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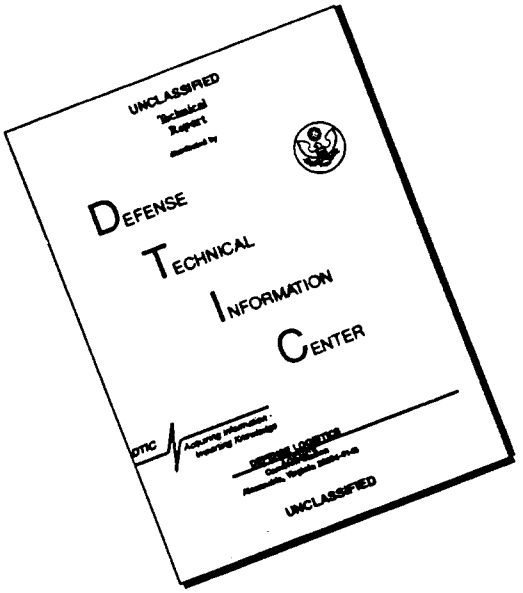

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To determine if TNFalpha plays a physiological role in normal mammary gland development, TNF and TNF receptor expression were measured in epithelial cells (MEC) isolated from mammary glands of virgin, pregnant, lactating, and post-lactational (day 7 of involution) rats. TNF mRNA increased significantly during pregnancy, then decreased during lactation and involution. The 26-kDa, transmembrane form of TNF protein, undetectable in MEC from virgin rats, increased throughout pregnancy and lactation and disappeared during involution. In contrast, p55 TNF receptor (TNFR) mRNA levels peaked in early lactation and declined thereafter, while p75 TNFR mRNA levels rose steadily through lactation. Using specific agonistic antibodies, the p55 TNFR was found to be the sole mediator of TNF-induced proliferation. Intriguingly, the two receptors had opposing effects on functional differentiation (casein accumulation), with inhibition occurring through the p55 receptor and stimulation through p75. Lastly, in DMBA- and NMU-induced mammary tumors, both TNF and TNFR mRNA levels were found to be decreased in comparison to normal MEC. Taken together, these results suggest that TNF plays a role in the growth and development of the mammary gland, and that both TNF receptors are important for TNF function and may mediate different effects.

14. SUBJECT TERMS Breast Cancer, Tumor Necrosis Factor-Alpha, Hormonal Regulation, Growth Factor, TNF Receptors, Development, Mammary Epithelial Cells

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FOREWORD

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Linda M. Varela 7/24/96

PI - Signature Date

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INTRODUCTION

I. Nature of problem: The risk that a woman in the US will develop breast cancer in her lifetime has now increased to an unprecedented one out of eight women, and breast cancer is currently the most common cancer among women. It is therefore crucial that a better understanding of those factors leading to the development of breast cancer and of the metastatic phenotype be achieved so that more appropriate strategies for its prevention and/or treatment can be applied. This can be achieved by a thorough investigation of the normal mammary gland, and through a subsequent comparison of this with malignant breast cells, important clues as to how breast cancer evolves may be discovered. While the fundamental causes for breast cancer remain elusive, a growing body of data suggests that the major risk factors may be inherently biological, such as natural hormone or growth factor production (1). Thus, by examining the cyclical variations in hormone levels, the complex hormonal regulation of proliferation and differentiation, and the changes in gene expression that occur as the mammary gland progresses through the different stages of development, the prospects for prediction, prevention, and treatment of breast cancer may be heightened. A possible key player in this intricate network is tumor necrosis factor - alpha (TNFα). TNFα has been shown by our laboratory to play a significant role in directing both the proliferation as well as the morphological and functional differentiation of mammary epithelial cells (2). This regulator may reach the MEC not only via traditional endocrine and local paracrine routes, but there may also be autocrine synthesis of TNFα by the MEC as well. Normally, there is strict control of the expression of this cytokine; however, it is possible that any disruption of this control has the potential to markedly affect the degree of both growth and differentiation and may confer on the cell a transformed phenotype. Thus, it is critically important to determine the physiological role of TNFα in the growth and development of the mammary gland and how this role differs in transformed cells, so that more appropriate strategies for the prediction, prevention, and treatment of breast cancer may be developed.

II. Address to Statement of Work:

The following aspects of the Statement of Work were addressed:

A. *Investigation of TNFα expression during mammary gland development.* The focus of this aim was to determine whether TNFα was produced by normal MEC, and to investigate changes in TNFα mRNA and protein expression during the various stages of normal *in vivo* mammary gland development.

B. *Identification of TNF receptor expression by normal MEC.* The purpose of these studies was to determine which TNF receptors were present on normal MEC, and to evaluate changes in expression of these receptors during the various stages of *in vivo* mammary gland development. The functional roles of the receptors were also identified.

C. *Investigation of TNFα expression in transformed MEC in DMBA- and NMU-induced mammary tumors.* The goal of these studies was to determine whether TNFα and TNF receptor expression were altered in transformed MEC as compared to normal MEC.

III. Background: Tumor necrosis factor - α (TNFα) is a multifunctional cytokine that was originally defined by its ability to cause the hemorrhagic necrosis of tumors *in vivo*. It is now

known, however, that TNFα affects the growth, differentiation, and/or function of virtually all cell types, either by acting alone or in concert with a variety of other cytokines, hormones or growth factors (3-6). On the first level, this complex physiology may be the result of different forms of TNFα, each of which has significant activity (7,8). Specifically, TNFα is first synthesized as a 26-kDa transmembrane precursor which is then proteolytically cleaved to release the 17-kDa soluble cytokine. In addition, TNFα production is under strict regulation; expression is controlled by numerous variables, including hormones and cytokines, and there is also stringent post-transcriptional regulation (9-11). Lastly, the pleiotropic effects of TNFα may be mediated through different receptors. Two distinct cell surface TNF receptors of 55- and 75-kDa (p55 and p75, respectively) have been identified in varying proportions on the membranes of virtually all cells so far examined (12-14). Although these two receptor forms have some sequence homology in their extracellular domains, the absence of significant homology in their intracellular regions suggests that the two receptors may activate different signal transduction pathways and ultimately induce different responses (15). For example, cytotoxicity in numerous cell types and up-regulation of epidermal growth factor receptor mRNA in epithelial cells have been attributed to the p55 receptor, while signals for thymocyte proliferation and up-regulation of transforming growth factor α mRNA in epithelial cells are transduced via p75 (16-18).

Previous studies demonstrated the presence of TNF receptors on both normal and malignant mammary cells (19) and determined that human breast cancer cells in culture were sensitive to either the cytostatic or cytotoxic effects of TNFα (5,19,20). In contrast, earlier work by our laboratory demonstrated that TNFα *stimulated* the growth and morphological development and regulated the function of normal rat mammary epithelial cells (MEC) in culture (2). These studies used a primary culture model system developed by our laboratory in which MEC from immature rats, cultured in the presence of a defined, serum-free medium, proliferate and morphologically and functionally differentiate to an extent comparable to that of the lactating mammary gland (21-23). Specifically, TNFα stimulated MEC proliferation under optimal medium conditions as well as in medium deficient in epidermal growth factor (EGF), a major mitogen for MEC in culture. TNFα had no effect on the morphological differentiation of MEC under optimal conditions, but had a marked stimulatory effect in medium either lacking or deficient in EGF. Interestingly, in optimal medium, TNFα inhibited casein accumulation, but in the absence of EGF its effects on functional differentiation were biphasic, with low concentrations of TNFα stimulating and higher concentrations inhibiting casein. Whether the stimulatory effect was direct or secondary to the TNFα-induced alveolar morphogenesis seen under these suboptimal conditions has not yet been resolved. These experiments did suggest, however, that TNFα may play a physiological role in normal mammary gland development. If so, it would be expected that either the MEC or the stromal cells of the mammary gland would produce TNFα, one or both TNF receptors would be present on MEC, and that expression of both TNFα and its receptors would be developmentally (and thus hormonally) regulated. In addition, there may also be alterations in both TNFα and TNF receptor expression during the process of mammary carcinogenesis.

To test these hypotheses, the current studies were designed to examine the expression of TNFα and its two receptors in freshly isolated rat MEC during puberty, pregnancy, lactation, and post-lactational mammary gland involution. Additionally, using specific agonistic antibodies, the functions of the p55 and p75 TNF receptors were each investigated in rat MEC in primary culture. Lastly, TNFα and TNF receptor expression were examined in DMBA- and NMU-induced mammary tumors and compared to expression in normal MEC.

EXPERIMENTAL METHODS

Materials

Dispase II powder (neutral protease), leupeptin, glycine and guanidine hydrochloride were purchased from Boehringer-Mannheim (Indianapolis, IN), collagenase class III from Worthington Biochemical (Freehold, NJ), liquid dispase (5,000 caseinolytic units) from Collaborative Biomedical Products (Bedford, MA), MOPS from Calbiochem (Cambridge, MA), and newborn calf serum (NCS), gentamicin, phenol red-free RPMI-1640, and Trizol from GIBCO BRL (Grand Island, NY). Diaminobenzidine (DAB), fatty acid-free bovine serum albumin (BSA), phenol red-free Dulbecco's Modified Eagle's Medium-F12 (DMEM-F12) containing 12 mM HEPES, dithiothreitol (DTT), urea, agarose, trichloroacetic acid (TCA), sodium phosphate, aprotinin, pepstatin A, benzamidine, iodoacetamide, sodium deoxycholate, sodium azide, glutamine, and diethyl pyrocarbonate (DEPC) were from Sigma (St. Louis, MO). Ovine prolactin (NIDDK-oPL-20) was a gift of NIDDK-NIH (Bethesda, MD) and mouse epidermal growth factor (EGF) was purchased from Upstate Biotechnology (Lake Placid, NY). Goat anti-rabbit biotinylated immunoglobulin G and streptavidin-peroxidase were products of Chemicon International, Inc. (El Segundo, CA), and chloroform, formaldehyde, and isopropanol of Fisher Scientific (Fair Lawn, NJ). Peroxidase conjugated goat anti-hamster immunoglubulin G and biotinylated goat anti-hamster immunoglobulin G were procured from Caltag (So. San Francisco, CA), and streptavidin-peroxidase (for the TNF receptor Western blot analysis) from Zymed (So. San Francisco, CA). [methyl-^{3}H]-Thymidine, [^{125}I]-Bolton-Hunter labeled TNFα and [α-^{32}P]-deoxycytidine 5'-triphosphate were purchased from DuPont NEN (Boston, MA). The Multiprime labeling kit used to radiolabel cDNA probes was purchased from Amersham (Arlington Heights, IL). The cDNA probe for recombinant human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Clontech (Palo Alto, CA). Recombinant human TNFα (2.5 x 10^6 U/mg), recombinant murine TNFα (3.1 x 10^6 U/mg) and plasmid pRtac1 containing a 1 kb rat TNFα cDNA insert (24) were generous gifts of Asahi Chemical Industry Co. (Fuji, Shizuoka, Japan). Plasmids containing the 1.8 kb and ~1.0 kb cDNA inserts corresponding to the murine p55 and p75 TNF receptors, respectively (15), were gifts of Genentech (San Francisco, CA), as were the two agonistic antibodies specific for either the p55 or p75 TNF receptor (18). The cDNA probe for the rat cyclophilin gene (p1B15) (25) was generously provided by Dr. Henry Thompson (AMC Cancer Research Center, Denver, CO). The monoclonal hamster anti-mouse p55 TNF receptor antibody was a generous gift of Dr. Robert Schreiber (Washington University, St. Louis, MO), and the anti-human p55 TNF receptor antibody was kindly provided by Dr. M. Brockhaus (Hoffmann-LaRoche, Basel, Switzerland). WEHI 164 mouse fibrosarcoma cells were a gift of M. Jane Ehrke and Dr. Enrico Mihich.

Animals

For primary culture of MEC, virgin, 50- to 55-day-old female Sprague-Dawley Crl:CD BR rats purchased from Charles River (Wilmington, MA) were used as the source of mammary glands. For isolation of MEC for analysis of TNFα and TNF receptor mRNAs, pregnant Sprague-Dawley Crl:CD BR rats were purchased from Charles River, and either sacrificed during pregnancy or allowed to give birth. Rats were then sacrificed on days 5, 10 or 15 of lactation, or either 12 hours or 1 week after the pups had been removed at day 21 of lactation. Virgin rats, 50-55 days of age, were used as controls. Female CD2F1 mice, purchased from NCI-Frederick Cancer Research Facility, Biological Testing Branch (Frederick, MD), were used to carry the Engelbreth-Holm-Swarm (EHS) sarcoma, which was the source of the reconstituted basement

membrane (RBM) matrix for the primary culture of the MEC organoids. Virgin rats and mice were fed rat (6% fat) or mouse chow diets, respectively, while rats during pregnancy and lactation were fed chow diets containing 10% fat (Teklad, Madison, WI). All animals were fed *ad libitum* and had free access to water. Animal rooms were air conditioned and humidity controlled, with light cycles of 14 h on-10 h off (rats) or 12 h on-12 h off (mice). Care and use of the animals was in accordance with NIH guidelines and Institute Animal Care and Use Committee regulations.

Preparation of reconstituted basement membrane

The RBM matrix used for primary culture of the MEC was extracted from the EHS sarcoma as previously described (21).

Mammary epithelial cell organoid isolation

For analysis of TNFα and TNF receptors in freshly isolated MEC, mammary glands were excised from virgin, mid-pregnant (day 13-14), lactating (days 5, 10, and 15) or post-lactational (either 12 hours or day 7 of involution) female rats, mechanically minced, placed in digestion solution (10 ml/g wet wt) consisting of 0.2% (w/v) collagenase type III, 0.2% (w/v) dispase II, 5% (v/v) NCS, and 50 μg/ml gentamicin in phenol red-free RPMI 1640, and incubated at 37°C for approximately 13.5 hours for virgin glands or 3-4 hours for pregnant, lactating and post-lactational glands. The resultant epithelial organoids were pelleted by centrifugation, washed once with DMEM-F12, resuspended in DMEM-F12, and separated from single cells and stromal contaminants by differential filtration through 530- and 60-μm nitex filters (Tetko, Depew, NY). The epithelial organoids retained on the 60μm filter were then rinsed off, refiltered through the 530μm filter, and incubated on tissue culture plastic in a solution of 10% (v/v) NCS and 50 μg/ml gentamicin in phenol red-free DMEM-F12 for ~2.5 hours at 37°C; this allowed the remaining stromal cells to adhere to the plastic and be removed from the non-adherent mammary epithelial organoids. Lastly, the number of MEC within the organoids was enumerated by counting of nuclei as previously described (2).

RNA isolation and Northern blot analysis

The freshly isolated epithelial organoids were pelleted by centrifugation at 500 x *g* for 10 min and homogenized in Trizol (10^7 cells/ml), a phenol-based reagent capable of extracting both RNA and protein from the same cells. Total cellular RNA and protein (see below) were then prepared following the protocol of the manufacturer, and poly A^+ mRNA was subsequently fractionated using the PolyATract mRNA Isolation System (Promega). Denatured poly A^+ mRNA (approximately 1.0 - 1.5 μg dissolved in RNase-free water) was separated by electrophoresis on a 1% (w/v) agarose gel containing 1 X MOPS and 2.2 M formaldehyde and transferred to Hybond N nylon membranes (Amersham). The RNA was then crosslinked to the membranes by UV irradiation (UV Stratalinker 1800, Stratagene, La Jolla, CA). Northern blots were prehybridized for 2 hrs at 65°C in 0.5 M Na phosphate, pH 7.2, 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA according to the method of Church and Gilbert (26), and probed overnight at 65°C in the same buffer using $2x10^6$ cpm/ml of random-primed ^{32}P-dCTP-labeled cDNA probes for either TNFα or the p55- or p75-TNF receptors. Blots were washed twice in 40 mM Na phosphate, pH 7.2, 0.5% (w/v) BSA, 5% (w/v) SDS, and 1 mM EDTA for 20 min at 65°C and twice in 20 mM Na phosphate, pH 7.2, 1% (w/v) SDS, and 1 mM EDTA for 20 min at 65°C. They were then exposed to film (Kodak X-Omat AR) at -80°C and subsequently placed into Phosphorimager cassettes (Molecular Dynamics) at room temperature. Quantifications from

the Phosphorimager exposures were performed using ImageQuant software (Molecular Dynamics). Hybridization with both the GAPDH and cyclophilin (p1B15) cDNA probes was used for normalization.

TNFα and casein Western blot analyses

Proteins from the Trizol homogenates of the MEC organoids isolated from virgin, pregnant, lactating, and post-lactational rats were precipitated with isopropanol and washed with 0.3 M guanidine-hydrochloride in 95% (v/v) ethanol according to the manufacturer's directions. Dried protein pellets were solubilized in 10 M urea containing 50 mM DTT for 1 hour, boiled, sonicated, diluted with 4x Laemmli sample buffer (27) and reboiled for 4 minutes. Fifty μg (for TNFα) or 2.5 μg (for casein) of total protein (as determined by Biorad protein assay) (28) was electrophoresed, respectively, on either 15% (TNFα) or 12.5% (casein) polyacrylamide-SDS gels according to the method of Laemmli (27), transferred to nitrocellulose, and blocked overnight at 4°C with either 5% (w/v) (TNFα) or 1% (w/v) (casein) Blotto in TBS buffer (150 mM NaCl, 10 mM Tris, pH 7.4 at 25°C). Membranes were washed in TBS containing 0.1% (v/v) Tween 20 and incubated for 2 hrs at room temperature with rabbit polyclonal antiserum against either TNFα (Genzyme, 1:100 dilution) or the rat casein proteins (22) (1:2000 dilution) in TBS containing 0.5% (w/v) BSA. Blots were washed, incubated with biotin-labeled goat anti-rabbit antiserum (1:1000 dilution) for 90 min at room temperature, washed again, and incubated 30 min at room temperature with streptavidin peroxidase (1:400). Membranes were then developed using the enhanced chemiluminescence system (ECL; Amersham) and/or DAB.

Cell culture

For primary culture studies *in vitro*, mammary epithelial cell organoids from virgin rats were isolated as described above and cultured as previously described (2). Briefly, the non-adherent epithelial organoids were pelleted by centrifugation at 500 x *g* for 10 min and resuspended in ice-cold RBM matrix at a concentration of 3 x 10^5 cells/0.2 ml. Two hundred microliters of RBM matrix containing epithelial organoids was then plated on top of 200 μl pre-gelled, cell-free RBM matrix per well of a 24-well plate and allowed to gel for 3 hours at 37°C. One milliliter of medium was then added to each well. The optimal, serum-free medium consisted of phenol red-free DMEM-F12 (1:1) containing 10 μg/ml insulin, 10 ng/ml EGF, 1 μg/ml progesterone, 1 μ g/ml hydrocortisone, 1 μg/ml prolactin, 5 μg/ml transferrin, 5 μM ascorbic acid, 1 mg/ml fatty acid-free BSA, and 50 μg/ml gentamicin. Suboptimal medium was identical except that EGF was decreased from 10 to 0.1 ng/ml.

^{3}H-Thymidine incorporation assay. MEC organoids from virgin rats were cultured until day 5 in suboptimal medium. On day 5 of culture, the medium was changed and EGF (10 ng/ml), TNFα (40 ng/ml), or various dilutions of the agonistic antibodies specific for either the p55 or p75 TNF receptor were added and the cultures were incubated for 48 hrs at 37°C. For the last 4 hours, the MEC organoids were pulse-labeled with [^{3}H]-thymidine (5 μCi/well). The medium was then removed and the RBM was digested away using 1% (v/v) dispase for 2 hours at 37°C. After washing, the acid-insoluble fraction of the MEC organoids was precipitated overnight at 4° C with 5% (v/v) TCA. Pellets were washed twice with 5% TCA, solubilized with 0.1N NaOH containing 0.1% (v/v) Triton X-100, neutralized with 1N HCl, and [^{3}H]-thymidine incorporation was determined by liquid scintillation counting.

Casein experiments. MEC organoids from virgin rats were cultured until day 5 in optimal serum-free medium. On day 5, the medium was changed and TNFα (40 ng/ml) or various dilutions of the agonistic antibodies specific for either the p55 or p75 TNF receptor were added and the cultures were incubated for 48 hrs. Samples were harvested as previously described for the casein ELISA (2), but casein levels were instead determined using Western blot analysis since the anti-TNF receptor antibodies interfered with the ELISA. Unless otherwise stated, each sample was a mixture of the triplicate wells for each treatment group. Eight μg of sample (adjusted to equivalent protein content following Biorad analysis) was electrophoresed, blotted and incubated with the polyclonal antibody against the rat casein proteins as described above.

Analysis of bioactive TNFα levels (Proprietary Information)

The level of biologically active TNFα in extracts of whole mammary glands was assayed using a modified version of the WEHI 164 fibroblast cytotoxicity assay (29). Mammary glands were excised from virgin, mid-pregnant (day 13-14), lactating (days 5, 10 and 15) or post-lactational (day 7 of involution) female rats and homogenized in RPMI-1640 containing 2mM glutamine (1 ml per 100 mg tissue). The homogenate was then centrifuged at 1000 x*g* for 30 min, followed by centrifugation at 13,000 rpm for 20 min at 4°C and filtration through a 0.45 μm filter. Serial dilutions (100 μl per well) of the samples were then plated in quadruplicate, and 100 μl of WEHI 164 cells (5×10^4 cells in RPMI-1640 containing 10% FCS, 2mM glutamine, and 0.003 mg/ml gentamicin) were added into each well in the presence of 0.5 μg/ml actinomycin D (Sigma, St. Louis, MO). After incubation for 22 hrs at 37°C, 180 μl of supernatant was removed from each well and replaced with 180 μl of fresh culture medium containing 0.5 μg/ml actinomycin D. Twenty microliters of MTT (5 mg/ml) (Sigma, St. Louis, MO) was then added to every well. After further incubation at 37°C for 4 hrs, 150 μl of supernatant was removed from every well, and 100 μl of 0.04 N HCl/isopropanol was added. Plates were then wrapped in aluminum foil with damp paper towels and stored overnight at room temperature. Absorbance at 570 nm was determined using a Bio-Tek (Winooski, VT) model EL 311 plate reader. A standard curve ranging from 0.01 to 1000 U/ml TNFα was set up for each experiment and used to calculate the concentration of TNFα.

Analysis of DMBA- and NMU-induced mammary tumors (Proprietary Information)

Mammary carcinomas were induced in virgin, 50- to 60-day-old pathogen free female Sprague-Dawley Crl:CD BR rats purchased from Charles River (Raleigh, NC) and were generously provided by Dr. Clement Ip. Tumors were generated by administration of 9,10-dimethyl-1,2-benz-anthracene (DMBA) (Sigma, St. Louis, MO) (in corn oil) p.o. using standard protocols (30) or via i.p. injection of 1-methyl-1-nitrosourea (NMU) (Ash Stevens, Inc., Detroit, MI) (10 mg in 0.5 ml saline). Animals were palpated weekly to determine the size and location of tumors, and the experiment was terminated 25 weeks after carcinogen administration. Tumors were excised from the mammary glands, cleared of surrounding tissue, rinsed in phosphate buffered saline, quick-frozen in liquid nitrogen and stored at -80°C until use. Four tumors of each type were pooled, pulverized in liquid nitrogen at 4°C, and homogenized in Trizol (100 mg per ml) using a PCU-2-110 Polytron (Brinkmann Instruments). Total cellular RNA and protein were then prepared following the standard protocol for Trizol, and poly A^+ mRNA was subsequently fractionated using the PolyATract mRNA Isolation System (Promega). Northern blot analysis for TNFα and TNF receptor mRNA and Western blot analysis for TNFα were performed as described above.

Statistics

Statistical significance was determined using a one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test for pairwise multiple comparisons. $P < 0.05$ was judged to be statistically significant.

RESULTS

Characterization of freshly isolated mammary epithelial cell organoids

Since the focus of this investigation was to determine the capability of the *epithelial* cells to synthesize and respond to TNFα, it was first necessary to remove the stromal elements in the mammary gland since these cell types (e.g. adipocytes, fibroblasts) may also produce TNFα. Figures 1 and 2 demonstrate that the cells used to assess steady state levels of TNFα and the TNF receptors were truly representative of epithelial cell organoids at the various *in vivo* developmental stages. The absence of loosely associated stromal cells and connective tissues around the organoids should be noted (Figure 1). Moreover, cross-sectional analysis of the epithelial organoids demonstrated that the structures were composed of well polarized columnar epithelial cells organized in a classical mammary-specific pattern (Verstovsek, Darcy and Ip, unpublished observations). In the pubescent, virgin gland, the MEC organoids were pale-colored, end bud-like ductal structures (Figure 1A) which did not express measurable amounts of casein (Figure 2). As the gland developed during pregnancy (Figure 1B), the MEC differentiated into dark-colored, multilobular alveoli which had already begun to accumulate significant amounts of the α, β, and γ forms of casein (Figure 2). This alveolar structure was maintained throughout lactation; however, the organoids were slightly lighter in color due to distention with milk (Figures 1C-1F). As expected, casein levels peaked at this time (Figure 2). After one week of involution, the MEC organoids were smaller and darker, but still retained most of their alveolar appearance (Figure 1G). By this time, casein production had essentially ceased (Figure 2).

Expression of TNFα mRNA and protein

Although our laboratory recently reported that TNFα could serve as a growth and differentiation factor for MEC organoids in culture (2), it is not known whether TNFα may play a physiological role in directing the overall growth and development of the mammary gland *in vivo*. Studies were therefore undertaken to determine whether TNFα was produced by MEC, and if so, to determine whether expression levels changed during mammary gland development. As can be seen in the Northern blot in Figure 3A, a single 1.9 kb mRNA transcript was detected in MEC from all stages of development. Normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) indicated that TNFα mRNA levels dramatically increased during mid-pregnancy and then steadily declined through lactation and involution; however, expression remained elevated relative to TNFα mRNA levels during puberty.

Since there appeared to be some variation in GAPDH levels even though equivalent amounts of mRNA were thought to have been loaded for each sample, we considered whether these variations were due to changes in GAPDH during development or the result of other factors. First, prior to isolation of poly A^+ mRNA, the integrity of the 18 and 28S RNA bands was examined in each sample and the RNA was found to be somewhat degraded during lactation (not shown). Thus, we believe that the variation in GAPDH was reflective of our

inability to accurately quantify the RNA prior to loading. In addition, previous studies have reported that GAPDH expression levels are relatively constant during development (31). To confirm our results, the blots were stripped and re-probed with a second, constitutively expressed housekeeping gene, cyclophilin (p1B15) (25). As is shown in Figure 3C, normalization of TNFα mRNA levels to cyclophilin was equivalent to GAPDH. Lastly, Northern blot analysis with several, independent sets of mRNA confirmed these developmental changes in TNFα.

In conjunction with mRNA expression, TNFα protein levels were also examined in MEC to determine if there was a correlation between the two. The 17-kDa soluble form of TNFα was barely detectable and did not appear consistently between experiments; however, this was not unexpected since we were examining cell extracts, and soluble TNFα would be lost during the digestion of the gland and/or subsequent washes and filtration of the organoids. The major form of TNFα detected in MEC was the 26-kDa transmembrane cytokine (Figure 4). While undetectable in MEC from virgin rats, this protein was induced during pregnancy, and, in contrast to its message, was highest during lactation, where it remained at a relatively uniform, peak level before disappearing during involution. The band at 25-kDa, immediately below the 26-kDa band for TNFα, may be TNFβ, which shows significant homology to TNFα; alternatively, the doublet could represent native and post-translationally modified forms of TNFα (32).

Expression of TNF receptor mRNA

Our previous studies demonstrating the effect of TNFα on MEC in primary culture suggested that TNF receptors must be present on the epithelial cells; however, as with TNFα, there is little information on TNF receptor expression in normal MEC, and no data on changes in receptor levels during the development of the mammary gland. Northern blot analysis showed that MEC expressed transcripts corresponding to both the p55 and p75 TNF receptors, and the mRNA levels of each were independently and specifically regulated during puberty, pregnancy, lactation, and involution. For the p55 TNF receptor, a single 2.3 kb mRNA species was detected in MEC from all stages (Figure 5). Upon normalization to GAPDH, p55 mRNA was found to increase steadily through pregnancy and into lactation, peak at day 10 of lactation, and then rapidly decrease through involution. In contrast, p75 mRNA was expressed as three distinct mRNA transcripts of 2.8, 4.3 and 5.7 kb, and normalization to GAPDH indicated only a modest increase during pregnancy followed by a marked elevation throughout lactation and involution (Figure 6). Northern hybridization analysis with several separate sets of mRNA confirmed these developmentally regulated expression patterns for both receptors. Most notably, p75 mRNA remained high at the end of lactation and during involution, while both TNFα and p55 mRNA levels were decreased at these times.

Investigation of p55 TNF receptor protein expression (Proprietary information)

In conjunction with TNF receptor mRNA expression, studies were also undertaken to determine if receptor protein expression followed the same developmental patterns. Initial efforts focused on the p55 TNF receptor because expression levels were higher than p75. First, Western blot analysis of membrane protein fractions of MEC extracts was performed with a monoclonal hamster anti-murine p55 TNF receptor antibody from Dr. Robert Schreiber (33). No specific bands were identified. The p55 receptor was then immunoprecipitated from MEC extracts (using the method of Sheehan and Schreiber) (33) and ligand blotting with ^{125}I-TNFα was attempted according to the method of Grazioli et al. (34). Even after several different concentrations of the ligand were used, no proteins were identified. Lastly, immunoprecipitation and Western blot analysis of the p55 receptor was performed according to the method of

(Proprietary information)
Sheehan et al. (33). A band at 55-kDa was identified; however, normal Armenian hamster serum identified the same protein, thus demonstrating that it was non-specific (data not shown). Further studies with this antibody were considered futile. A second p55 TNF receptor antibody (against the human p55 TNF receptor) (35) also failed to identify any specific bands in rat membrane protein samples. Due to the inability to detect the p55 receptor, no attempts were made to identify the p75 receptor via immunoprecipitation and/or Western blot analysis.

As a final attempt to profile TNF receptor expression on normal MEC, Scatchard analysis was performed on isolated MEC from mid-lactation using ^{125}I-TNFα. The results of two attempts were inconclusive, and further studies were not pursued due to the exorbitant cost of iodinated TNFα.

Determination of individual TNF receptor function

The studies presented above, in conjunction with our previous studies on TNFα, indicated that TNF receptors were present on normal MEC. In order to determine the specific functions of each TNF receptor in MEC, experiments were performed on MEC organoids in primary culture using agonistic antibodies that were specific to either the p55 or p75 TNF receptor (18). Using a [^{3}H]-thymidine incorporation assay, the ability of each individual TNF receptor antibody to stimulate cellular proliferation was determined. Under suboptimal (low EGF, 0.1 ng/ml) medium conditions, EGF, TNFα, or the 1:1000 dilution of the p55 antibody significantly stimulated thymidine incorporation approximately 3-fold by MEC, while neither the three higher dilutions of the p55 antibody nor the p75 antibody had any effect (Figure 7). The inability of the p75 antibody to affect MEC organoid growth was in direct contrast to its ability to stimulate mouse (18) and rat (Varela, Ehrke, and Ip, unpublished observations) thymocyte proliferation at these same concentrations. Murine TNFα (32 ng/ml) also had the identical stimulatory effect as described above for human TNFα (data not shown).

To determine how each TNF receptor affected the functional differentiation of the MEC, the effects of the agonistic antibodies on the ability of the MEC organoids to accumulate each of the casein isoforms was measured using Western blot analysis. In both suboptimal (data not shown) and optimal (10 ng/ml EGF) media conditions (Figure 8), human TNFα decreased the accumulation of the α_1, α_2, β, and γ forms of casein by MEC, with the primary effect on α_2, β, and γ (compare first and second lanes). Murine TNFα (32 ng/ml) had the identical effect (data not shown). Surprisingly, the two TNF receptors were found to have opposing effects on functional differentiation, with <u>inhibition</u> of casein accumulation occurring in response to the p55 antibody and <u>stimulation</u> by the p75 receptor antibody. MEC organoids treated with the 1:10^3 dilution of the p55 receptor antibody showed a <u>decreased</u> accumulation of α_2, β, and γ casein (Figure 8A, compare third and fourth lanes to lane 1, and Figure 8B), albeit to a lesser extent than TNFα, while treatment with the 1:10^4, 1:10^5 and 1:10^6 dilutions of the p75 antibody <u>increased</u> the accumulation of all four forms of casein by the MEC organoids (Figure 8A, compare seventh, eighth and ninth lanes to lane 1, and Figure 8B).

TNFα protein expression and bioactivity in whole mammary glands (Proprietary information)

The aforementioned studies indicate that mammary epithelial cells produce TNFα mRNA and protein; however, cells present in the mammary stroma, such as adipocytes and fibroblasts, may also synthesize and secrete TNFα, and it is not known whether mammary-derived TNFα is physiologically active. Studies were therefore undertaken to compare TNFα protein expression in whole mammary glands with that in isolated MEC, and to determine whether TNFα produced by the mammary gland was biologically active. When mammary gland

(proprietary information)
extracts from virgin, mid-pregnant, lactating and post-lactational rats were analyzed in a modified WEHI 164 cytotoxicity assay (established by Ignatowski et al. (29)), bioactive TNFα levels were found to be very low (< 0.5 U/ml), with little variability between developmental stages, except for a minor increase at day 7 post-lactation (data not shown).

As in isolated MEC, the 17-kDa soluble form of TNFα was not detectable in whole mammary gland extracts by Western blot analysis (Figure 9). This was somewhat unexpected, as it was believed that examination of whole glands would facilitate the detection of the soluble cytokine. Expression of the 26-kDa transmembrane form of TNFα followed the same pattern as in isolated MEC; however, the overall level of this protein appeared to be lower in whole mammary glands. It should be noted that different methods were used to prepare the MEC and whole gland samples; however, this apparent decrease in TNFα protein levels may also reflect the presence of non-TNFα producing cells in the mammary stroma. In contrast to the isolated MEC, a very distinct 32-kDa protein was uniformly expressed in whole gland extracts from all stages of development, with only a minor fluctuation in expression on day 21 of lactation. This band may represent a high molecular weight aggregate form of TNFα; however, it was not believed to be physiologically significant because of the lack of TNFα activity in the bioassay.

TNFα and TNF receptor expression in DMBA- and NMU-induced mammary tumors (Proprietary information)

Previous studies on TNFα and TNF receptor expression in breast cancer are somewhat conflicting and have been performed primarily in human tissue. In order to determine whether TNFα and TNF receptor expression change during the process of mammary carcinogenesis, expression in DMBA- and NMU-induced rat mammary carcinomas was examined. Northern blot analysis showed that TNFα mRNA was barely detectable in both DMBA- and NMU-induced rat mammary tumors, and normalization to GAPDH demonstrated that expression was greatly decreased when compared to normal, age-matched MEC (Figure 10). In conjunction, neither form of TNFα protein (17- or 26-kDa) was detectable in either tumor type or in normal, age-matched MEC (data not shown). For the p55 TNF receptor, a single, 2.3 kb mRNA transcript was detected in both DMBA- and NMU-induced mammary carcinomas as in normal MEC; however, normalization to GAPDH demonstrated that p55 TNF receptor mRNA expression was markedly lower in both tumor types than in normal MEC (Figure 11). In contrast, p75 TNF receptor mRNA was not detectable in either tumor type (data not shown).

DISCUSSION

The experiments presented herein demonstrate that normal rat MEC produce TNFα and strengthen our previous data suggesting that TNFα may play a physiological role in directing the growth and development of the mammary gland. Our studies are the first to directly demonstrate TNFα and TNF receptor mRNA expression by freshly isolated, purified normal rat MEC and to obtain sizes for these mRNA species. Moreover, we show direct evidence for the existence of the 26-kDa, membrane-bound form of TNFα in MEC. Most importantly, these studies demonstrate that TNFα and TNF receptor expression are independently and specifically regulated during mammary gland development. When taken in conjunction with previous reports by our laboratory (2), this suggests that TNFα, acting in concert with various other hormones and growth factors, is a physiologic regulator of normal mammary gland development. Lastly, our studies indicate that both TNFα and TNF receptor expression are decreased in both DMBA- and NMU-induced rat mammary carcinomas compared to normal MEC. In contrast to our studies in rats, Pusztai and co-workers (36), using

immunohistochemical techniques, failed to detect TNFα or p75 TNF receptors in normal human breast tissue from non-pregnant patients. In a separate study using *in situ* hybridization as well as immunohistochemistry in human tissue, Miles and co-workers (37) localized both TNFα and TNF receptor expression exclusively to the mammary stroma. Basolo et al. (38), however, detected TNFα protein in alveolar and ductal epithelial cells using immunohistochemistry, and, using RT-PCR, detected TNFα mRNA in isolated, cultured human MEC. Finally, using radioligand binding, Dollbaum et al (19) demonstrated receptor expression on normal mammary epithelium, but did not determine which receptor species was present. The differences in expression patterns observed in these various studies most likely result from the use of different methodologies with varying sensitivities.

Potential roles in development

Puberty. Given the relatively high proliferative rate of the MEC during puberty, and the fact that TNFα stimulates growth and ductal morphogenesis *in vitro*, we predicted that TNFα levels would be elevated as the epithelium proliferates and invades the mammary gland fat pad during this developmental stage. Our Northern data, however, indicates low TNFα levels in 50-day old virgin rats. We cannot exclude the possibility that TNFα is expressed at higher levels in younger rats. Alternatively, TNFα production may be primarily stromal rather than epithelial at this time; indeed, immunohistochemical studies of TNFα in whole glands support this hypothesis (Stangle, Varela, and Ip, unpublished observations). Thus, TNFα would still be present to stimulate branching morphogenesis but its levels would not be elevated in the epithelial cells themselves.

Pregnancy. The pronounced increase in TNFα mRNA during pregnancy suggests a role for TNFα in promoting both the proliferation and morphogenesis of mammary epithelial cells during this stage. To support this theory, we have previously demonstrated that TNFα stimulated MEC proliferation and induced extensive morphological differentiation in primary culture (2). Moreover, p55 TNF receptor mRNA levels significantly increased during pregnancy, and functional studies of the individual TNF receptors indicated that p55 was the sole mediator of TNFα-induced proliferation in MEC. This suggests that during pregnancy, TNFα-induced stimulation of growth and morphogenesis is signaled primarily via the p55 receptor. In addition, TNFα can stimulate angiogenesis (39,40), so it may also play a role in increasing vascularization during pregnancy. Lastly, casein production is inhibited during this time (41). We have previously reported that TNFα (40 ng/ml) reduced accumulation of casein proteins by MEC (2), and functional studies presented herein demonstrated that signaling through p55 also decreased casein accumulation. Thus, in concert with other hormones such as progesterone, TNFα may act via the p55 receptor to inhibit casein production until the onset of lactation. It must be noted, however, that only one time point during gestation was examined, so it cannot be ruled out that other, significant changes in TNFα production and/or effects may occur at other times during pregnancy.

Lactation. As opposed to the high levels of TNFα mRNA observed during pregnancy, TNFα mRNA levels appeared to decline throughout lactation. It could be argued that this is a dilutional effect due to high-level milk gene expression; however, p55 and p75 expression did not show the same pattern, so the decline in TNFα mRNA is believed to be specific. Concurrently, the 26-kDa transmembrane form of TNFα protein was expressed at high levels, which may reflect an increased protein half-life, or, alternatively, a decreased cleavage of the membrane form and thus decreased release of the soluble cytokine. Consequently, instead of

widespread, systemic action within the mammary gland, as may occur during the proliferative phase of pregnancy, TNFα would now only be able to act in a very localized, cell-to-cell manner. Alternatively, this may indicate that the 17-kDa form of TNFα is not present, and it is instead the 26-kDa cytokine that is the physiologically active protein in the mammary gland. Numerous reports have documented the difference in activity between the soluble and membrane forms of TNFα, and the two forms may also act via different TNF receptors (7,8,42). Thus, expression of transmembrane TNFα during lactation, in combination with other hormones and growth factors, may act to stimulate casein production and/or secretion by the MEC. A specific, 32-kDa protein was also uniformly expressed in extracts of whole mammary glands from all stages of development; however, this protein is probably not physiologically significant because it was not developmentally, and thus hormonally, regulated.

In contrast to TNFα mRNA, p55 mRNA levels continued to increase into early lactation, thereafter declining and remaining low into involution. Since we believe that TNFα stimulates growth and inhibits casein via p55 during pregnancy, this receptor may no longer be needed after the proliferative burst in early lactation (43). At this time, however, p75 mRNA expression was increased and remained elevated throughout the remainder of lactation, and functional studies showed that signaling via p75 actually stimulated casein accumulation by MEC. When considered in conjunction with a previous report suggesting that the membrane form of TNFα acts primarily via p75 (42), it is tempting to speculate that the transmembrane TNFα we observe during lactation acts via the p75 receptor to stimulate casein production. Thus, we hypothesize that soluble TNFα works via the p55 receptor during pregnancy, while membrane TNFα acts via p75 during mid- and late-lactation, with both receptors playing significant yet different roles in early lactation. This disparity in the functions of the two receptors is well documented, and may be due to differences in the location of the receptors, either within one cell or between different cell types (12,44). For example, p55 receptors may predominate on actively dividing epithelial cells, such as those found in newly branching ducts during puberty and pregnancy, while p75 receptors may only be present on the differentiated, milk-producing cells. This will be addressed in follow-up studies using immunohistochemistry or *in situ* hybridization. Unfortunately, due to the low receptor protein levels and inability to obtain an appropriate rat reactive antibody for Western blotting, it was not possible to examine receptor expression by Western blot analysis.

Involution. Since TNFα can stimulate apoptosis in various cell types (45), TNFα may also be involved in mammary gland involution. While TNFα mRNA levels were not significantly elevated at one week of involution, we cannot rule out the possibility of an increase in TNFα during other (especially earlier) stages of involution, or within the stromal cells of the mammary gland. However, the level of TNFα mRNA in MEC during involution was higher than that in virgin, and the 26-kDa TNFα protein disappeared at this time. Although undetectable by Western blot, this may reflect increased production and cleavage to release the soluble, 17-kDa cytokine. Thus, instead of localized activity, TNFα may again have widespread, systemic activity throughout the gland. In addition, the bioactivity assay indicated that TNFα levels may be slightly increased at one week of involution; however, the most pronounced apoptosis and regression of the mammary gland occurs from days 2-7 post-lactation (46), so TNFα levels may have been higher at this time. A role for TNFα in apoptosis would be seemingly contradictory to its proposed role as a growth and differentiation factor during mammary gland development; however, it could be hypothesized that an inhibitor may be produced during development which could block the cytotoxic activity of TNFα. Alternatively, proteins required for downstream TNFα signaling in the apoptotic pathway may only be present during involution.

Stromal effects during development. In addition to serving as an autocrine growth factor for MEC during mammary gland development, TNFα may also have significant effects on the mammary stroma. Since other groups have detected TNF receptors on fibroblasts and adipocytes (47-50), these cell types are obvious targets for MEC-derived TNFα. In other systems, TNFα has been shown to stimulate adipocyte lipolysis (51), so TNFα may be a key mediator of mammary adipocyte lipolysis during lactation (52). In contrast, however, TNFα has also been shown to stimulate hepatic lipogenesis (53). Since it is well documented that TNFα serves different roles in different cells, it could be hypothesized that TNFα stimulates lipid mobilization in adipocytes while concurrently stimulating lipogenesis in MEC. Lastly, as our preliminary immunohistochemical data suggests, the cells within the mammary stroma may themselves produce TNFα which could act in either an autocrine and/or paracrine fashion to modulate growth and development.

Potential role in carcinogenesis

The decreases in both TNFα and TNF receptor expression found in mammary carcinomas were not unexpected, given that TNFα is either cytotoxic or cytostatic to many breast cancer cells. However, the overall significance of these findings is, as yet, unclear. Since our previous data indicated that TNFα stimulates the proliferation and morphogenesis and regulates the function of normal MEC, it is crucial to determine when the function of TNFα in MEC changes during mammary carcinogenesis, and what other factors are involved. In addition, changes in the production and function of TNFα in the mammary stroma during tumor formation must also be identified.

In conclusion, we have demonstrated for the first time that normal MEC produce TNFα and express mRNA for both the p55 and p75 TNF receptors. Furthermore, the levels of all three are both specifically and independently regulated during the various stages of mammary gland development. TNFα, working through the p55 receptor, stimulated the growth of MEC *in vitro* and may induce mammary gland growth and development during pregnancy. In addition, the two TNF receptors may work in opposition to each other to regulate milk production during pregnancy and lactation, with inhibition being signaled via p55 and stimulation via p75. Although the exact functions of TNFα in the mammary gland are still speculative, the data presented herein strongly suggests that TNFα regulates several key processes during mammary gland development.

CONCLUSIONS

1. Normal MEC from all developmental stages express mRNA for both TNFα and its two receptors. The levels of all 3 are developmentally regulated.

2. The 26-kDa transmembrane form of TNFα increased significantly during pregnancy and lactation.

3. The p55 TNF receptor is the sole mediator of TNFα-induced proliferation of MEC in primary culture.

4. Casein accumulation by MEC in primary culture is inhibited in response to the p55 antibody and stimulated in response to the p75 antibody.

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APPENDICES

FIGURE LEGENDS

FIG. 1. Morphological appearance of freshly isolated mammary epithelial organoids. Epithelial cell organoids were isolated from the mammary glands of virgin, pregnant, lactating and post-lactational rats using mechanical and enzymatic digestion followed by differential filtration. A, puberty, age 50 days; B, day 14 pregnancy; C, day 5 lactation; D, day 10 lactation; E, day 15 lactation; F, day 21 lactation; G, one week post-lactation. Magnification in all pictures is the same (40.9X).

FIG. 2. Western blot of casein protein in freshly isolated mammary epithelial organoids. Equivalent amounts of protein (2.5 μg) were loaded into each lane. Lane 1, puberty, age 50 days; lane 2, day 14 pregnancy; lane 3, day 5 lactation; lane 4, day 10 lactation; lane 5, day 15 lactation; lane 6, day 21 lactation; lane 7, one week post-lactation.

FIG. 3. Expression of TNFα mRNA in normal MEC. A, Northern blot of TNFα mRNA. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated MEC was loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled TNFα cDNA probe was used for hybridization. The blot was exposed to either a Phosphorimager screen or x-ray film for 48 hours. As a positive control, total RNA from LPS-stimulated J774A.1 macrophage cells was also probed and demonstrated the same electrophoretic mobility (data not shown). This Northern is representative of 4 experiments. B, Normalization of TNFα to GAPDH. (Represents the

experiment shown in A.) C, Normalization of TNFα to p1B15. (Represents the experiment shown in A.)

FIG. 4. Expression of TNFα protein in normal MEC. This figure shows a representative (of 4 experiments) Western blot of TNFα protein. Equivalent amounts of protein (50 μg/lane) from freshly isolated MEC were loaded into each lane. Lane 1, puberty, age 50 days; lane 2, day 14 pregnancy; lane 3, day 5 lactation; lane 4, day 10 lactation; lane 5, day 15 lactation; lane 6, day 21 lactation; lane 7, one week post-lactation. Duplicate blots incubated either without primary antiserum or with normal rabbit serum demonstrated that the TNFα bands were specific. Purified, soluble mouse TNFα was used as a positive control and found to be 17-kDa (data not shown). The upper arrow indicates 26 kDa transmembrane TNFα, and the lower arrow indicates 17 kDa soluble TNFα. This Western is representative of 4 experiments.

FIG. 5. Northern blot analysis of p55 TNF receptor mRNA from normal MEC. A, Northern blot of p55 TNF receptor mRNA. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated MEC were loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled p55 TNF receptor cDNA probe was used for hybridization. The blot was exposed to either a Phosphorimager screen or film for 48 hours. As a positive control, total RNA from Wehi-164 cells was also probed and demonstrated the same electrophoretic mobility (data not shown). This blot is representative of 4 experiments. B, Normalization of p55 mRNA levels to GAPDH. (Represents the experiment shown in A.)

FIG. 6. Northern blot analysis of p75 TNF receptor mRNA from normal MEC. A, Northern blot of p75 TNF receptor mRNA. Approximately 1.0 - 1.5 μg of poly A^+ mRNA from freshly isolated

MEC were loaded into each lane, and 2 x 10^6 cpm/ml of ^{32}P-labeled p75 TNF receptor cDNA probe was used for hybridization. The blot was exposed to either a Phosphorimager screen or film for 7 days. As a positive control, total RNA from Wehi-164 cells was also probed (data not shown). This blot is representative of 4 experiments. B, Normalization of p75 mRNA levels to GAPDH. (Represents the experiment shown in A.)

FIG. 7. Effect of TNF receptor agonistic antibodies on [^{3}H]-thymidine incorporation by normal mammary epithelial cells in primary culture. Various dilutions of the two agonistic antibodies specific for either the p55 or p75 TNF receptor were tested for their ability to affect [^{3}H]-thymidine incorporation by MEC. EGF (10 ng/ml), TNFα (40 ng/ml), and the 1:1000 dilution of the p55 receptor antibody induced statistically significant increases in thymidine incorporation after 48 hours (*Significantly different than 0.1 ng/ml EGF control, $P<0.05$). Each group represents the mean $\pm$ SEM of triplicate wells. The graph is representative of 4 experiments.

FIG. 8. Effect of TNFα or TNF receptor agonistic antibodies on casein accumulation by MEC in primary culture in optimal (10 ng/ml EGF) medium. A, Western blot analysis of casein protein accumulation in extracts of cells plus EHS gel. Equivalent amounts of protein (8 μg) were loaded into each lane. Each sample represents a combination of triplicate wells for each group. The blot is representative of 2 experiments. B, Quantitation of casein accumulation by MEC. The relative intensities of all 4 (α_1, α_2, β and γ) casein bands were quantified by densitometric analysis. Each group represents the mean $\pm$ SEM of triplicate wells. (*Significantly different than serum-free control, $P<0.05$).

FIG. 9. Expression of TNFα protein in whole mammary glands. This figure shows a Western blot of TNFα protein. Equivalent amounts of protein (50 μg/lane) from whole mammary gland

extracts were loaded into each lane. Lane 1, puberty, age 50 days; lane 2, day 14 pregnancy; lane 3, day 5 lactation; lane 4, day 10 lactation; lane 5, day 15 lactation; lane 6, day 21 lactation; lane 7, one week post-lactation. A duplicate blot incubated without primary antiserum demonstrated that the TNFα bands were specific.

FIG. 10. TNFα mRNA expression in mammary carcinomas. Equivalent amounts of poly A^+ mRNA (~ 1.5 μg) from freshly isolated virgin MEC or DMBA- or NMU-induced mammary tumors were probed as previously described for TNFα mRNA. The blot was exposed to film for 5 days, and TNFα mRNA expression was normalized to GAPDH. The level of the 1.9 kb transcript was greatly reduced in both tumor types when compared to normal virgin MEC.

FIG. 11. Expression of p55 TNF receptor mRNA in mammary carcinomas. Equivalent amounts of poly A^+ mRNA (~ 1.5 μg) from freshly isolated virgin MEC or DMBA- or NMU-induced mammary tumors were probed as previously described for p55 TNF receptor mRNA. The blot was exposed to film for 5 days. The graph shows normalization of p55 receptor mRNA levels to GAPDH. p55 mRNA expression was reduced in both tumor types when compared to normal virgin MEC.

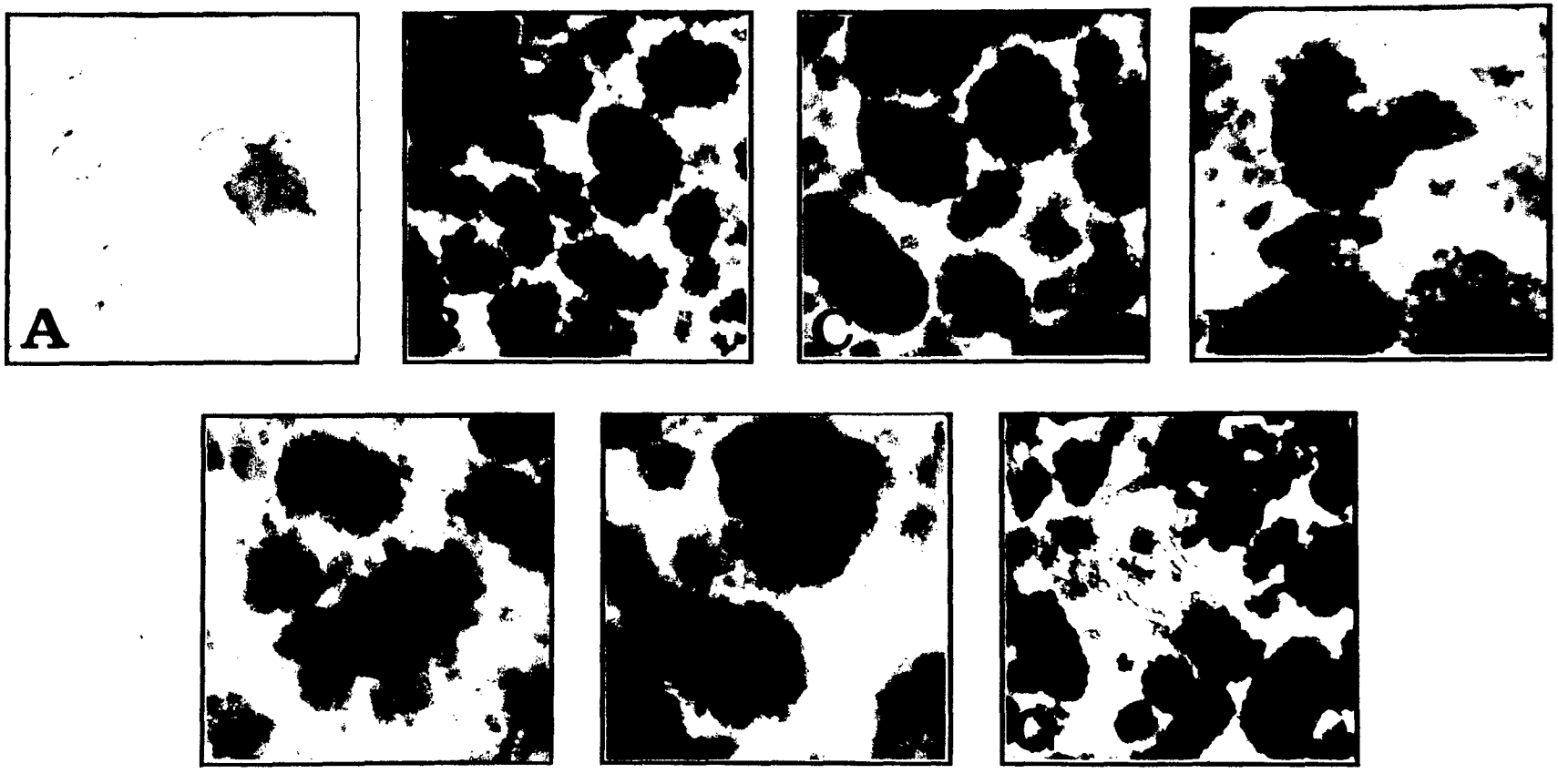

Figure 1.
Varela and Ip
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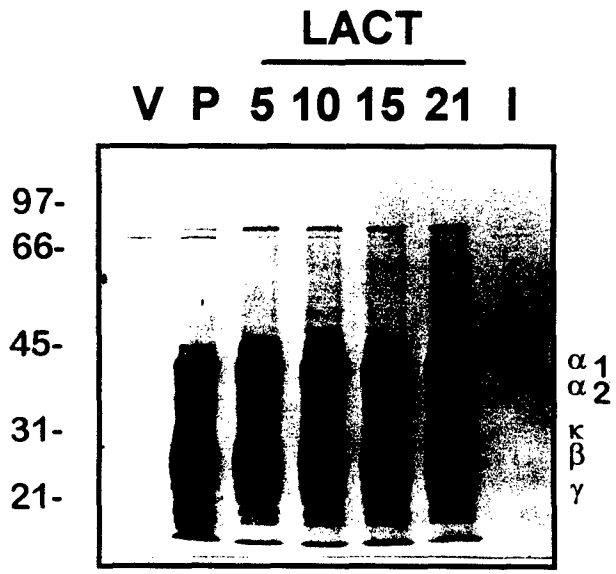
Varela,L.M.



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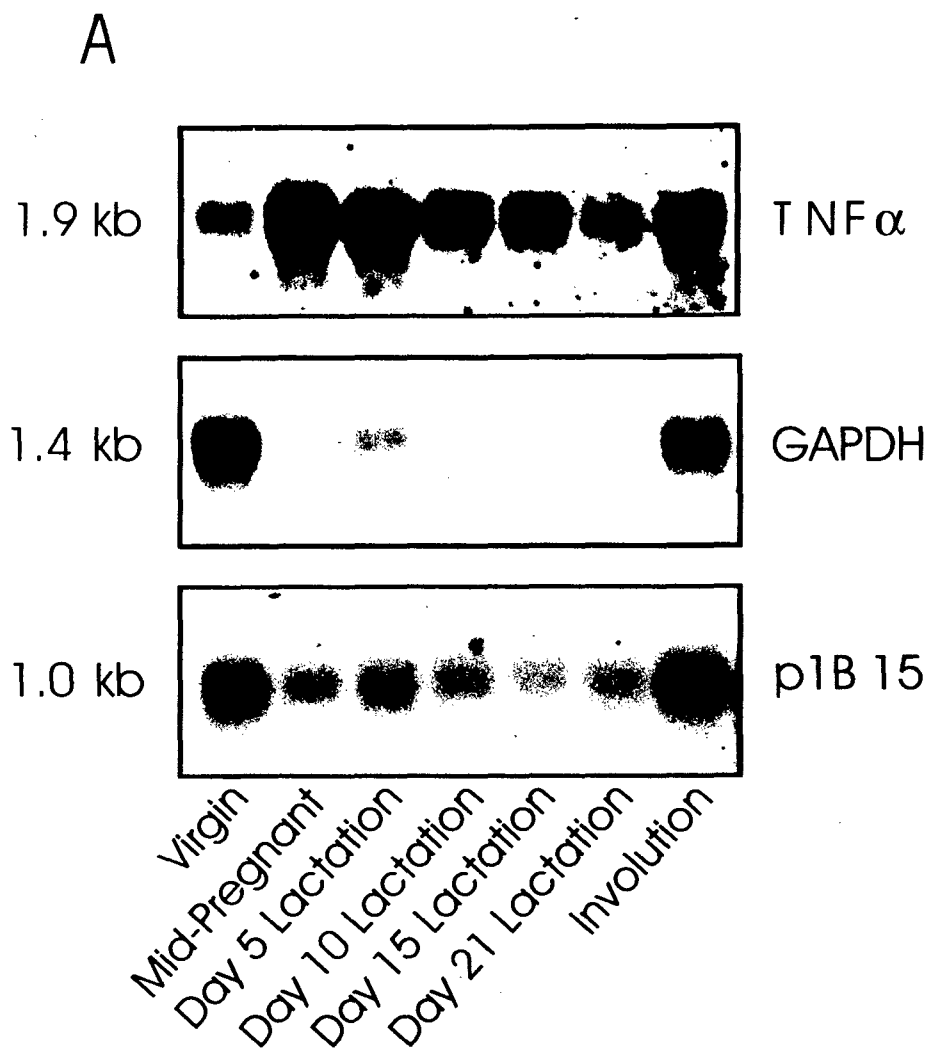

Figure 3A.
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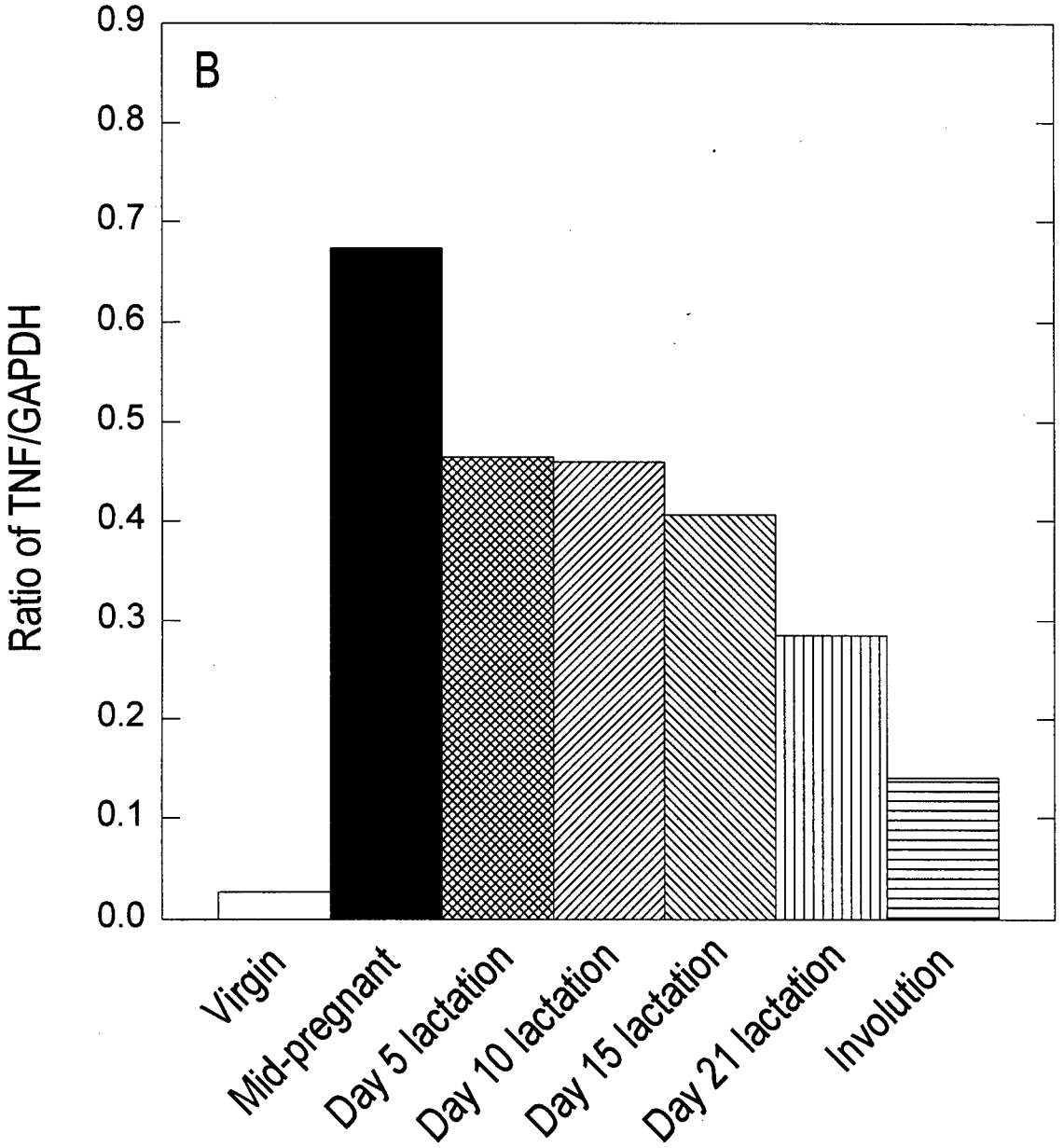

Figure 3B.
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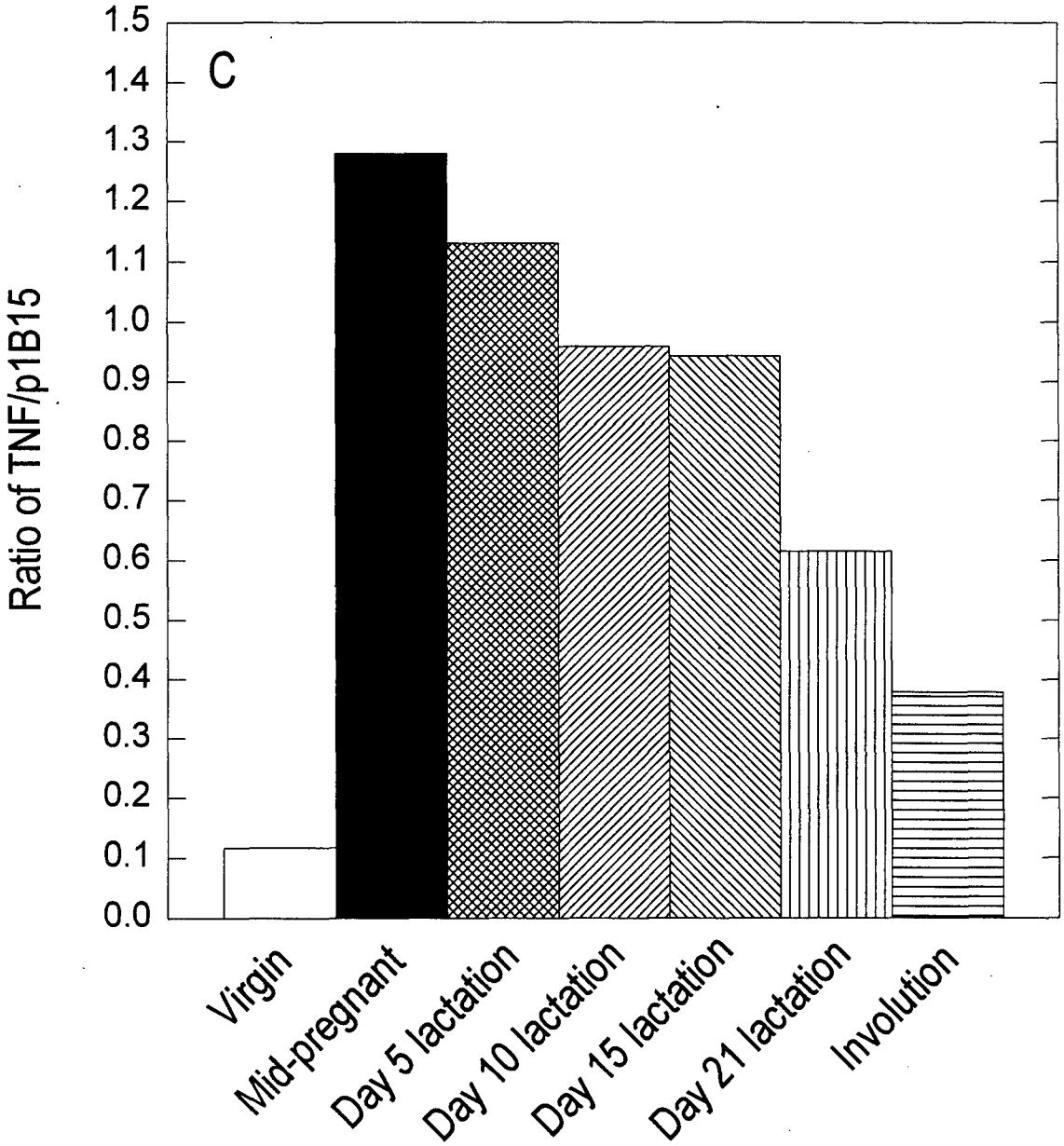

Figure 3C.
Varela and Ip
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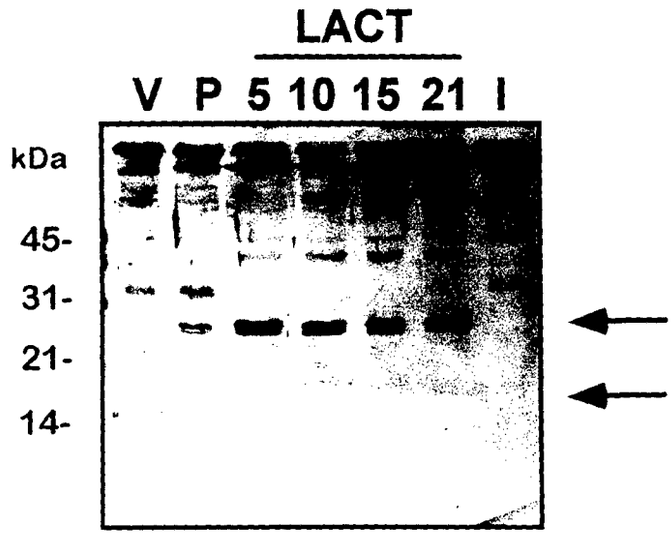
Varela,L.M.



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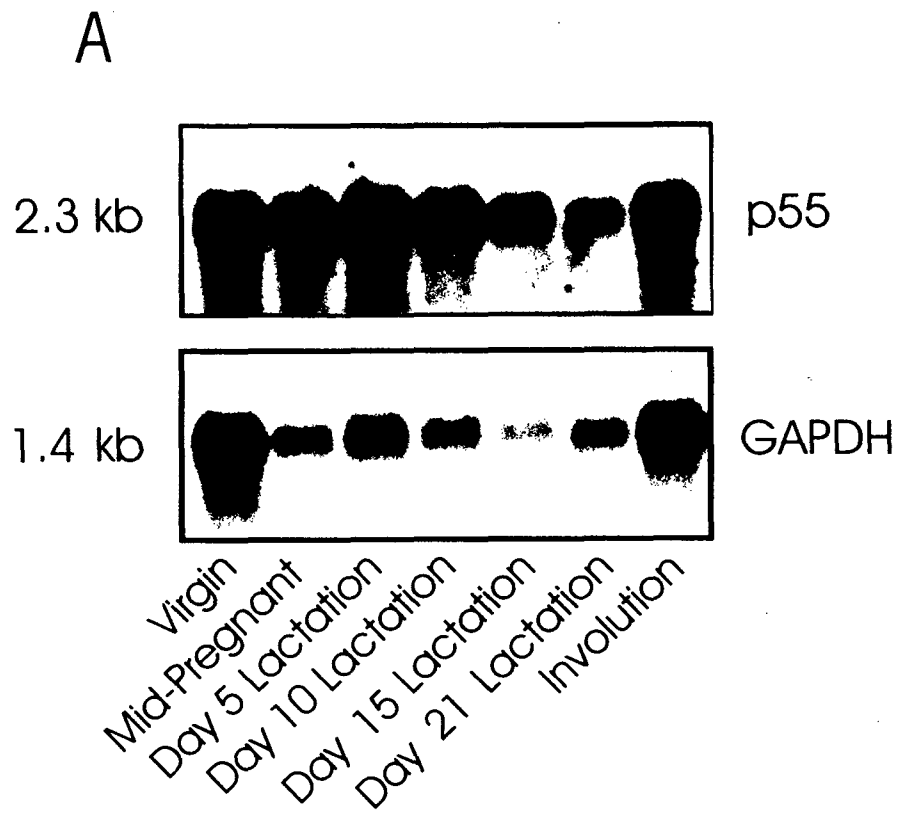

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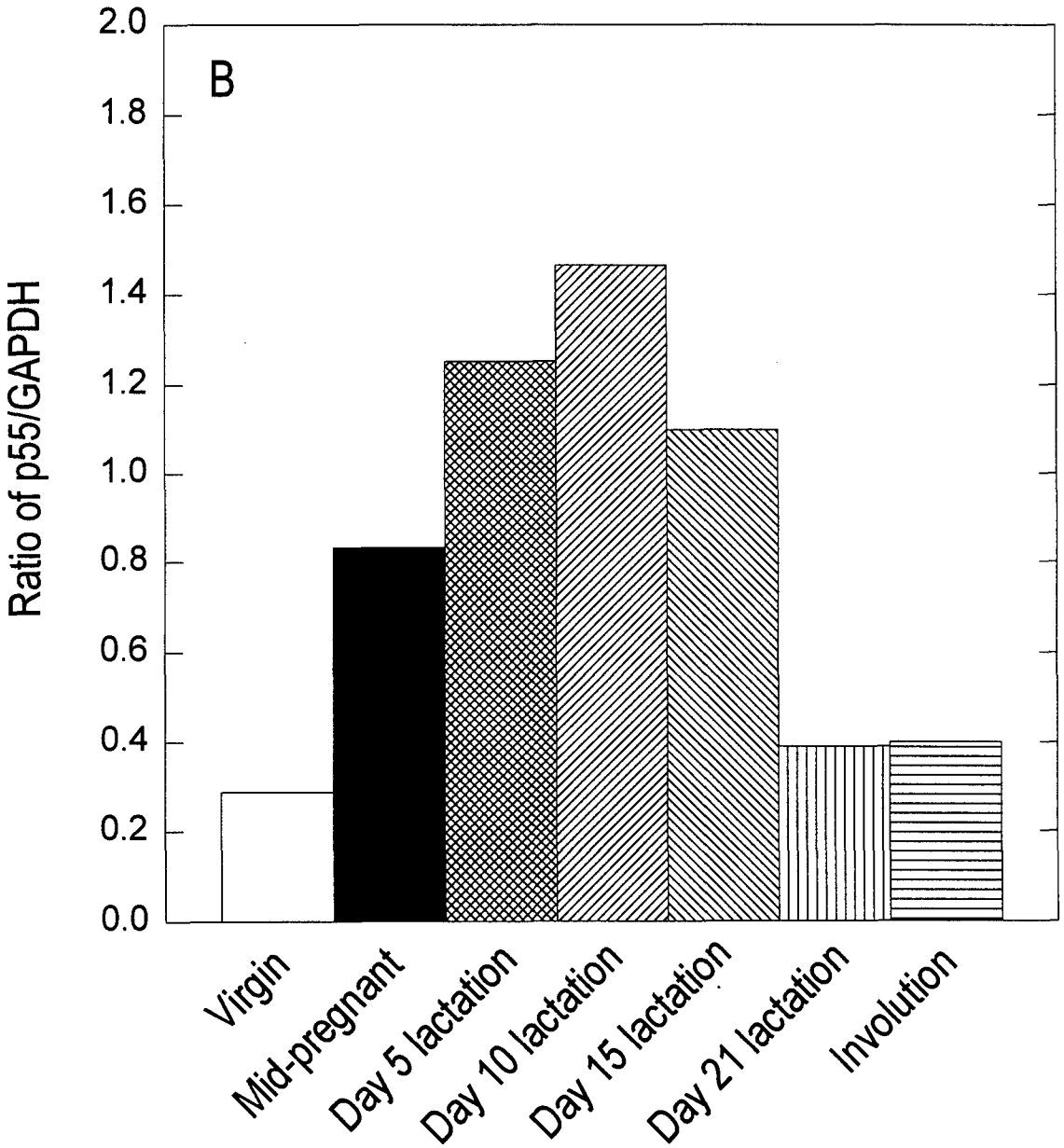

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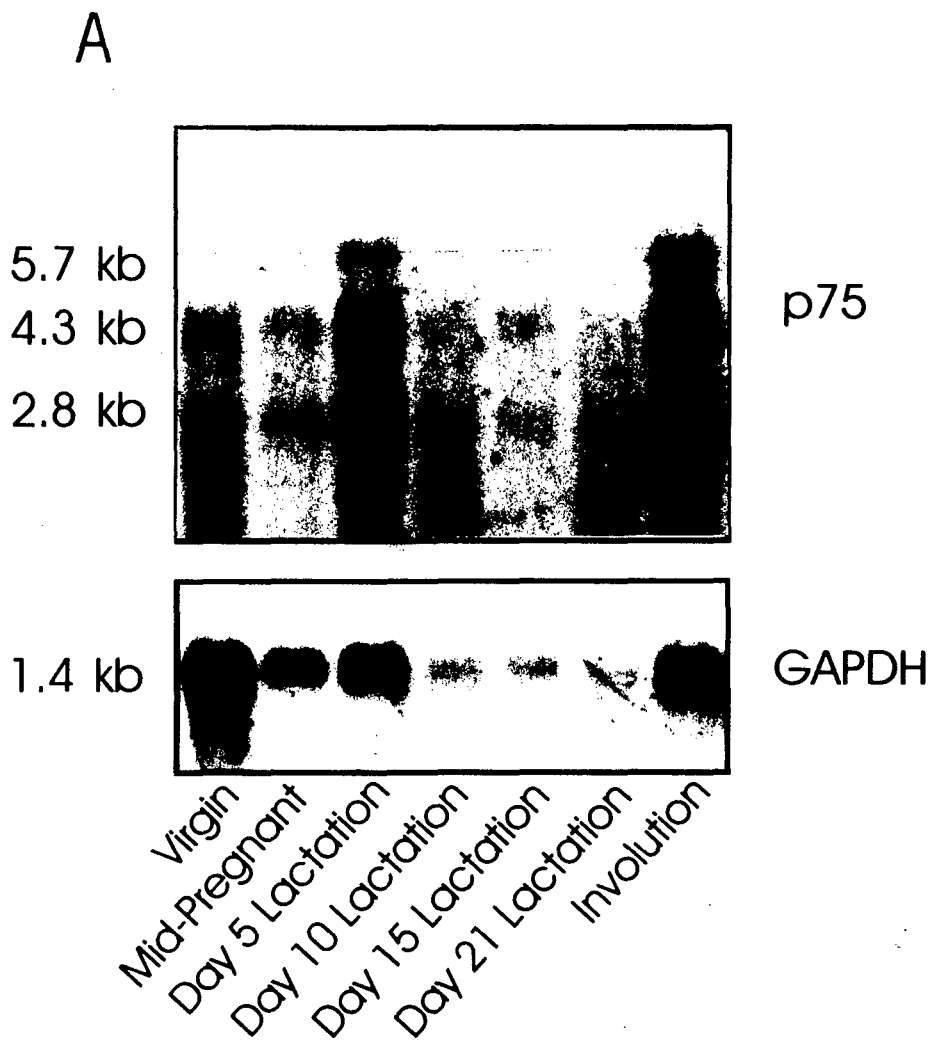

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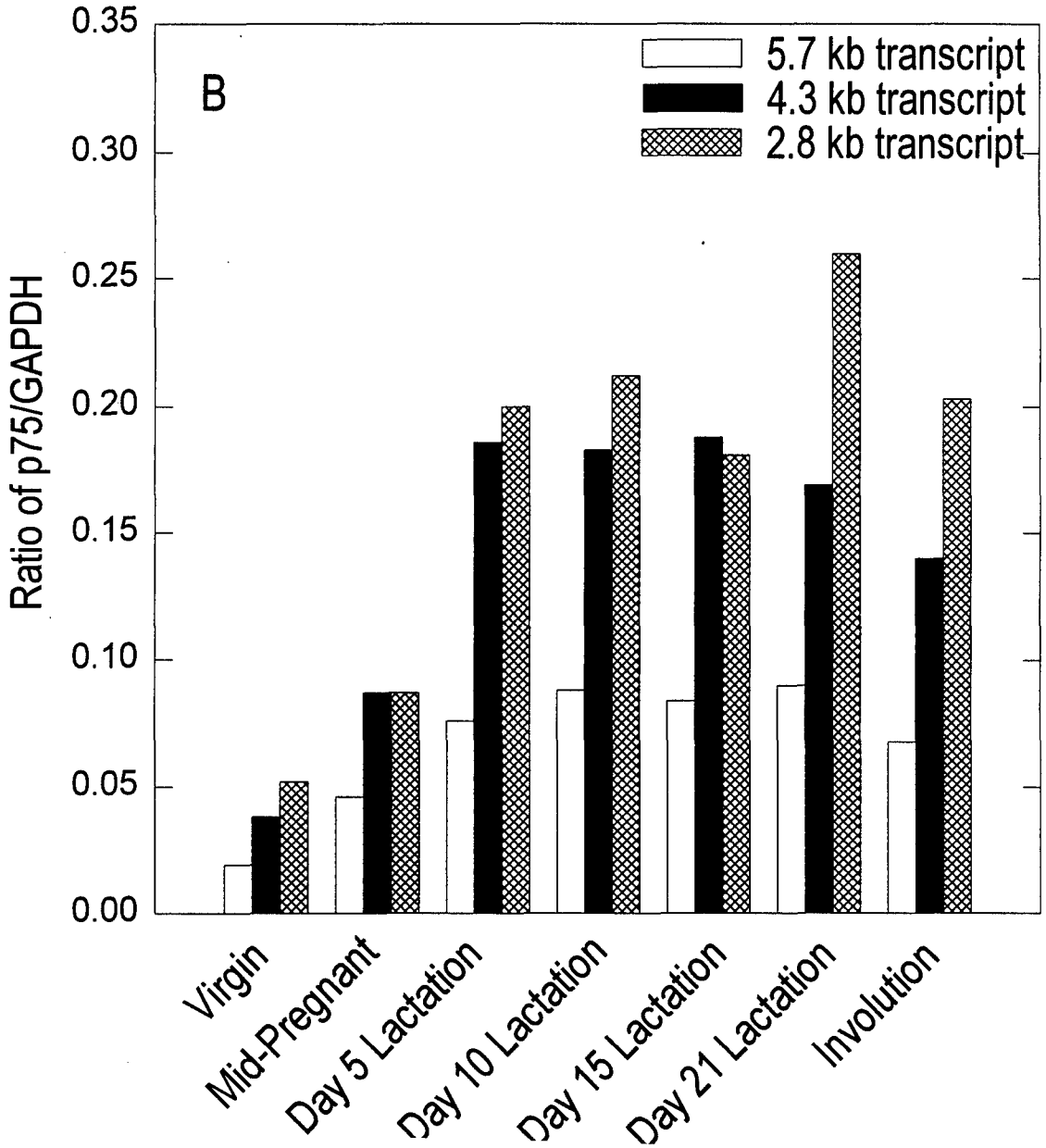

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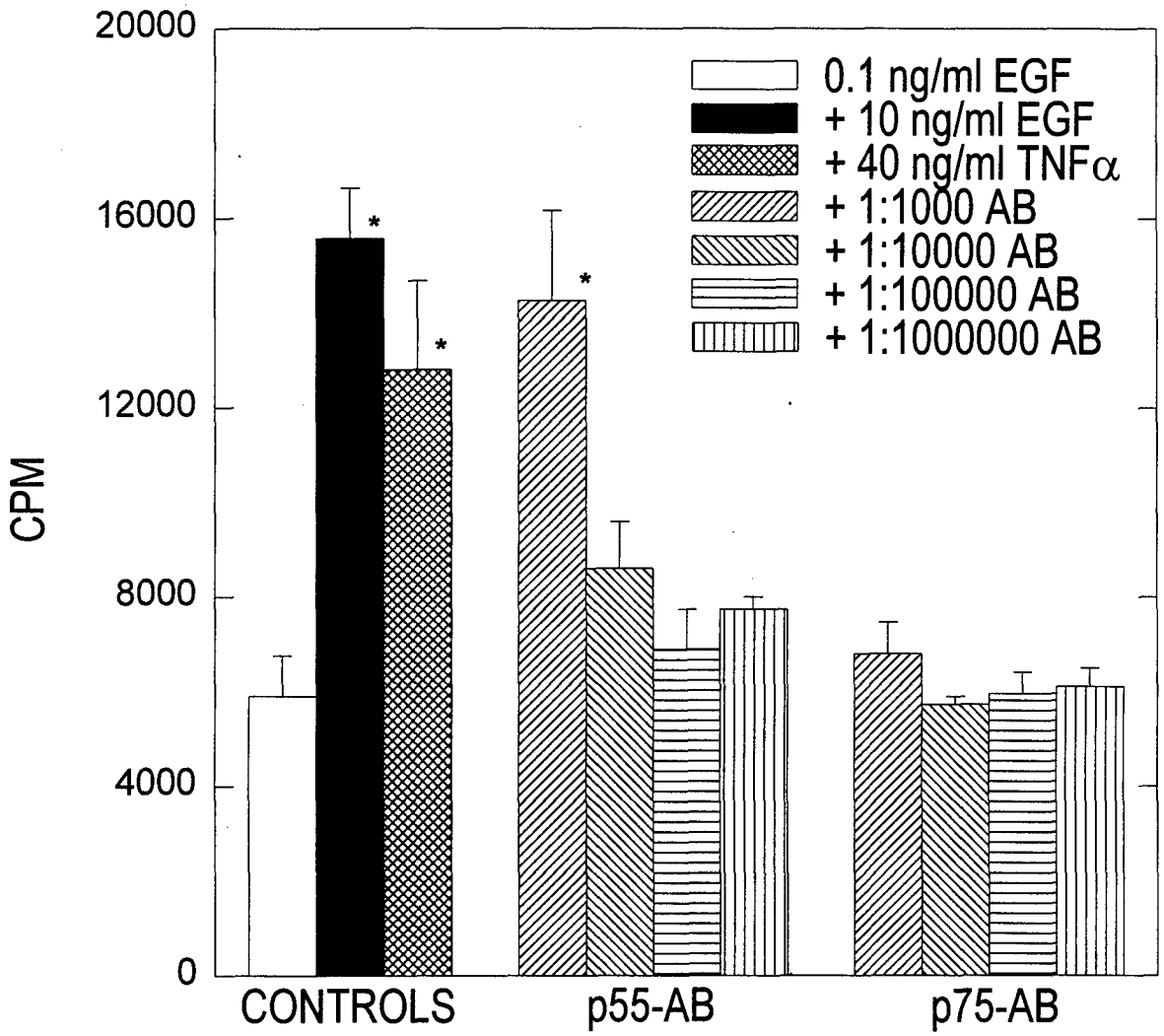

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Varela and Ip
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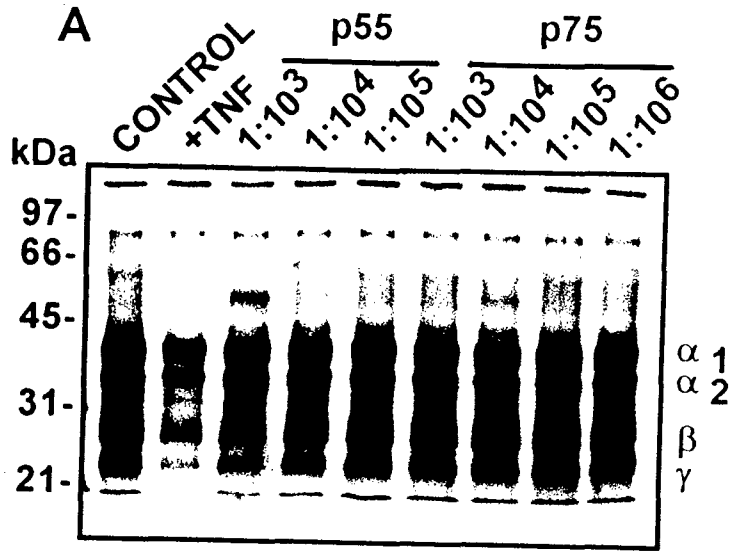

Figure 8A.
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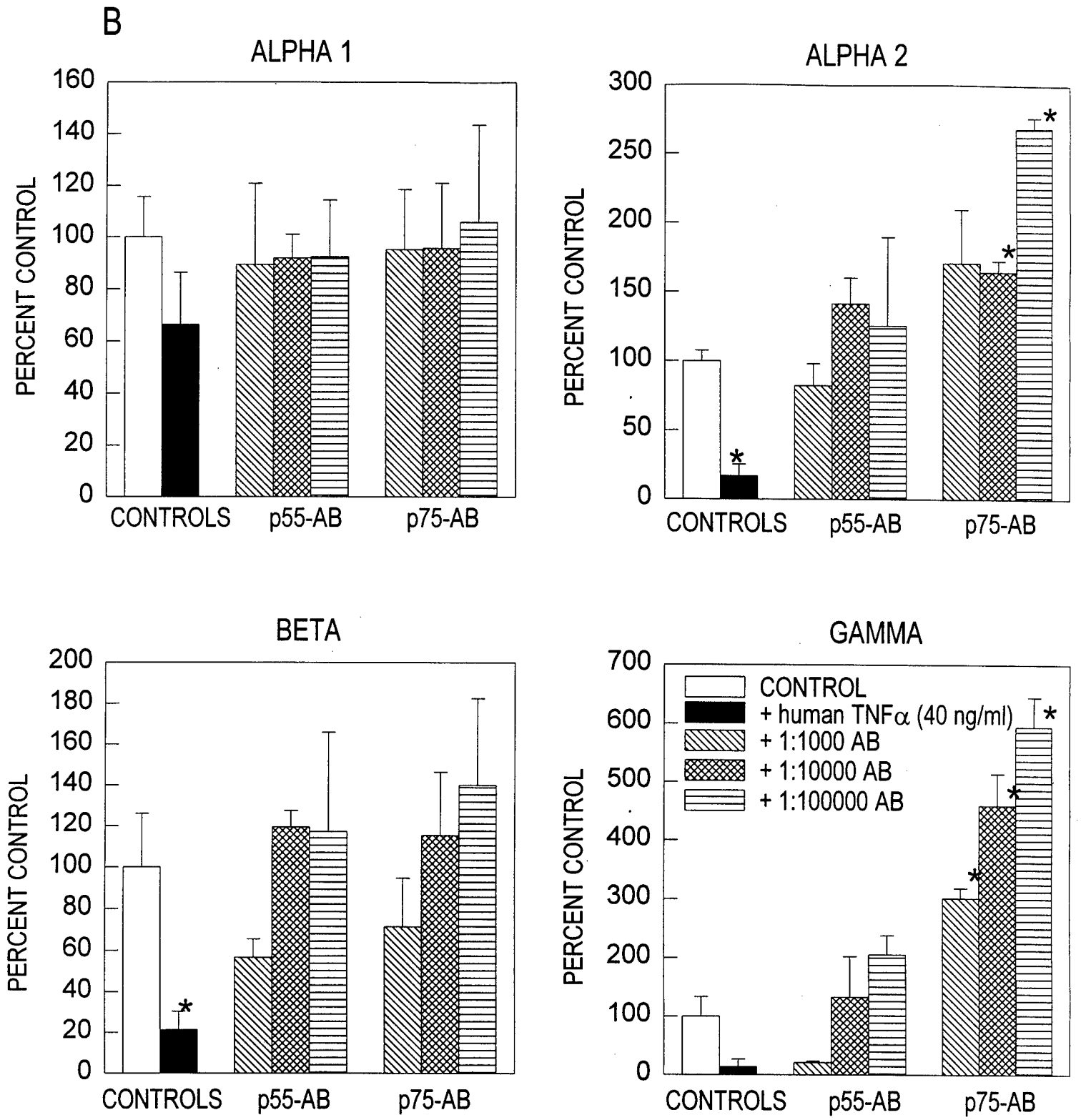

Figure 8B.
Varela and Ip
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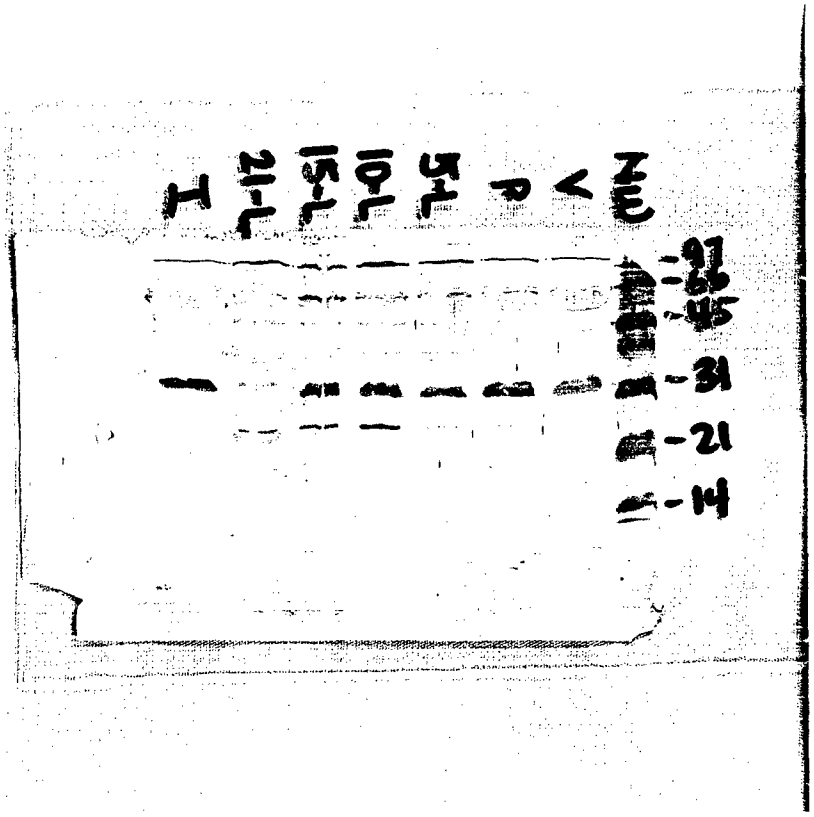
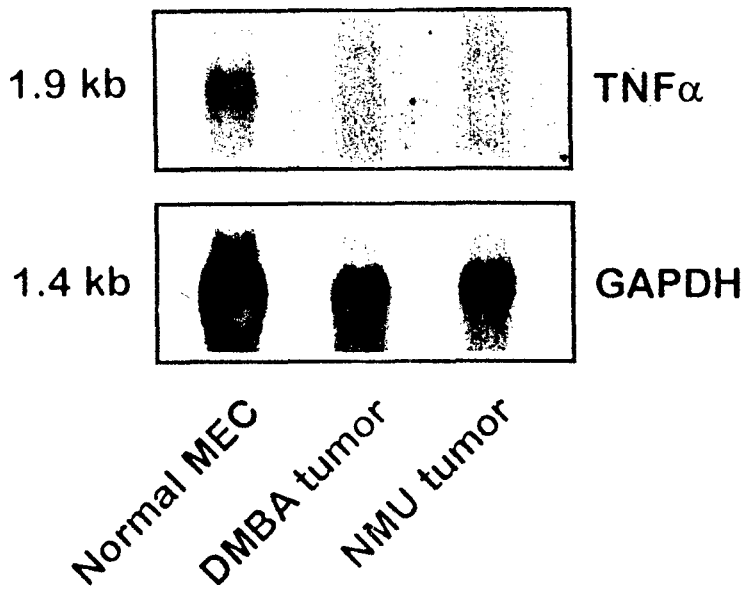


Figure 9
Varela and Ip



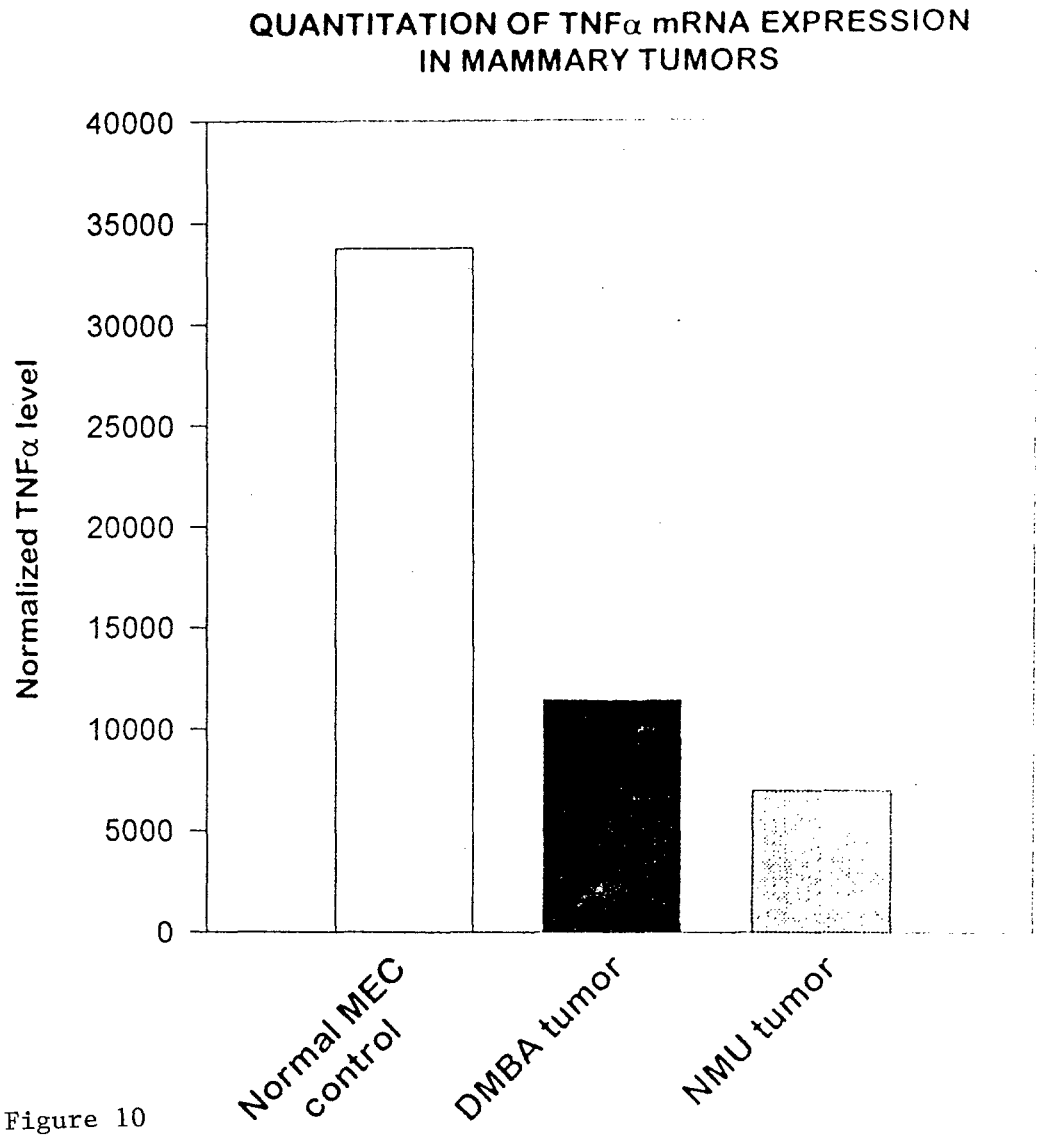


Figure 10
Varela and Ip

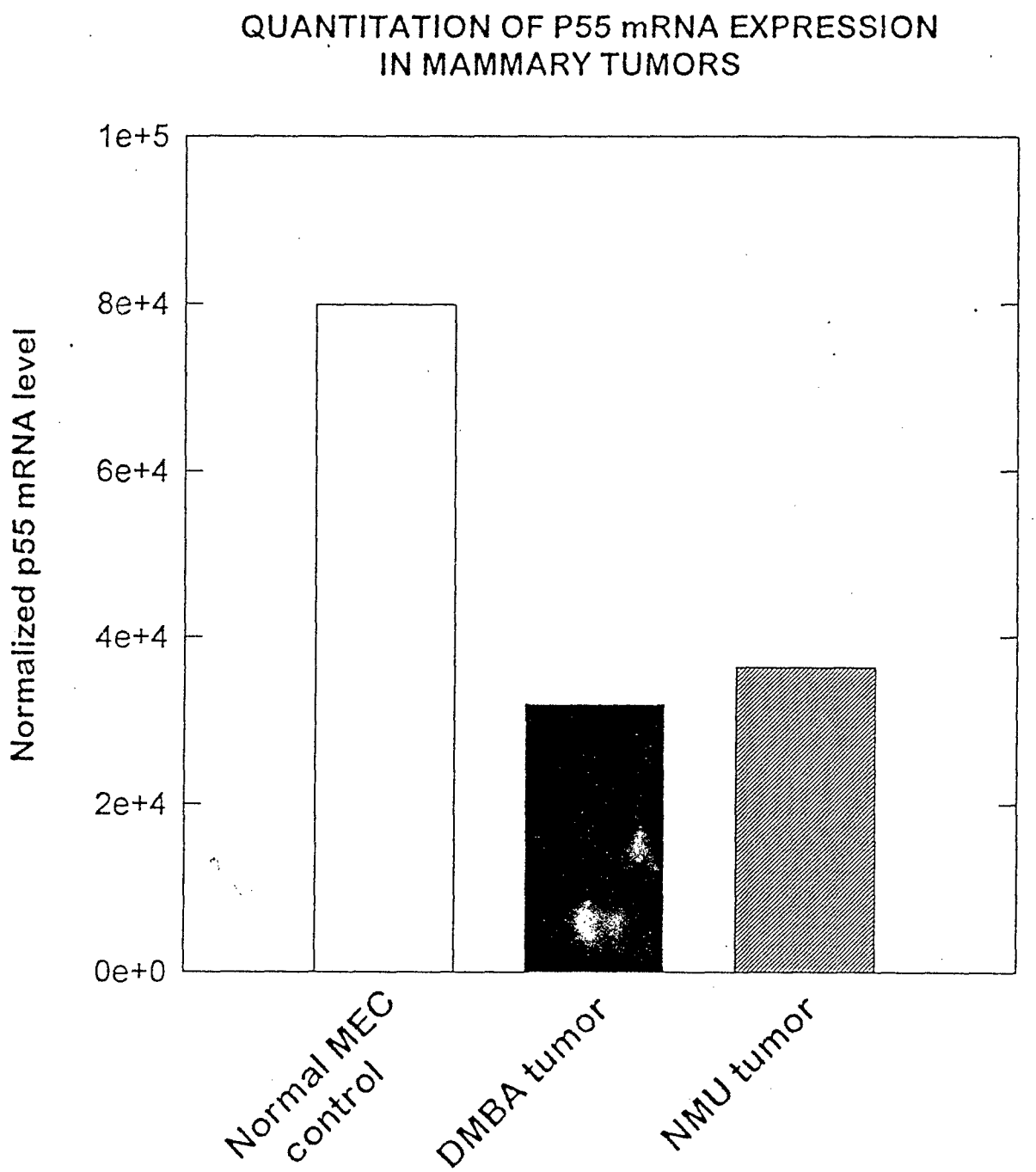


Figure 11
Varela and Ip

MEETING ABSTRACTS:

1. Poster presented at American Association for Cancer Research Meeting in Washington, D.C. Abstract entitled "Regulation of TNFα and its receptors during mammary gland development", Varela,L.M. and Ip,M.M., AACR Program, *87*: 72, 1996. Recipient of travel award from Women in Cancer Research.

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) 6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, 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 written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
DAMD17-94-J-4030	ADB215484
DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864
DAMD17-94-J-4267	ADB216187
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562
DAMD17-94-J-4156	ADB216186
DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management