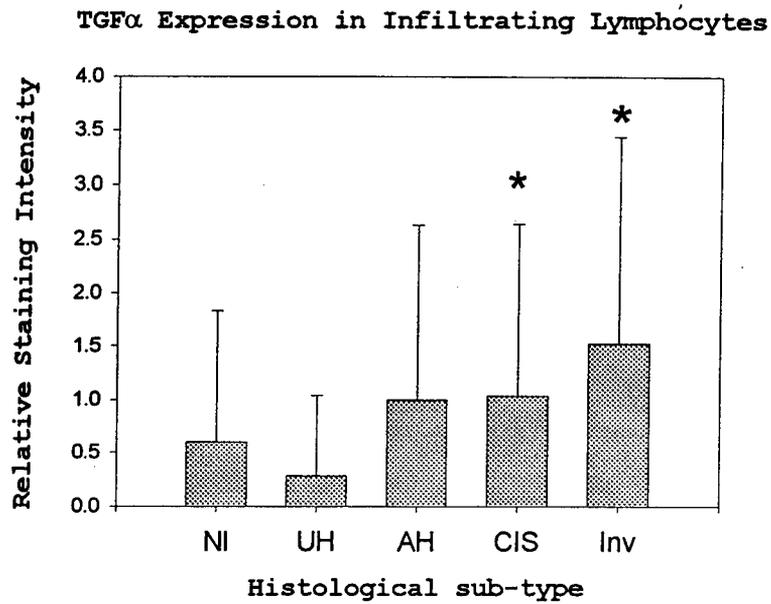


Figure 3 shows mean staining intensity for TGF α \pm the standard deviation in leukocytes adjacent to the listed histologic types of epithelium. The * means statistically significant relative to normal tissue ($P < 0.001$).

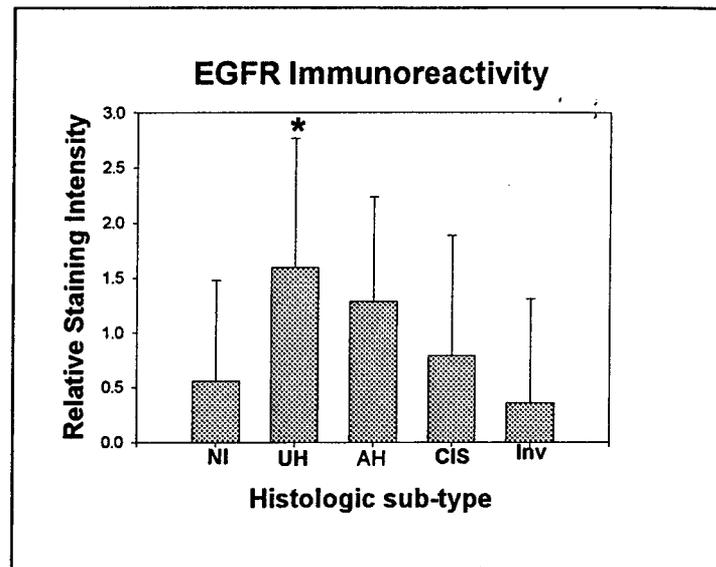


Figure 4 shows the mean \pm the standard deviation of EGFR immunoreactivity for each histologic subtype. The * indicates values which are statistically different from normal epithelium ($p=0.005$).

	DME	LME
Normal	49%	37%
UH	50%	44%
AH	64%	57%
CIS	66%	67%

Table 1. Shown are the percent of cases positive for TGF α in ductal and lobular myoepithelium (DME = ductal myoepithelium, LME = lobular myoepithelium).

	Leukocytes
Normal	21%
UH	13%
AH	39%
CIS	28%
Inv	39%

Table 2 shows the percent of cases positive for TGF α expression in infiltrating leukocytes during tumor progression.

	LE
Normal	33%
UH	93%
AH	80%
CIS	33%

Table 3 shows the percent of cases positive for EGFR immunoreactivity in the luminal epithelium (LE).

PANEL NOT REPRODUCIBLE

PANEL NOT REPRODUCIBLE

Figure 5. Panel a shows the pattern of EGFR immunoreactivity in normal epithelium.
Panel b shows UH. Note the redistribution of EGFR immunoreactivity around the
circumference of the luminal epithelial cells.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

A handwritten signature in black ink, appearing to read "Phyllis M. Rinehart".

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl

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