

Tumor necrosis factor deficiency inhibits mammary tumorigenesis and a tumor necrosis factor neutralizing antibody decreases mammary tumor growth in neu/erbB2 transgenic mice

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Abstract

Tumor necrosis factor- α (TNF- α) is a pleiotropic cytokine that is synthesized and secreted by cells of the immune system, as well as by certain epithelia and stroma. Based on our previous studies demonstrating TNF-stimulated proliferation of normal and malignant mammary epithelial cells, we hypothesized that TNF might promote the growth of breast cancer *in vivo*. To test this, we generated bigenic mice that overexpressed activated neu/erbB2 in the mammary epithelium and whose TNF status was wild-type, heterozygous, or null. Mammary tumorigenesis was significantly decreased in TNF-/- mice ($n = 30$) compared with that in TNF+/+ mice ($n = 27$), with a palpable tumor incidence of 10.0% and 44.4%, and palpable tumors/mouse of 0.10 ± 0.06 and 0.67 ± 0.17 , respectively. Tumorigenesis in the heterozygous group fell between that in the TNF+/+ and TNF-/- groups, but was not significantly different from either of the homozygous groups. The decreased tumor development in the TNF-/- mice was associated with a decreased proliferative index in the lobular and ductal mammary epithelium. To further investigate the role of TNF in breast cancer, mammary tumor-bearing mice whose tumors overexpressed wild-type neu/erbB2 were treated with a TNF-neutralizing antibody or a control antibody for 4 weeks ($n = 20$ /group). Mammary tumor growth was significantly inhibited in mice

treated with the anti-TNF antibody compared with the control antibody. Together, these data show a stimulatory role for TNF in the growth of breast tumors and suggest that TNF antagonists may be effective in a subset of patients with breast cancer. [Mol Cancer Ther 2009;8(9):2655–63]

Introduction

In women, breast cancer is the most common form of cancer, and is the second leading cause of cancer deaths. Although advances in therapy have increased survival, advanced disease remains difficult to treat, necessitating new treatment options. One possibility is to use a biological approach, in which a naturally occurring growth-promoting molecule in the tumor microenvironment is targeted. A potential candidate is tumor necrosis factor- α (TNF- α), which is expressed in the mammary epithelium and stroma (1, 2), as well as the immune cells which are recruited to the mammary gland stroma under various physiologic or pathologic conditions. This cytokine has been shown to have pleiotropic effects on cells, with the outcome of cell death, growth, or differentiation being concentration-dependent and context-dependent (3). For example, at high doses, TNF has antitumor properties, which result, at least in part, from its cytotoxic effects on the tumor vasculature (4). At more physiologic concentrations, however, TNF exerts a growth-promoting effect on certain normal epithelia, including that in the mammary gland (5), intestine (6), ovary (7), and liver (8). Pharmacologically, high doses of TNF can be exploited clinically, although its use is limited to regional treatment, for example, isolated limb perfusion for soft tissue sarcoma (4) because of systemic toxicity at high doses. The opposite approach, i.e., a TNF blockade, should be considered to combat the growth-promoting effects of locally produced TNF on the tumor epithelium.

TNF has been shown to increase the growth of both normal and malignant mammary epithelial cells in experimental models. In a physiologically relevant three-dimensional model system, TNF stimulated the proliferation and branching morphogenesis of rat mammary epithelial organoids, and modulated functional differentiation (1, 5, 9–12). These effects appear to be physiologically relevant because expression of TNF and its two receptors is regulated throughout normal mammary gland development (1). Furthermore, TNF also stimulated the growth and morphogenesis of organoids derived from 1-methyl-1-nitrosourea-induced rat mammary tumors (13), demonstrating the potential for TNF-induced growth of mammary tumors *in vivo*. TNF

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may also play a role in metastasis, as it stimulates matrix metalloproteinase-9 secretion from mammary epithelial cells (10, 11) as well as the surrounding stroma (14). Clinically, TNF expression is increased in the stroma of invasive breast carcinoma tissue compared with benign tissue (2). Moreover, the number of cells expressing TNF increased with tumor grade (2), and serum TNF concentration showed a positive correlation with breast tumor stage (15).

Based on the above studies, we hypothesized that TNF might promote the growth of breast cancers *in vivo*. Two approaches were used to test this hypothesis. In the first, we crossed mice which overexpress activated neu/erbB2 in the mammary epithelium, a mouse model which gives rise to spontaneous mammary tumors (16), with TNF null mice (17), and compared mammary tumor development in TNF null and wild-type bigenic mice. ErbB2 has been shown to be overexpressed in 20% to 30% of human breast cancers, and is associated with poor outcome (18). Secondly, we asked whether anti-TNF therapy would inhibit the growth of established mammary tumors. Data from both studies support the notion that anti-TNF therapy might play a role in the treatment of breast cancer.

Materials and Methods

Animals

Mouse mammary tumor virus (MMTV)/neu (NDL2-5) FVB transgenic mice were bred in-house from breeder pairs generously provided by Dr. William Muller (McGill University, Montreal, Quebec). These mice overexpress a constitutively activated neu protein in the mammary epithelium (16), and were used to generate mice for the tumorigenesis study, and for the generation of tumors used in the primary culture experiment. Breeder pairs of FVB/N-Tg (MMTVneu)202Mul/J mice, which overexpress wild-type neu in the mammary epithelium (19), were obtained from The Jackson Laboratory, and used to generate the mice used in the anti-TNF therapeutic study. TNF null mice (17), which lack TNF in all tissues, were generously provided by Dr. George Kollias (Biomedical Sciences Research Center "Alexander Fleming," Vari, Greece) at the sixth-generation backcross into C56BL/6 mice, and were mated with C57BL/6J mice (The Jackson Laboratory) to create the seventh-generation backcross. After crossing the heterozygotes, the resultant TNF^{-/-} and TNF^{+/+} mice were bred in-house and maintained at this backcross generation. Mice were maintained in microisolator cages in a temperature-controlled and humidity-controlled environment with a 12 h light/12 h dark cycle, and were given chow and water *ad libitum*. The care and use of the animals were in accordance with NIH guidelines and Institute Animal Care and Use Committee regulations.

Mammary Tumorigenesis Study

The breeding strategy for this study was as follows. Seventh-generation TNF^{-/-} female mice ("parental") were mated with MMTV/neu (NDL2-5) male mice (cross 1), and the resultant TNF^{+/-} : neu-positive progeny bred with either the TNF^{-/-} parental mice to generate TNF^{+/-} : neu-

positive and TNF^{-/-} : neu-positive progeny (cross 2A), or with C57BL/6J (TNF^{+/+}) mice to generate TNF^{+/-} : neu-positive and TNF^{+/+} : neu-positive mice (cross 2B). Mice were monitored for palpable mammary tumor development, and were euthanized when a tumor reached 18 to 20 mm in the longer diameter. The remaining mice were sacrificed at 78 wk of age. At sacrifice, mammary glands and tumors were removed and prepared for whole mount analysis and/or fixed in formalin. Lungs of the euthanized mice were perfused with India ink through the trachea, removed, and fixed as described previously (20).

PCR

The genotype of each mouse was verified using tail DNA, isolated using kits from either Promega or Sigma-Aldrich. Primers, which were obtained from Integrated DNA Technologies were as follows: TNF, 5'-AAC CAG GCA GGT TCT GTC CC-3' (sense); 5'-GCT TCC CAG CAA GCA TCT ATG-3' (antisense) and Neu, 5'-TTC CGG AAC CCA CAT CAG GCC-3' (sense); 5'-GTT TCC TGC AGC AGC CTA CGC-3' (antisense).

Preparation of Mammary Whole Mounts, Detection of Metastases, Histology, and Immunohistochemistry

Whole mounts of the mammary glands were prepared as described previously (20). Using photographic images of the whole mounts, with verification under the 4× objective of the microscope, the number of hyperplasias/lesions in mammary glands 1 to 5 on one side of each mouse was counted in three groups according to size (0.2–0.5, 0.5–1.0, and >1.0 mm in diameter). Lung metastases, H&E staining, Ki67 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immunohistochemistry, and photography were as described previously (20), with the exception that the Ki67 antibody (RM-9106-S1) was purchased from Neomarkers/Lab Vision. The percentages of Ki67-positive or TUNEL-positive cells were quantified in 117 to 120 lobules, ducts, or microscopic hyperplasias/putative neoplastic lesions in the mammary epithelium. In the mammary tumors, Ki67 staining was evaluated separately by two pathologists, scoring both staining intensity (0–3) and percentages of Ki67-positive neoplastic cells which was classified as follows: 0% (scored as 1); <5% (scored as 2); 5% to 25% (scored as 3); 26% to 50% (scored as 4); and >50% (scored as 5). The immunohistochemical index was calculated by multiplying the intensity score by the Ki67 positivity score.

Isolation and Primary Culture of Mammary Tumor Organoids

Tumor epithelial organoids were isolated from mammary tumors generated in MMTV/neu (NDL2-5) FVB transgenic mice according to the protocol developed by our laboratory (21), with the following modifications: digestion time was reduced to 2.75 h by placing the digestion flasks in a 37°C shaking water bath; the collagenase and dispase solutions were each 1% (w/v). The organoids were embedded within an Engelbreth Holmes Swarm sarcoma-derived reconstituted basement membrane (as described previously; ref. 21) and cultured in a serum-free medium consisting of phenol red-free DMEM-F12 (50:50, v/v) with 10 ng/mL

of epidermal growth factor, 10 $\mu\text{g}/\text{mL}$ of insulin, 1 $\mu\text{g}/\text{mL}$ of progesterone, 1 $\mu\text{g}/\text{mL}$ of hydrocortisone, 1 $\mu\text{g}/\text{mL}$ of prolactin, 5 $\mu\text{g}/\text{mL}$ of transferrin, 5 μM of ascorbic acid, 1 mg/mL of fatty acid-free bovine serum albumin, and 50 $\mu\text{g}/\text{mL}$ of gentamicin, with or without recombinant mouse TNF (100 ng/mL ; Biosource International). Ovine prolactin (NIDDK-oPRL-21) was obtained from Dr. A.F. Parlow at the National Hormone & Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA. To evaluate the growth of the tumor epithelial organoids, the organoids (5×10^5 cells) were cultured in a 24-well plate within the Engelbreth Holmes Swarm sarcoma-reconstituted basement membrane in 1 mL of serum-free medium with or without TNF for the times indicated. The viable cell number was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (5) and is presented as absorbance at 570 nm.

Effect of TNF Status on Carcinogen Sensitivity of Mammary Glands in Organ Culture

Carcinogen sensitivity of mammary glands from TNF $+/+$ and TNF $-/-$ mice was evaluated by an adaptation of the procedure of Banerjee et al. (22), and is described in the on-line Supplementary Materials.

Anti-TNF Therapy of Mammary Tumor-Bearing FVB/N-Tg (MMTVneu)202Mul/J Mice

As mice developed palpable mammary tumors of 3 to 4 mm in diameter, they were randomly divided into two groups of 20 mice per group, and treated with either the antimouse TNF antibody (CNT02213; also referred to as cV1q), or the isotype control monoclonal antibody (CNT01322; Centocor R&D, Inc.), at a dose of 1 mg/wk , i.p., for 4 wk. cV1q is an antimouse TNF- α antibody which is a chimeric (rat variable regions, mouse constant regions) monoclonal antibody (23). The properties of cV1q with regard to specificity, affinity, and neutralization of murine TNF are analogous to those seen for the human TNF-specific antibody, infliximab (Remicade).

Statistical Analysis

Log rank analysis was used to statistically evaluate the Kaplan-Meier curves, with the χ^2 test with 1 *df* used to compare the TNF null versus TNF wild-type groups, and the TNF null or wild-type with each of the TNF $+/-$ groups. Differences in tumor incidence at 78 wk of age were evaluated using Fisher's exact test. Differences in mammary tumor number per mouse in the tumorigenesis study were evaluated by Kruskal-Wallis one-way ANOVA on ranks; the difference between the TNF wild-type and TNF null groups was evaluated by the Mann-Whitney rank sum test. Differences in tumor incidence at sacrifice were compared using the Fischer exact test; lung metastasis differences were compared by the same method. Body weight and spleen weight differences were evaluated by Kruskal-Wallis ANOVA on ranks. Ki67 and TUNEL staining of tissues from TNF $-/-$ and TNF $+/+$ mice were compared using Student's *t* test. In the primary culture experiment, Student's *t* test was used to examine the statistical significance of the differences between the control and TNF-treated groups at each time point. In the anti-TNF therapeutic study, a repeated

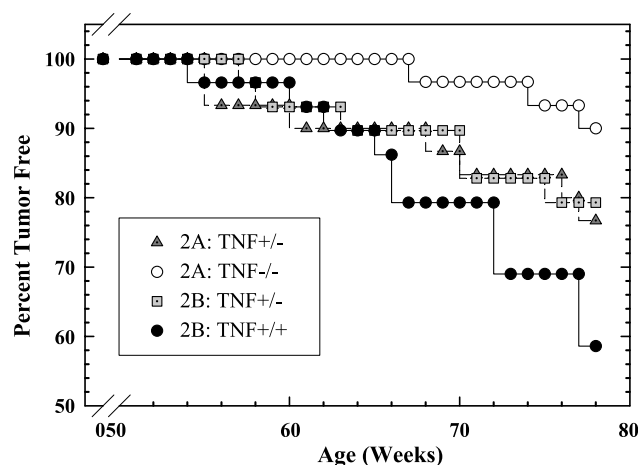


Figure 1. Mammary tumorigenesis is significantly decreased in TNF null mice. Neu/erbB2-overexpressing mice which were TNF $-/-$, TNF $+/-$, or TNF $+/+$ were generated in the 2A and 2B crosses as described in Materials and Methods, and the time at which a palpable tumor was detected in each mouse was recorded. Kaplan-Meier plot showing the percentage of tumor-free mice at different ages in each group. The TNF null group (*open circles*) is significantly different from the TNF wild-type group (*closed circles*; $P < 0.05$); however, neither of the TNF $+/-$ groups (*dotted triangles*, cross 2A or *dotted squares*, cross 2B), is significantly different from the TNF $-/-$ or TNF $+/+$ groups. The 2A and 2B identifiers refer to the cross number, as described in Materials and Methods. The number of mice in each group was as follows: 2A (TNF $+/-$), 29; 2A (TNF $-/-$), 30; 2B (TNF $+/-$), 27; and 2B (TNF $+/+$), 27.

measures statistical model was fit to the tumor diameter data at various time points assuming a first-order autocorrelation covariance structure. Natural splines were used to flexibly model the curvature of trends in the time profiles, and a log scale was used to better satisfy underlying statistical model assumptions of variability and normal distribution shape. A pairwise comparison between the two groups was made at each of the time points. For all the evaluations, $P < 0.05$ was deemed statistically significant.

Results

TNF Stimulates the Growth of erbB2-Overexpressing Mammary Tumor Cells *In vitro*

We previously showed that TNF stimulated the growth of normal rat and mouse mammary epithelial cells, as well as 1-methyl-1-nitrosourea-induced rat mammary tumor cells, in a three-dimensional primary culture model (1, 5, 13, 24). These observations suggested that TNF might stimulate mammary tumorigenesis *in vivo*. To examine this, we chose a transgenic mouse model in which neu/erbB2 was overexpressed in the mammary epithelium. We further chose to use a model, NDL2-5, in which neu/erbB2 is constitutively active because of an in-frame deletion (16), reasoning that because we would be breeding the NDL2-5 mice into the relatively tumor-resistant C57BL/6 strain (25), tumorigenesis would be greater than if we were to use the somewhat less aggressive wild-type neu/erbB2 model (16). Prior to undertaking the extensive breeding required to test our hypothesis, we first asked whether mammary tumor cells from NDL2-5 mice were sensitive

to TNF. Mammary tumor organoids were suspended within the Engelbreth Holmes Swarm sarcoma-reconstituted basement membrane, and cultured in serum-free medium in the absence or presence of 100 ng/mL of TNF. As can be seen in Supplementary Fig. S1, the mammary tumor cells were growth-stimulated at all time points tested (up to 3 weeks in culture). Based on these data, we then proceeded with developing the TNF null and wild-type erbB2-overexpressing mice.

Mammary Tumorigenesis Is Decreased in TNF Null Mice

NDL-2 neu/erbB2 transgenic mice were crossed with TNF null or TNF wild-type mice to generate mice that overexpressed neu/erbB2 in the mammary epithelium, and which were TNF^{+/+}, TNF^{+/-}, or TNF^{-/-} in all tissues. As seen in Fig. 1, the development of palpable mammary tumors was significantly delayed in TNF^{-/-} mice when compared with TNF^{+/+} mice ($P < 0.05$). Palpable tumor development in the two TNF^{+/-} groups was comparable, and fell between that of the TNF null and wild-type groups, although neither TNF^{+/-} group was significantly different from TNF wild-type or null mice. Compared with the parental FVB NDL-2 transgenic mice in which the average age of mammary tumor onset in nulliparous mice is 23 weeks (16), tumor development was slower in the TNF^{+/+} FVB:C57BL/6 mice of the current study, with palpable tumor incidence of only 44.4% at 78 weeks of age (Table 1). The corresponding percentages for the other groups at this age were 10.0% for the TNF^{-/-} group ($P < 0.015$ compared with TNF^{+/+}), and 20.7% and 22.2% for the TNF^{+/-} 2A and TNF^{+/-} 2B crosses, respectively. The number of visible tumors per mouse at sacrifice was also significantly different between the TNF^{-/-} and TNF^{+/+} groups (0.10 and 0.67 tumors/mouse, respectively; $P < 0.019$), with the two TNF^{+/-} groups falling between the homozygous mice

(Table 1). Together, these data suggest a potential dose-dependent effect of TNF for primary tumor development. In contrast, no significant differences in lung metastasis were noted among the groups (Table 1).

Because of the low palpable tumor incidence, we considered the possibility that microscopic mammary lesions had developed which had not progressed to palpable tumors. We therefore prepared whole mounts from mammary glands 1 to 3 and 4 to 5 on one side of each mouse to determine the extent to which these putative lesions had developed, and whether TNF status altered their number. Extensive hyperplasia was noted in the mammary glands of mice from all groups (see example in Fig. 2A and B). We quantified these hyperplastic and putative preneoplastic lesions within three different size groupings (0.2–0.5, 0.5–1.0, and >1.0 mm in diameter), but found no statistically significant effect of TNF gene dosage (Table 1). In cross-section, large hyperplastic ductal-lobular structures were observed in both TNF^{+/+} and TNF^{-/-} mice, the majority of which were composed of a single layer of epithelial cells surrounding a lumen (TNF^{-/-}, Fig. 2C; TNF^{+/+}, Fig. 2D). Additionally, areas of both lobular carcinoma *in situ* (Fig. 2E), as well as ductal carcinoma *in situ* (Fig. 2F) were observed, some of which were within the large structures, as is illustrated in Fig. 2E.

Finally, it should be noted that all the transgenic mice were healthy, although the TNF^{-/-} mice were slightly smaller than the TNF^{+/+} mice (Table 1). Spleen weights were not significantly different among the groups, although in the TNF^{+/+} group, tumor-bearing mice had larger spleens than non-tumor-bearing mice (Table 1). Because of the small number of mice developing tumors in the TNF^{-/-} group, it was not possible to definitively determine whether this biological response was observed in TNF-deficient mice.

Table 1. Effect of TNF status on mammary tumor development, lung metastasis, and mouse weight

	Group			
	TNF ^{-/-} 2A	TNF ^{+/-} 2A	TNF ^{+/-} 2B	TNF ^{+/+} 2B
N	30	29	27	27
Tumor incidence (mice with tumors visible at sacrifice, %)	10.0 ^a	20.7 ^{a,b}	22.2 ^{a,b}	44.4 ^b
No. of tumors visible at sacrifice*	3	8	11	18
Visible tumors per mouse [†]	0.10 ± 0.06 ^a	0.28 ± 0.11 ^{a,b}	0.41 ± 0.18 ^{a,b}	0.67 ± 0.17 ^b
Microscopic hyperplasias/MG ₁₋₅ ^{‡,§} (0.2–0.5 mm diameter)	95.9 ± 13.5 ^{a,c}	85.6 ± 12.8 ^a	190.1 ± 29.7 ^{b,c}	150.1 ± 25.5 ^{a,c}
Microscopic hyperplasias/MG ₁₋₅ ^{‡,§} (0.5–1.0 mm diameter)	1.17 ± 0.33 ^a	1.28 ± 0.39 ^a	1.04 ± 0.38 ^a	1.30 ± 0.36 ^a
Microscopic hyperplasias/MG ₁₋₅ ^{‡,§} (>1.0 mm diameter)	0.33 ± 0.27 ^a	0.66 ± 0.19 ^a	0.33 ± 0.12 ^a	0.22 ± 0.08 ^a
Lung metastases, #/N (%) [§]	3/28 (10.7%) ^a	2/25 (8.0%) ^a	4/23 (17.4%) ^a	4/27 (14.8%) ^a
Body weight [†] (g)	25.3 ± 0.6 ^a	27.0 ± 0.6 ^{a,b}	27.1 ± 0.7 ^{a,b}	28.2 ± 0.7 ^b
Spleen weight (mg/100 g body weight; N)				
All mice	479 ± 71 (30) ^a	313 ± 12 (29) ^a	453 ± 55 (26) ^a	426 ± 50 (26) ^a
Non-tumor-bearing mice	434 ± 55 (27) ^{a,1}	308 ± 12 (23) ^{a,1}	387 ± 47 (21) ^{a,1}	317 ± 20 (15) ^{a,1}
Tumor-bearing mice	883 ± 524 (3) ^{a,1}	330 ± 37 (6) ^{a,1}	728 ± 169 (5) ^{a,2}	573 ± 100 (11) ^{a,2}

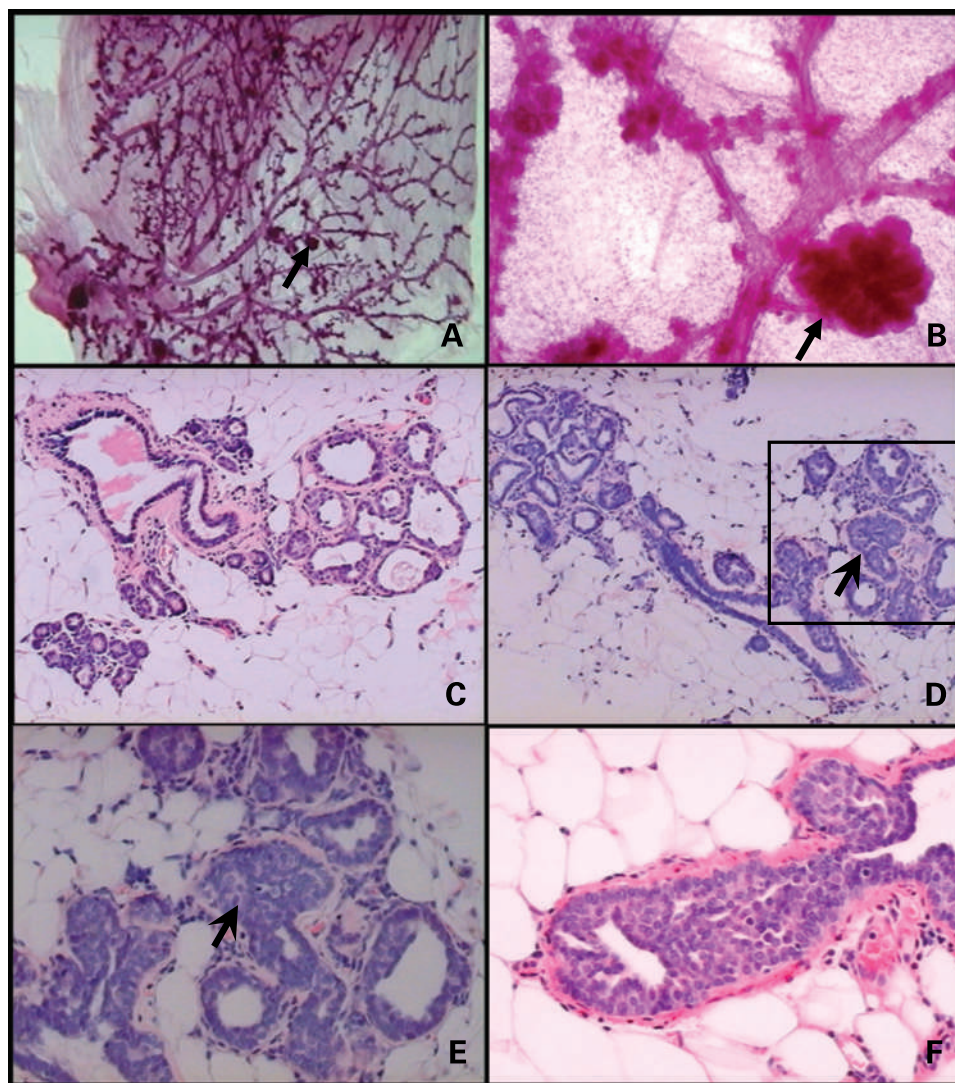
*Total visible tumors per group.

[†]Microscopic hyperplasias/lesions were counted in mammary glands (MG) 1 to 5 on one side of each mouse. Numbers presented are per mouse.

[‡]Mean ± SE. Means or percentages in a row without a common superscript letter, or in a column without a common superscript number, are statistically different.

[§]Metastases were found in both visible tumor-bearing (TB) and non tumor-bearing (NTB) mice as follows: TNF^{-/-} 2A, TB 1, NTB 2; TNF^{+/-} 2A, TB 1, NTB 1; TNF^{+/-} 2B, TB 2, NTB 2; TNF^{+/+} 2B, TB 2, NTB 2.

Figure 2. Hyperplasia as well as microscopic carcinomas are observed in mammary glands of FVB: C57BL/6 mice which overexpress neu/erbB2 in the mammary epithelium. **A** and **B**, whole mount of the extensive hyperplasia that developed in these mice. *Black arrow*, a putative preneoplastic lesion from a TNF^{-/-} mouse. Photos were taken under the 1× objective (**A**) or the 4× objective (**B**). **C** and **D**, H&E-stained paraffin sections of ductal-lobular hyperplasia in mammary glands of TNF^{-/-} (**C**) and TNF^{+/+} (**D**) mice. Both photos were taken under the 10× objective (*inset* in **D** is shown enlarged in **E**). **E**, H&E-stained paraffin section of lobular carcinoma *in situ* within the large ductal-lobular structure in **D** (TNF^{+/+} mouse, 20× objective; *arrows* in **D** and **E** point to the same structure). **F**, H&E-stained paraffin section of ductal carcinoma *in situ* from a TNF^{-/-} mouse (20× objective). Overall, similar morphologies were seen in the other groups.



Proliferation Is Decreased in the Mammary Epithelium of TNF Null Mice

Ki67 immunohistochemistry was used as a preliminary step to investigate the mechanism by which TNF status might alter mammary tumorigenesis. We first looked at the Ki67 immunohistochemistry score (see Materials and Methods) of the mammary tumors, but found no significant difference between the TNF wild-type ($n = 10$) and TNF null ($n = 4$, three palpable and one microscopic) tumors (immunohistochemical indices of 10.8 ± 0.4 and 11.3 ± 1.0 , respectively). Because this analysis was limited by the small number of mammary tumors in the TNF null mice, we therefore examined the proliferative status of the mammary epithelium per se. As seen in Table 2, the proliferative rate was high in both the TNF null and wild-type groups as compared with what would be expected in the epithelium of a nontransgenic mouse of the same age. Notably, however, the percentages of Ki67-positive nuclei was significantly decreased in both the ductal and lobular mammary epithe-

lium of the TNF null mice, when compared with that of the TNF wild-type mice (Table 2). Apoptosis was concurrently decreased in the lobular, but not the ductal mammary epithelium of TNF^{-/-} mice, but overall, the percentages of cells undergoing apoptosis was only ~15% of those which were undergoing proliferation (Table 2).

Effect of TNF Status on Carcinogen Sensitivity of Mammary Glands in Organ Culture

The increased tumor latency in the TNF null mice could result from the systemic absence of TNF because TNF is deleted in all tissues, and/or from the absence of TNF in the mammary gland. Because TNF is expressed in both the epithelial and stromal compartments of the mammary gland (1),⁴ the role of mammary gland TNF on tumorigenic sensitivity is not readily addressable in an *in vivo* study. We therefore used the *in vitro* mammary gland organ

⁴ N.C. Stangle-Castor, L.M. Varela, and M.M. Ip, unpublished.

Table 2. Effect of TNF status on proliferation and apoptosis in the mammary epithelium of neu/erbB2 transgenic mice

Proliferation or apoptosis within mammary ducts and lobules	TNF ^{-/-}	TNF ^{+/+}
Ducts: Ki67-positive nuclei (%)	33.2 ± 1.6 ^a	45.2 ± 1.7 ^b
Lobules: Ki67-positive nuclei (%)	43.3 ± 1.4 ^a	54.1 ± 1.2 ^b
Ducts: TUNEL-positive cells (%)	6.7 ± 1.5 ^a	7.3 ± 1.7 ^a
Lobules: TUNEL-positive cells (%)	5.7 ± 0.7 ^a	9.9 ± 1.8 ^b

NOTE: Values are mean ± SE from 10 mice per group. A total of 119 to 120 ducts or lobules were counted in each group. Means in a row without a common letter are statistically different.

culture model described in the Supplementary Materials to ask whether mammary glands from TNF null mice had an altered sensitivity to the carcinogen 7,12-dimethylbenz (a)anthracene (DMBA). Previous studies in BALB/c mice (22) had shown that the culture conditions used permitted the normal lobuloalveolar epithelium which develops during the first 9 days of culture to regress when placed in an involution medium; however, preneoplastic nodule-like alveolar lesions arising in the DMBA-treated mammary glands did not regress, and thus, could be quantified. In our studies, in which the glands from one side of the mouse were treated with DMBA, and those from the other side with the DMSO vehicle, we were unable to identify nodule-like alveolar lesions ($n = 9$ TNF^{-/-} mice and $n = 7$ TNF^{+/+} mice). Specifically, although all the glands underwent extensive morphologic development, in each case, the regressed mammary glands from the control and DMBA-treated cultures could not be distinguished (Supplementary Fig. S2, A versus B and C versus D). This failure to detect preneoplastic lesions might be related to the decreased sensitivity of C57BL/6 mice (the strain background of our TNF null and wild-type mice) to carcinogen-induced mammary tumors, when compared with BALB/c mice (26). We did not test higher DMBA concentrations due to its reported cytotoxicity (27). In spite of the lack of effect of DMBA in these studies, these experiments did show that the regressed mammary glands were morphologically similar in the TNF null and wild-type mice (Supplementary Fig. S2; data not shown; $n = 7-9$).

Mammary Tumor Growth Is Inhibited in Mice Treated with a TNF-Neutralizing Antibody

As a result of our observation that mammary tumorigenesis was delayed and decreased in TNF null mice, we hypothesized that anti-TNF therapy would inhibit the growth of established mammary tumors. To examine this, mice overexpressing wild-type neu/erbB2 in the mammary epithelium were randomized into two groups and treated with the TNF-neutralizing antibody or the control antibody for 4 weeks. As seen in Fig. 3, the anti-TNF antibody significantly inhibited the growth of established mammary tumors, when compared with controls. This treatment did not seem to be deleterious to the mice because neither body weight (Table 3) nor liver weight (data not shown) were altered. Interestingly, however, the tumor-induced increase in spleen weight (see above), was partially, although signifi-

cantly, reversed, suggesting a biological effect of the TNF antibody on this organ. A similar observation was made in a previous study in which the same TNF-neutralizing antibody was shown to partially block the increase in spleen weight resulting from immunization with sheep RBC (23).

The proliferative index in the tumors, as measured by Ki67 immunohistochemistry, was not significantly different in the two groups, with immunohistochemical indices of 12.0 ± 0.3 ($n = 19$) and 12.4 ± 0.3 ($n = 20$) in the control antibody-treated and TNF antibody-treated groups, respectively. It is important to note, however, that Ki67 staining was quite variable in different regions of the tumor, and the immunohistochemical index for each tumor represents an average for the section. In contrast to the tumor, TNF antibody therapy did have an inhibitory effect on proliferation in the surrounding mammary epithelium in the tumor-bearing mice. As seen in Table 3, mice treated with the neutralizing TNF antibody had significantly decreased proliferation in the lobular epithelium ($P < 0.008$). Proliferation was also decreased in the ducts, although this did not achieve statistical significance ($P = 0.123$). Unexpectedly, no change in proliferation was noted within the microscopic hyperplasias and ductal carcinoma *in situ*/lobular carcinoma *in situ* lesions. We also used TUNEL immunohistochemistry to determine whether the TNF antibody treatment altered the apoptotic index within the tumors. However, essentially all of the tumor cells were TUNEL-negative, even though TUNEL-positive cells could be seen in the adjacent "normal" epithelium (data not shown). TNF antibody treatment had no significant effect on the apoptotic index within the ductal and lobular epithelium, nor on the small neoplastic (ductal carcinoma *in situ*/lobular carcinoma *in situ*) or hyperplastic lesions (Table 3).

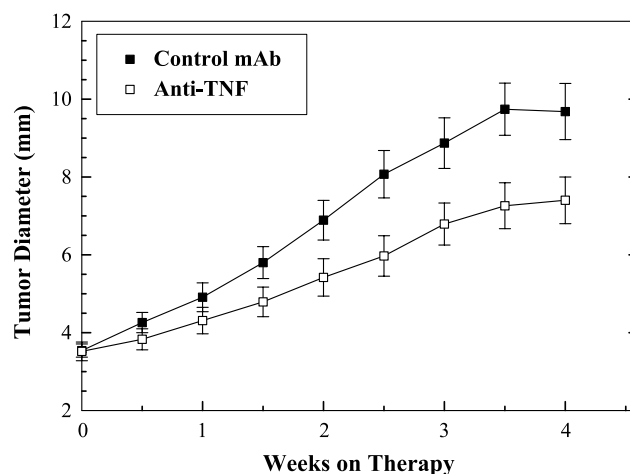


Figure 3. The TNF-neutralizing cV1q antibody inhibits the growth of neu/erbB2-overexpressing mouse mammary tumors. FVB/N-Tg (MMTVneu)202Mul/J mice were randomly divided into two groups when they developed a tumor of 3 to 4 mm in diameter, and treated with either the neutralizing anti-TNF (cV1q, □) or control (cVam, ■) antibodies, at a dose of 1 mg/wk, i.p., for 4 wks. Points, mean of 20 mice per group; bars, SE. The two groups are significantly different from the 2-wk time period onward.

Table 3. Effect of anti-TNF therapy on tumor variables, as well as body and spleen weights

Tumor weight, body weight, and spleen weight	Control monoclonal antibody	Anti-TNF
Tumor diameter (mm)*	9.38 ± 0.75 ^a	7.21 ± 0.64 ^b
Tumor weight (g)*	0.51 ± 0.10 ^a	0.34 ± 0.06 ^a
Body weight (g)*	26.3 ± 0.4 ^a	25.4 ± 0.4 ^a
Spleen weight (mg/100 g body weight)*	564 ± 26 ^a	492 ± 12 ^b
Proliferation or apoptosis within mammary ducts and lobules	Control monoclonal antibody	Anti-TNF
Ducts: Ki67-positive nuclei (%) [†]	54.0 ± 2.4 ^a	48.5 ± 2.8 ^a
Lobules: Ki67-positive nuclei (%) [†]	40.5 ± 1.6 ^a	34.9 ± 2.1 ^b
Lesions: Ki67-positive (%) [†]	44.8 ± 1.8 ^a	45.7 ± 1.6 ^a
Ducts: TUNEL-positive cells (%) [†]	7.7 ± 1.0 ^a	8.5 ± 1.2 ^a
Lobules: TUNEL-positive cells (%) [†]	10.7 ± 1.0 ^a	8.6 ± 0.8 ^a
Lesions: TUNEL-positive (%) [†]	11.1 ± 1.4 ^a	11.2 ± 1.2 ^a

*Values are mean ± SE from 20 mice per group.

[†]Values are mean ± SE from 10 mice per group. A total of 117 to 120 ducts, lobules, or hyperplasias/putative preneoplastic lesions were counted in each group. Means in a row without a common letter are statistically different.

Discussion

The present study provides strong support for the hypothesis that TNF plays a stimulatory role in the growth of breast cancer. Specifically, mammary tumorigenesis was decreased in TNF null mice which overexpressed activated neu/erbB2 in the mammary epithelium, when compared with the corresponding TNF wild-type mice. Moreover, treatment of mammary tumor-bearing mice with an antibody that neutralizes TNF inhibited tumor growth. Although these studies were conducted using the neu/erbB2 transgenic mouse model, based on our previous data demonstrating that TNF increases the proliferation of primary malignant mammary cells from NMU-induced rat mammary tumors, as well as normal rat and mouse mammary epithelial cells (1, 5, 13, 24), we believe that the observed effects are more general in nature, and are not limited to neu/erbB2-overexpressing breast cancers.

Growth of Several Cancer Types Is Modified by TNF Status

TNF is not required for normal mouse development, but splenic follicular architecture fails to develop normally in TNF null mice, and these mice do not develop a normal humoral immune response, are susceptible to infection, and have an altered inflammatory response (17, 28). This phenotype suggested that TNF status might affect tumorigenesis, as indeed was found to be the case. Most of the work in this regard has been done using the DMBA/12-O-tetradecanoylphorbol-13-acetate two-stage skin carcinogenesis model, in which a significant decrease in tumor development was observed in TNF^{-/-} mice (29–31). In this model, TNF did not exert its effect on initiation, but rather as a tumor promoter (32); moreover, although both TNF receptors (TNFR) contributed to the effect of TNF, TNFR1 was found to be more critical (33). Mice deficient in TNFR1 or TNFR2 were also shown to be resistant to UV-induced skin carcinogenesis (34). In addition to its stimulatory role in skin carcinogenesis models, TNF promotes the growth of ovarian cancer. Unlike normal ovarian epithelium, ovarian tumor cells secrete TNF (35), which acts in an autocrine or paracrine manner by

stimulating an increased release of chemokines, cytokines, and vascular endothelial growth factor, all potentially involved in tumor growth and neovascularization (36). Furthermore, ovarian tumors from TNF knockdown cells had reduced growth, metastasis, and vascularization *in vivo*, compared with TNF^{+/+} or mock-transfected cells (36). Finally, hepatic carcinogenesis induced by a choline-deficient, ethionine-supplemented diet, as well as carcinogen-induced colorectal carcinogenesis, were found to be decreased in TNFR1 null mice (37, 38). We suspect that TNFR1 plays a predominant role in TNF-stimulated mammary tumorigenesis as well, based on our previous studies demonstrating that TNFR1, but not TNFR2, mediated the stimulatory effect of TNF on the proliferation of normal mammary epithelial cells (1).

At least two studies have examined the role of TNF in models of inflammation-linked cancer. In a transgenic mouse model in which cyclooxygenase-2 and microsomal prostaglandin E synthase-1 are overexpressed in the gastric epithelium, producing an inflammatory phenotype, Oshima et al. showed that TNF deficiency decreased both inflammation and gastric cancer incidence (39). Moreover, in the Mdr2-null mouse model of inflammation and hepatocellular carcinoma, TNF-neutralizing antibodies inhibited the activation of the RelA subunit of nuclear factor- κ B (NF κ B) and induced the apoptosis of dysplastic hepatocytes (40). Because tumorigenesis in the neu/erbB2 mammary model is driven by the overexpressed neu/erbB2, it is unlikely that TNF plays a proinflammatory role in driving tumor development in the current experiments. Rather, based on our previous studies (24), the higher proliferative rate we observed in the mammary epithelium of TNF wild-type mice is most likely driven, at least in part, through TNF induction of the NF κ B2/RelB or NF κ B1/RelB transcription factors, and subsequent binding of the NF κ B complex to the cyclin D1 promoter.

Inhibitors of TNF Activity Have Anticancer Efficacy

The above studies show that physiologic levels of TNF are stimulatory to the growth of certain types of cancer,

and suggest that agents which interfere with TNF or with TNF signaling, may be a useful therapeutic option. Indeed, we found that an antibody which neutralizes TNF significantly inhibited the growth of established neu/erbB2-overexpressing mammary tumors. Although this effect was relatively modest, it is likely that more frequent dosing (e.g., more than the once per week regimen we used here), or a higher dose, would have a greater effect. To our knowledge, only one other group has examined the effect of TNF neutralization on the growth of mammary tumors. In that study, anti-TNF treatment was initiated 1 day prior to transplantation of 410.4 mouse mammary tumor cells, and continued on a weekly basis (31). Tumor growth was significantly inhibited in this model, although it should be pointed out that because treatment was started prior to tumor transplantation, it is possible that neutralization of TNF could have inhibited tumor take, as well as tumor growth. In contrast, our study used a strictly therapeutic approach, in which treatment began only after a tumor was present, such as would be the case in the clinic.

Neutralization of TNF during 12-*O*-tetradecanoylphorbol-13-acetate promotion was shown to inhibit skin papilloma development in DMBA-treated mice (31), suggesting a role for endogenous TNF in this model of skin carcinogenesis. Interestingly, the TNF antibody was equally effective whether it was given once weekly for only the first 6 weeks of promotion, or whether it was given for the full 15 weeks, implying that TNF is involved in the early stages of tumor promotion. Additionally, a recent report showed that the TNF antagonist, etanercept, a soluble TNFR2 fusion protein, inhibited colitis-associated colon tumorigenesis when administered after completion of the tumor induction protocol, suggesting that it may have partially reversed tumorigenesis at a time when tumors were already present (38). Our own data suggest a promotion role for TNF both during neu/erbB2-driven mammary tumorigenesis, as well as during the growth of established mammary cancers. The mechanism by which the promotion occurs remains to be determined. Although it is clear in the current study that TNF can stimulate the proliferation of the preneoplastic neu-erbB2-overexpressing mammary epithelium, neither the number of neu/erbB2-driven hyperplasias nor the proliferative rate of the tumors per se were different between the TNF+/+ and TNF-/- mice. A possible explanation for this observation is that TNF may stimulate vasculogenesis, as shown by Kulbe et al. (36) and Li et al. (41) in other models, thus providing the hyperplasias and tumors with an environment more favorable for expansion. Furthermore, in the absence of TNF, or in the presence of the TNF-neutralizing antibody, the vasculature may lose this positive stimulus, and thus, tumor growth is slowed, as we show here.

TNF Inhibitors Are in Use Clinically

Currently, there are three TNF inhibitors in routine clinical use, with more in development (42). These include the TNF-neutralizing antibodies infliximab (Remicade) and adalimumab (Humira), and the TNFR2 fusion protein etanercept (Enbrel), which are used in the treatment of

inflammatory diseases including rheumatoid and psoriatic arthritis; infliximab and adalimumab are also effective in treating Crohn's disease (42). Preliminary reports have also shown that infliximab and etanercept are safe and well-tolerated in patients with advanced cancer; moreover, there was some indication of biological efficacy in these phase I/II studies, including disease stabilization (43–46). To our knowledge, only one study has examined the effect of a TNF inhibitor in patients with breast cancer. In that study, 1 out of 16 patients with metastatic breast cancer treated with etanercept had a brief period of disease stabilization (44). It is tempting to speculate that treatment might be more effective in patients with earlier disease stage. Although these first-generation drugs are not without problems (42), our data demonstrating the role of TNF in mammary tumorigenesis, as well as the efficacy of a TNF-neutralizing antibody in inhibiting the growth of established aggressive mammary tumors, suggests that TNF inhibitors may be effective in at least a subset of patients with breast cancer. Moreover, we suggest that appropriate candidates in this regard may be selected based on an elevated TNF concentration in their breast cancer and/or in the adjacent stromal and epithelial tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Correction: Tumor Necrosis Factor Deficiency Inhibits Mammary Tumorigenesis and a Tumor Necrosis Factor Neutralizing Antibody Decreases Mammary Tumor Growth in neu/erbB2 Transgenic Mice

In this article (Mol Cancer Ther 2009;8:2655–63), which was published in the September 1, 2009 issue of *Molecular Cancer Therapeutics* (1), the name of the fourth author was spelled incorrectly. The correct name is Wiam Bshara. The online article has been changed to reflect this correction and no longer matches the print.

Reference

1. Warren MA, Shoemaker SF, Shealy DJ, Bshara W, Ip MM. Tumor necrosis factor deficiency inhibits mammary tumorigenesis and a tumor necrosis factor neutralizing antibody decreases mammary tumor growth in neu/erbB2 transgenic mice. Mol Cancer Ther 2009;8:2655–63.

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Tumor necrosis factor deficiency inhibits mammary tumorigenesis and a tumor necrosis factor neutralizing antibody decreases mammary tumor growth in neu/erbB2 transgenic mice

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