

# Anti-Transforming Growth Factor (TGF)- $\beta$ Antibodies Inhibit Breast Cancer Cell Tumorigenicity and Increase Mouse Spleen Natural Killer Cell Activity

## Implications for a Possible Role of Tumor Cell/Host TGF- $\beta$ Interactions in Human Breast Cancer Progression

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### Abstract

TGF- $\beta$  effects on angiogenesis, stroma formation, and immune function suggest its possible involvement in tumor progression. This hypothesis was tested using the 2G7 IgG2b, which neutralizes TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3, and the MDA-231 human breast cancer cell line. Inoculation of these cells in athymic mice decreases mouse spleen natural killer (NK) cell activity. Intraperitoneal injections of 2G7 starting 1 d after intraperitoneal inoculation of tumor cells suppressed intraabdominal tumor and lung metastases, whereas the nonneutralizing anti-TGF- $\beta$  12H5 IgG2a had no effect. 2G7 transiently inhibited growth of established MDA-231 subcutaneous tumors. Histologically, both 2G7-treated and control tumors were identical. Intraperitoneal administration of 2G7 resulted in a marked increase in mouse spleen NK cell activity. 2G7 did not inhibit MDA-231 primary tumor or metastases formation, nor did it stimulate NK cell-mediated cytotoxicity in beige NK-deficient nude mice. Finally, serum-free conditioned medium from MDA-231 cells inhibited the NK cell activity of human blood lymphocytes. This inhibition was blocked by the neutralizing anti-TGF- $\beta$  2G7 antibody but not by a nonspecific IgG2. These data support a possible role for tumor cell TGF- $\beta$  in the progression of mammary carcinomas by suppressing host immune surveillance. (*J. Clin. Invest.* 1993. 92:2569–2576.) **Key words:** breast neoplasms • transforming growth factor- $\beta$  • nude mice • immunologic surveillance • natural killer activity

### Introduction

The TGF- $\beta$ s comprise a family of several multifunctional structurally related polypeptides first identified for their ability to induce anchorage-independent growth of nontransformed fibroblasts (1, 2). The multiplicity of TGF- $\beta$  actions in nearly all cell types (3) suggests that this molecule has a pivotal role in several physiological and pathological processes. The escape of

many tumor cells from TGF- $\beta$ -mediated growth inhibition as well as the ability of this polypeptide to suppress the immune system, to induce angiogenesis, and to promote the formation of stroma have suggested a possible role of TGF- $\beta$  on the maintenance and progression of transformed cells in an intact host (4).

Expression of TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 mRNAs has been detected in human breast cancer cells in culture (5, 6). Exogenous TGF- $\beta$ 1 and - $\beta$ 2 inhibit the growth of most breast cancer cell lines in vitro (7–9). Furthermore, anti-TGF- $\beta$ 1 and - $\beta$ 2 neutralizing antisera stimulated the proliferation of hormone-independent human breast cancer cell lines and downregulated basal TGF- $\beta$ 1 mRNA levels (10), supporting a functional TGF- $\beta$ -mediated negative autocrine pathway in breast cancer cell culture systems. However, the biological and clinical implications of these in vitro data are unclear. For example, exogenous TGF- $\beta$ 1 fails to inhibit the growth of (in vitro sensitive) human breast cancer cells in athymic mice (11). Moreover, higher levels of TGF- $\beta$ 1 expression have been associated with states of enhanced breast cancer cell tumorigenesis or with a more aggressive breast cancer phenotype (12–19).

A causal association between higher levels of TGF- $\beta$ 1 expression and enhanced tumorigenicity of human breast cancer cells is supported by a recent report in which MCF-7 cells transfected with a TGF- $\beta$ 1 expression vector escaped from hormone dependence when inoculated in castrated athymic mice (20). Tumor formation by the MCF-7/TGF- $\beta$ 1 transfectants was blocked by the anti-TGF- $\beta$  2G7 monoclonal antibody that neutralizes TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 (21). Moreover, administration of human recombinant TGF- $\beta$ 1 transiently supported estrogen-independent growth of parental MCF-7 cells in castrated nude mice (20). To follow this recent observation, we now report a series of experiments using the naturally transformed MDA-231 human breast cancer cell line and anti-TGF- $\beta$  neutralizing antibodies in athymic mice. Our results show a clear dissociation between the in vitro and the animal model data but continue to support a causal association between TGF- $\beta$  and the progression of human breast carcinoma cells in vivo.

### Methods

**Cell line and antibodies.** The MDA-231 human breast cancer cell line was purchased from the American Type Culture Collection (Rockville, MD) and maintained in improved minimum essential medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (Hazelton, Lenexa, KS). These cells express high levels of TGF- $\beta$ 1 and - $\beta$ 2 (5, 6), and are inhibited by picomolar concentrations of TGF- $\beta$ 1 and - $\beta$ 2 in vitro (7–9). The 2G7 IgG2b and the 12H5 IgG2a were raised against human recombinant TGF- $\beta$ 1 and have been characterized previously (21). Both immunoprecipitated <sup>125</sup>I-TGF- $\beta$ 1 but only 2G7 did so with labeled TGF- $\beta$ 2 (21). 2G7 neutralized the growth inhibitory

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activity of TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3 on mink lung Mv1Lu epithelial cells but 12H5 was devoid of neutralizing activity (21).

**DNA synthesis.**  $4 \times 10^4$  MDA-231 cells/well were plated in 24-well plates and treated the following day with human recombinant TGF- $\beta$ 1 for 18 h. In some cases TGF- $\beta$ 1 was preincubated overnight at 4°C with different concentrations of 2G7 or a nonspecific IgG2 (Sigma Chemical Co., St. Louis, MO) before addition to the cells. Monolayers were then labeled with 0.5  $\mu$ Ci/ml [ $^3$ H]thymidine (82.2 Ci/mmol; NEN Products, Boston, MA) for 2 h, and the rate of DNA synthesis was estimated by measuring acid-precipitable radioactivity as described previously (8).

**Anchorage-independent growth assay.** A 1-ml top layer containing a single-cell suspension of  $10^4$  cells, 0.8 agarose (Sea-Plaque; FMC Corp. BioProducts, Rockland, ME), IMEM, 10% calf serum (CS; Hazleton), and 10 mM HEPES, with or without different concentrations of 2G7, was added to a 1-ml bottom layer of 0.8% agarose/10% CS in 35-mm dishes. Dishes were incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C, and colonies measuring  $\geq 50 \mu$ m were counted after 10–14 d using an Omnicon feature analysis stem model II image analyzer (Bausch & Lomb, Rochester, NY).

**Collection of conditioned medium (CM).**<sup>1</sup> MDA-231 cells were plated in 100-mm tissue culture dishes in growth medium and, when 50–75% confluent, washed twice with PBS and then changed to 7 ml of serum-free IMEM. After 24 h, the CM was collected, supplemented with pepstatin, aprotinin, and leupeptin (1  $\mu$ g/ml each), centrifuged at 3,000 rpm to remove cellular debris, and tested the same day on a human lymphocyte NK cytotoxicity assay (below).

**Experiments in athymic mice.** 3–4-wk-old female athymic mice (Harlan Sprague Dawley, Madison, WI) or 5–9-wk-old NIH-3 nude beige NK-deficient female mice (Taconic, Germantown, NY) were inoculated intraperitoneally or subcutaneously in the flank just caudal to the forelimb with  $5 \times 10^6$  exponentially growing MDA-231 cells. Mice were then injected serially intraperitoneally at the indicated intervals with the monoclonal antibodies 12H5 or 2G7 or PBS in a 0.5-ml volume using a 26-gauge needle. At the completion of the experiment, mice were killed by cervical dislocation. In those mice inoculated with tumor cells intraperitoneally, multiple specimens from different sites in the abdominal cavity and the lungs were collected, fixed in 10% formalin, paraffin embedded, stained with hematoxylin and eosin, and evaluated by light microscopy (M. D. Johnson and C. L. Arteaga). At least three histological sections were prepared from each lung, two from opposite sides and one from the middle of each paraffin block. Subcutaneous tumors were removed and processed similarly to the intraperitoneal tumors. Tumor diameters were measured with calipers and tumor volume calculated by the formula:

$$\text{volume} = \text{width}^2 \times \text{length} / 2.$$

**Factor VIII and collagen immunohistochemistry.** For in situ detection of factor VIII, 5- $\mu$ m-thick sections from formalin-fixed, paraffin-embedded MDA-231 subcutaneous xenografts were predigested with 0.1% trypsin/0.1% CaCl<sub>2</sub>. This was followed by a peroxidase-antiperoxidase (PAP) histochemical procedure using a 1:200 dilution of the anti-human von Willebrand factor purified Ig fraction of rabbit antiserum (Dakopatts, Glostrup, Denmark) as previously described (22). Nonimmune rabbit Ig fraction was used as control. For detection of collagen in the MDA-231 xenografts, similar sections were analyzed using a Masson's Trichrome histochemical stain procedure (23).

**Mouse spleen NK cell cytotoxicity assay.** Whole mouse spleens were aseptically removed and forced through a sterile stainless mesh to obtain a single-cell suspension. The Yac-1 mouse lymphoma cell line was used as target cells in the cytotoxicity assay.  $10^7$  Yac-1 cells in suspension culture were labeled with 200  $\mu$ Ci/ml of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham Corp., Arlington Heights, IL) for 60 min at 37°C as described (24). Unbound radioactivity was removed after two washes. Live effector spleen cells and target cells (in ratios of 10:1, 50:1, and 100:1) were added to microtiter wells in a final volume of 0.2 ml/well in quadrupli-

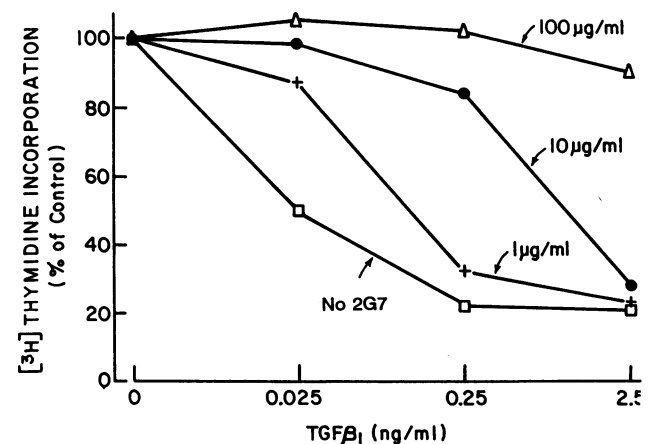
cate. Wells containing target cells alone served as controls for spontaneous <sup>51</sup>Cr release (SR), and 0.1 ml of labeled target cells was counted by  $\gamma$  scintigraphy to determine total potential release (TR) of radioactivity. The plates were incubated in a humidified 5% CO<sub>2</sub> incubator for 4 h at 37°C. After centrifugation at 750 g for 10 min at room temperature, 0.1-ml aliquots of the supernatants (SN) were removed for determination of radioactivity. Percent cytotoxicity was calculated from the formula:

$$100 \times \frac{[(\text{cpm in SN} \times 2) - (\text{SR} \times 2)]}{[\text{TR} - (\text{SR} \times 2)]}$$

**Human peripheral blood lymphocyte NK cytotoxicity assay.** Single donor peripheral blood lymphocytes were separated by Ficoll-Hypaque discontinuous gradient centrifugation, washed twice, and treated like mouse splenocytes to obtain a single-cell suspension. Cell suspensions were then treated in a 5% CO<sub>2</sub> incubator for 18 h at 37°C with different volumes of serum-free medium conditioned by MDA-231 cells. Human K-562 erythroleukemia cells were labeled with <sup>51</sup>Cr similar to the Yac-1 mouse lymphoma cells (above) and used as target in the cytotoxicity assay. After two washes, adjusted equal concentrations of live effector cells (lymphocytes) were added to <sup>51</sup>Cr-labeled K-562 cells for 4 h at 37°C. Lymphocyte-mediated NK activity was estimated as radioisotope release from target cells as described above.

## Results

**Neutralizing anti-TGF- $\beta$  antibodies stimulate growth of MDA-231 breast cancer cells in vitro.** Initially, we tested whether the 2G7 IgG2b blocked the inhibitory effect of exogenous TGF- $\beta$ 1 on sensitive MDA-231 cells. Picomolar concentrations of human recombinant TGF- $\beta$ 1 inhibited DNA synthesis in MDA-231 cells. Preincubation with 2G7 but not with a nonspecific IgG2 blocked this inhibition with total abrogation of the TGF- $\beta$ 1 inhibitory effect at 100  $\mu$ g/ml (Fig. 1). 2G7 by itself did not change the basal rate of [ $^3$ H]thymidine incorporation in these cells. Before the experiments in nude mice we examined the ability of anti-TGF- $\beta$  antibodies on basal growth of MDA-231 cells. Similar to previously reported anti-TGF- $\beta$  polyclonal antibodies (10), the 2G7 monoclonal induced a marked increase



**Figure 1.** Neutralization of TGF- $\beta$ 1-induced inhibition of DNA synthesis in MDA-231 cells with anti-TGF- $\beta$  antibodies. [ $^3$ H]Thymidine incorporation in MDA-231 cells was measured as described in Methods 18 h after the addition of human recombinant TGF- $\beta$ 1. TGF- $\beta$ 1 was preincubated overnight with different concentrations of 2G7 or 10  $\mu$ g/ml of a nonspecific IgG2 (No 2G7) before addition to the cell monolayers. Each data point represents the mean of triplicate wells. All SE were < 10%.

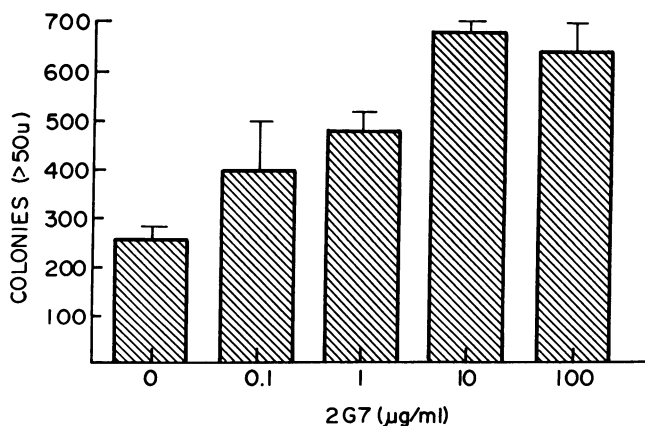
1. Abbreviation used in this paper: CM, conditioned medium.

in MDA-231 colony formation in a dose-dependent fashion (Fig. 2). Similar results were obtained in a monolayer growth assay in serum-free medium (not shown).

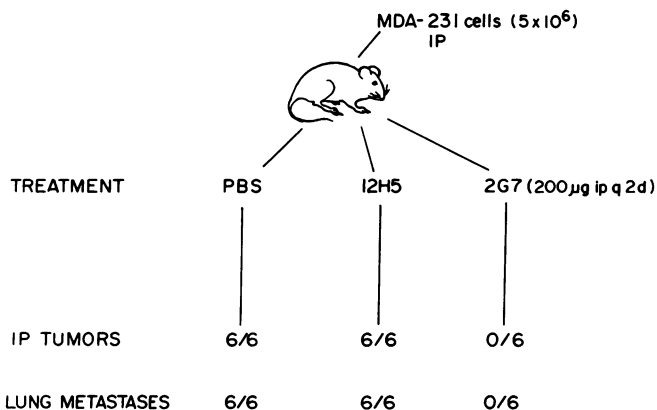
*Antibody-mediated TGF- $\beta$  blockade inhibits growth of human breast cancer cells in athymic mice.* For these experiments we inoculated the highly tumorigenic MDA-231 cells intraperitoneally. When injected intraperitoneally, this cell line rapidly forms extensive intraabdominal tumors and readily metastasizes to the lungs (C. L. Arteaga and C. K. Osborne, unpublished data). This site of injection was chosen initially for two reasons: first, to assure a high local concentration of anti-TGF- $\beta$  antibodies (also injected intraperitoneally) in the tumor cell environment; and second, to anticipate a possible differential effect of the antibodies on tumors at the injection site vs. metastatic tumors. Every-other-day intraperitoneal injections of a 200- $\mu$ g dose of 2G7 started 1 d after intraperitoneal inoculation of tumor cells suppressed the development of MDA-231 intraabdominal tumor and detectable lung metastases. We arbitrarily chose this initial dose of antibody because in preliminary experiments plasma from animals treated with 200  $\mu$ g 2G7 i.p. 48 h before blood collection inhibited binding of  $^{125}$ I-TGF- $\beta$ 2 to AKR-2B mouse fibroblasts (not shown). All animals treated with the nonneutralizing anti-TGF- $\beta$  12H5 IgG2a or PBS exhibited extensive omental seeding by tumor cells and metastases in both lungs at 3 wk (Figs. 3 and 4).

To confirm this result we performed a second experiment with different doses of 2G7 given every 3 d. As shown in Table I and Fig. 4, all mice treated with PBS or the lowest dose of 2G7 developed intraabdominal tumor and all but one exhibited large hematogenous lung metastases. Intravascular foci of tumor cells were identified in the lungs of one of four mice treated with 20  $\mu$ g of 2G7 every 3 d (Fig. 4, *third panel*). At the highest dose of antibody, only one of four mice developed a small focus of tumor cells in the abdominal wall and none exhibited histologically identifiable lung metastases at 3 wk.

We next studied the effect of TGF- $\beta$  blockade with systemic administration of 2G7 on established subcutaneous MDA-231 tumors. Treatment was started when subcutaneous tumors had reached a volume of  $\sim 100$  mm<sup>3</sup> (Fig. 5, day 8). Serial injections



**Figure 2.** Effect of 2G7 on MDA-231 colony formation. A single-cell suspension of  $10^4$  MDA-231 cells was plated in 0.8% agarose/10% calf serum/10 mM Hepes in the absence or presence of different concentrations of the 2G7 IgG2 as described in Methods. Colonies measuring  $\geq 50$   $\mu$ m were counted 10 d later with an automated image analyzer. Each bar represents the mean  $\pm$  SE of three dishes.

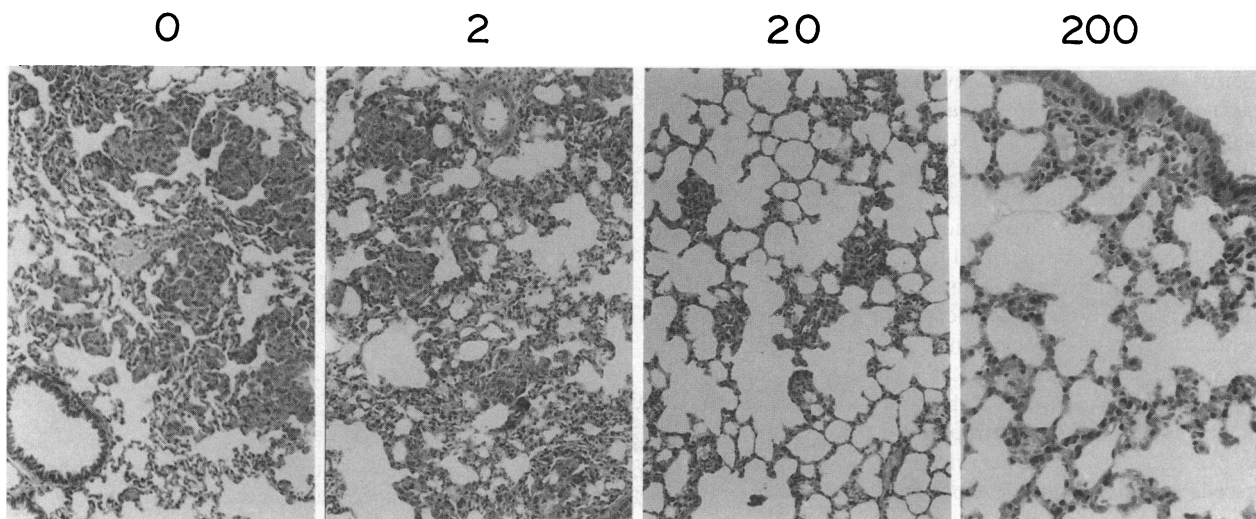


**Figure 3.** Neutralizing anti-TGF- $\beta$  antibodies suppress MDA-231 intraabdominal tumor and lung metastases. MDA-231 cells were inoculated intraperitoneally in female nude mice. The following day, every-other-day intraperitoneal injections of PBS or 200  $\mu$ g of the 2G7 or 12H5 monoclonal antibodies were started. Six mice were in each treatment group. After 10 doses (3 wk) mice were killed. The contents of the abdominal cavity and both lungs were examined macroscopically and microscopically for tumor formation as described in Methods.

tions of 2G7 at a distant site (intraperitoneally) every 3 d from days 8 to 20 transiently inhibited MDA-231 tumor growth compared with the nonneutralizing TGF- $\beta$  monoclonal 12H5 and PBS (Fig. 5,  $*P < 0.01$  on days 14 and 17, Student's *t* test). Two tumors from each of the three treatment groups were harvested on day 20 and processed for light microscopy as well as factor VIII and Masson's Trichrome stains. All tumors were moderately or poorly differentiated adenocarcinomas. Endothelial cells (identified by factor VIII immunostaining), presence of extracellular matrix and collagen (using Masson's Trichrome stain), and infiltration with mouse lymphocytes or other stromal cells appeared scarce in all tumors (data not shown). 2G7-treated tumors were histologically and immunohistochemically indistinguishable from 12H5- and PBS-treated tumors.

*2G7 upregulates mouse spleen NK activity and lacks an antitumor effect in NK-deficient nude mice.* Once we failed to recognize any differences in the degree of tumor angiogenic activity or tumor stroma induced by TGF- $\beta$  blockade, we examined the effect of 2G7 on mouse immune function, which, in turn, may explain the potent antitumor effect. Since T cell function is deficient while NK cell function is normal in athymic mice (25) and because of the reported effects of TGF- $\beta$  on NK cell activity (26), we focused on the latter. Three daily injections of 200  $\mu$ g of 2G7 induced a marked increase in mouse spleen NK activity compared with PBS or to a nonspecific control IgG2 in tumor-free athymic mice (Fig. 6). Furthermore, the sole presence of subcutaneous MDA-231 tumors inhibited basal mouse spleen NK activity 10 d after tumor cell inoculation (Fig. 6). Daily intraperitoneal injections of 2G7 on mice bearing subcutaneous MDA-231 tumors on days 7, 8, and 9 after tumor cell inoculation did not change the tumor-induced inhibition of NK activity (not shown).

We next tested whether coadministration of exogenous TGF- $\beta$ 1 could reverse the 2G7-induced stimulation of NK activity. In this second experiment, spleen NK activity was greater than sixfold higher in mice treated with 2G7 than in



**Figure 4.** Dose-dependent inhibition of MDA-231 lung metastases by anti-TGF- $\beta$  antibodies. 1 d after intraperitoneal inoculation of MDA-231 cells into nude mice, every-3-d doses of 2G7 (2, 20, and 200  $\mu$ g) or PBS were started and continued for 3 wk as described in Methods and Table I. After death of the animals, three histological sections were prepared from each lung and examined for the presence of tumor cells. Mice treated with PBS and the low dose of antibody exhibited dense infiltration of their lung parenchyma by large MDA-231 metastases. Small metastases were detected in one of four of the mice treated with 20  $\mu$ g of 2G7 every 3 d ( $\times 125$ ).

those treated with the nonneutralizing 12H5 control IgG2a (Fig. 7). Simultaneous administration of human recombinant TGF- $\beta$ 1 partially blocked 2G7-induced upregulation of NK cell activity while it did not affect those levels measured in 12H5-treated mice (Fig. 7).

Finally, we proposed that if the stimulation of NK activity were critical for the antitumor effect mediated by the neutralizing anti-TGF- $\beta$  monoclonal, the latter would not exert an antitumor effect in an NK-deficient host. A similar experiment to the one shown in Fig. 3 was performed in beige NK cell-deficient athymic mice. In a preliminary experiment, all six NK<sup>-</sup> animals rapidly developed intraabdominal tumors and lung metastases after an intraperitoneal inoculum of  $5 \times 10^6$  MDA-231 cells. In a second experiment 12 NK<sup>-</sup> mice were treated with either 2G7 or 12H5 (200  $\mu$ g i.p. every 2 d for eight doses;  $n = 6$  per group) starting 1 d after tumor cell inoculation. All 12 mice treated either with 2G7 or 12H5 developed extensive intraperitoneal tumors and large metastatic foci in the lungs. In contrast to the results with NK<sup>+</sup> athymic mice (Figs. 6 and 7), spleen NK cell activity against <sup>51</sup>Cr-labeled Yac-1 lymphoma

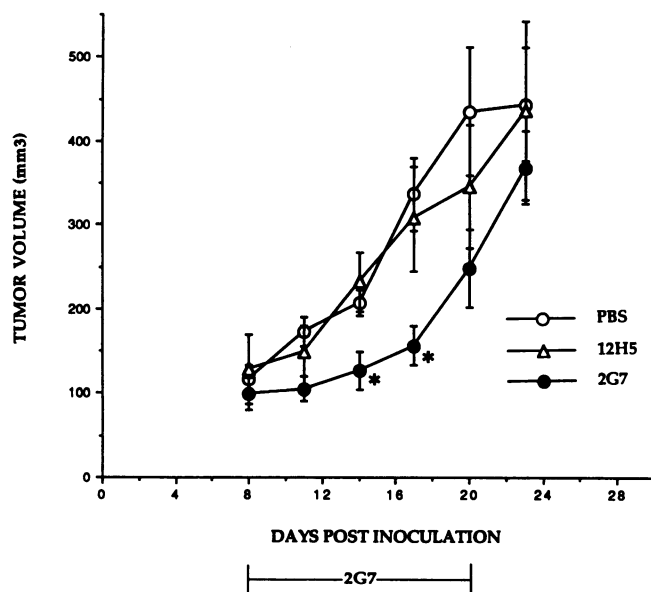
cells from both tumor-free and tumor-bearing beige NK<sup>-</sup> mice treated with either 2G7 or 12H5 was undetectable.

*MDA-231 cell-CM contains TGF- $\beta$  activity that inhibits human lymphocyte-mediated NK cell activity.* To follow the observed inhibition of mouse spleen NK cell activity induced

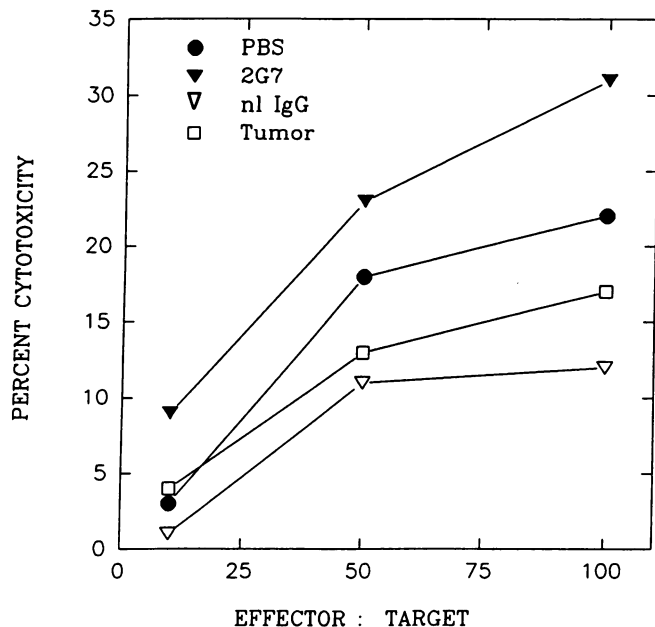
**Table I.** Dose-dependent Inhibition of MDA-231 Intraabdominal Tumor and Metastases Formation by Anti-TGF- $\beta$  Antibodies

Treatment	Primary tumor	Lung metastases
PBS	4/4	4/4
2 $\mu$ g 2G7	4/4	3/4
20 $\mu$ g 2G7	2/4	1/4
200 $\mu$ g 2G7	1/4	0/4

MDA-231 cells were inoculated intraperitoneally into female athymic mice. The following day serial intraperitoneal injections of PBS or 2G7 were initiated and continued every 3 d for a total of 3 wk ( $n = 4$  per group). At 3 wk, mice were killed and their abdominal contents and both lungs examined macro and microscopically for the presence of gross and/or microscopic tumor as described in Methods.

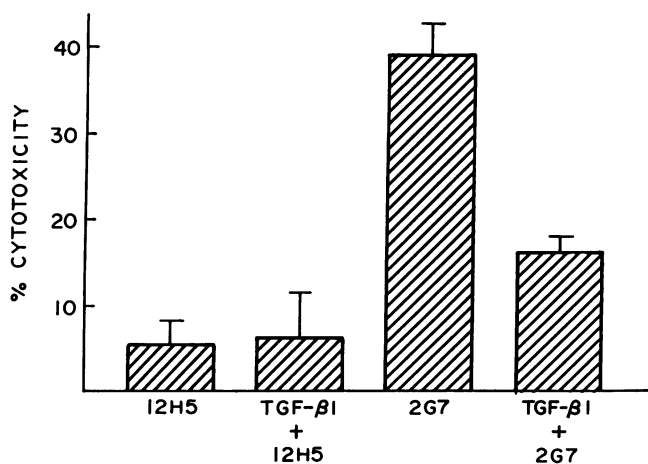


**Figure 5.** Neutralizing anti-TGF- $\beta$  antibodies transiently inhibit growth of established MDA-231 subcutaneous tumors. Nude mice were inoculated subcutaneously with MDA-231 cells as described in Methods. After 8 d all mice had tumors with an approximate volume of 100 mm<sup>3</sup>. Intraperitoneal administration of 200  $\mu$ g of 2G7 every 3 d from days 8 to 20 transiently inhibited MDA-231 tumor growth compared with the nonneutralizing TGF- $\beta$  antibody 12H5 and PBS ( $*P < 0.01$  on days 14 and 17, Student's  $t$  test). Each data point represents the mean  $\pm$  SE of six mice.



**Figure 6.** Effect of 2G7 and of MDA-231 tumors on mouse spleen NK cell activity. 5-wk-old female nude mice were inoculated subcutaneously with  $5 \times 10^6$  MDA-231 cells and tumors allowed to form for 10 d. In other cases, tumor-free mice were treated intraperitoneally three times daily with 200  $\mu$ g of 2G7 or a nonspecific IgG2 (Sigma Chemical Co.) or PBS. 1 d after the last injection or 10 d after subcutaneous tumor cell inoculation, mouse spleens ( $n = 3$  per group) were removed and tested in an NK cell cytotoxicity assay against syngeneic Yac-1 lymphoma cells as described in Methods. Mice injected with 2G7, normal IgG2, or PBS were tumor free.

by MDA-231 tumors (Fig. 6), we tested the effect of secreted factors by these cells on the NK activity mediated by human effector cells. For this purpose we collected serum-free CM



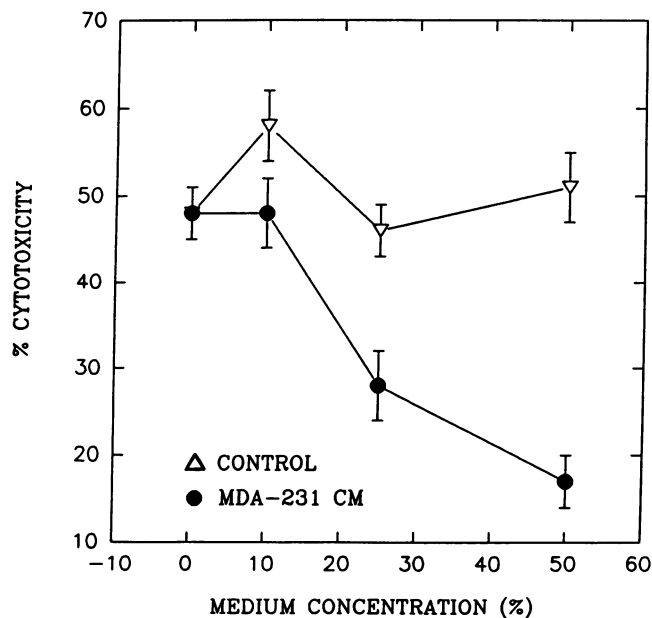
**Figure 7.** Exogenous TGF- $\beta$ 1 abrogates 2G7-induced upregulation of mouse spleen NK cell activity. 5-wk-old female nude mice were treated intraperitoneally three times daily with 200  $\mu$ g of 2G7 or 12H5. In some cases, the antibodies were administered simultaneously with 1  $\mu$ g of human recombinant TGF- $\beta$ 1. Two mice were in each treatment group. On day 4, spleens were removed and splenocyte NK cell activity was measured as described in Methods using syngeneic  $^{51}\text{Cr}$ -labeled Yac-1 lymphoma cells as target.

from MDA-231 cells and added it to human peripheral blood lymphocytes from a healthy donor. CM but not control medium induced a dose-dependent decrease in lymphocyte-mediated NK activity against syngeneic K-562 erythroleukemia cells (Fig. 8). Transient acidification followed by neutralization of the CM before addition to the effector lymphocytes enhanced the inhibitory effect on NK activity > 20-fold (not shown). This change induced by acidification was consistent with the possibility that the NK inhibitory activity present in the CM was mediated by TGF- $\beta$ .

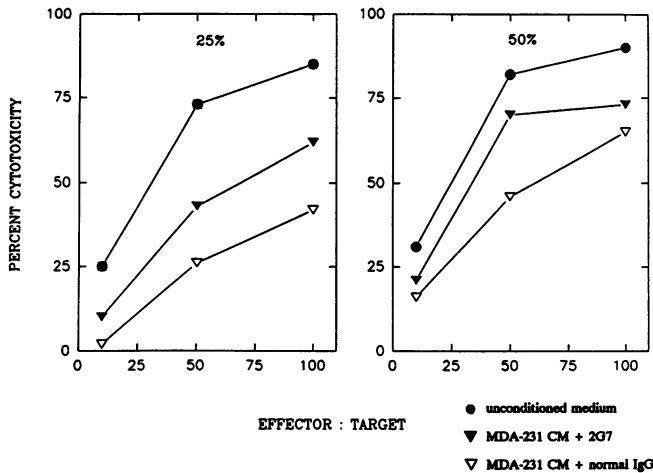
To confirm this latter possibility, we incubated the CM with the neutralizing anti-TGF- $\beta$  2G7 antibody before addition to the human lymphocytes. 2G7 but not a nonspecific IgG2 partially blocked the CM-induced inhibition of lymphocyte-mediated NK activity (Fig. 9). This result was consistent in both concentrations of CM tested (25 and 50%) and in all three effector/target cell ratios examined (Fig. 9).

## Discussion

We have presented a series of experiments that address the biological role of TGF- $\beta$  in the tumorigenicity of human breast cancer cells. Antibody-induced blockade of all three mammalian TGF- $\beta$  isoforms with the monoclonal antibody 2G7 inhibited MDA-231 breast cancer cells in athymic mice. Similar to a previous report (24), the establishment of tumors in the animals downregulated mouse spleen NK cell activity. However, administration of the neutralizing TGF- $\beta$  antibody enhanced this activity in tumor-free animals. 2G7-mediated tumor cell inhibition was not seen in NK-deficient nude mice. Taken to-



**Figure 8.** MDA-231 cell serum-free CM inhibits human lymphocyte-mediated NK cell activity. Human peripheral blood lymphocytes were incubated for 18 h at 37°C in a 5% CO<sub>2</sub> incubator with different concentrations of unconditioned (control) or conditioned medium from MDA-231 cells. Lymphocytes were then washed and tested in an NK cell cytotoxicity assay using syngeneic  $^{51}\text{Cr}$ -labeled K-562 erythroleukemia cells as target as described in Methods. Results using a 50:1 effector/target cell ratio are shown.

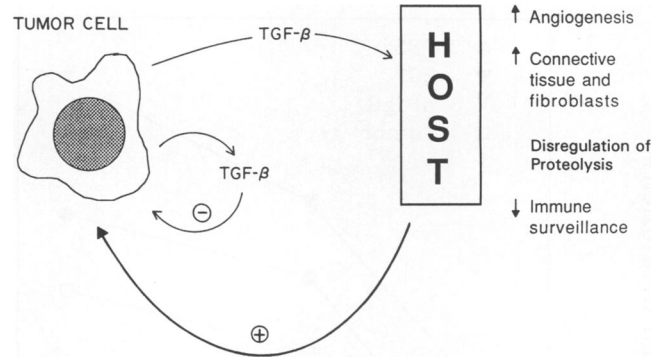


**Figure 9.** Neutralizing anti-TGF- $\beta$  antibodies partially block MDA-231 cell CM-induced inhibition of lymphocyte-mediated NK activity. Serum-free CM from MDA-231 human breast cancer cells was preincubated for 6 h at 4°C with 10  $\mu$ g/ml of 2G7 or a nonspecific IgG2. Control or conditioned medium, in concentrations of 25 and 50%, was then added to peripheral blood lymphocytes for 18 h as described above. After this incubation, lymphocyte-mediated NK activity against target K-562 erythroleukemia cells was measured as described in Methods.

gether these data support the possibility that an interaction between tumor cell TGF- $\beta$  with endogenous host TGF- $\beta$  is involved in the progression of human breast carcinoma cells in vivo.

The MDA-231 breast cancer cells used in this study are inhibited by exogenous TGF- $\beta$ 1 and - $\beta$ 2 (7–9). Although the majority of the TGF- $\beta$  activity secreted by these cells is in a “latent” form, it is likely that a TGF- $\beta$ -negative autocrine circuit is operative in them since polyclonal antibodies that neutralize mature TGF- $\beta$ 1 and - $\beta$ 2 stimulate their proliferation (10). Similar to these antisera, the 2G7 anti-TGF- $\beta$  IgG2b used in this study also stimulated MDA-231 cell growth in culture (Fig. 2). However, in the intact animal, 2G7 inhibited MDA-231 tumor formation. This result does not discard in any way an operational role for tumor cell TGF- $\beta$  on autocrine growth control since transient growth stimulation of MDA-231 cells could still have occurred with 2G7 in the animals. However, since in the end tumor formation was inhibited rather than stimulated, our data would suggest the possibility that the net effect of tumor cell TGF- $\beta$ 's interaction with the host is one of maintenance or progression of the transformed cells, thus subverting or masking autocrine growth inhibition (Fig. 10).

This experimental result underscores the potential lack of predictability of some in vitro tumor models, especially when studying multifunctional molecules with effects on many cell types. Nevertheless, the MDA-231 tumor inhibition by 2G7 concurs with several reports of an association between higher levels of TGF- $\beta$  expression and a more tumorigenic breast cancer phenotype. For example, MCF-7 cells transfected with v-Ha-ras escape estrogen dependence and secrete greater than fivefold TGF- $\beta$  activity compared with parental cells (12, 13). We recently reported that transfection and overexpression of a mouse TGF- $\beta$ 1 cDNA in MCF-7 tumors abrogated their estrogen dependence in athymic mice (20). In the same study, exogenous TGF- $\beta$ 1 supported wild-type MCF-7 tumor formation in



**Figure 10.** Schematic illustration of the proposed net effect of TGF- $\beta$  on tumor cells. Autocrine growth inhibition (–) is contravened by positive signals generated by host cells in response to tumor cell TGF- $\beta$  (+).

the absence of estradiol (20). Loss of estrogen sensitivity in T47D cells is associated with acquired sensitivity to stimulation with TGF- $\beta$ 1 and with a > 100-fold increase in TGF- $\beta$ 1 mRNA levels (14). TGF- $\beta$ 1 mRNA transcripts are more abundant in tumor than in nontumor mammary tissues (15), and 78% of estrogen receptor/progesterone receptor (ER/PgR)-negative but only 13% of ER/PgR-positive tumors exhibited a high level of immunoreactive TGF- $\beta$ 1 protein (16). TGF- $\beta$ 1 increases the proteolytic activity and metastatic potential of mammary tumor cells (17). In another study, TGF- $\beta$ 1 immunoreactivity was greater in invasive than in in situ ductal mammary carcinomas and was associated with higher tumor stage (18). Finally, Thompson et al. (19) reported a study in which breast tumors unresponsive to antiestrogen therapy expressed higher levels of TGF- $\beta$ 1 mRNA than responsive tumors.

As illustrated on Fig. 10, there are several possible mechanisms by which TGF- $\beta$  may indirectly contribute to the establishment and progression of tumor cells in an intact host. These include the induction of angiogenesis (27), the formation of connective tissue and deregulation of pericellular proteolysis (27–29), the recruitment of stromal cells (29, 30), and the suppression of several elements of the host's immune response (26, 31–33). Arrick et al. (34) recently confirmed some of these by transfecting human E1A-transformed 293 tumor cells with a human TGF- $\beta$ 1 cDNA encoding latent TGF- $\beta$ 1. The transfected cells were more tumorigenic than the parental cells in nude mice. In addition, they expressed higher levels of fibrinogen, plasminogen activator, and several  $\beta$ 1 integrin mRNAs, and displayed increased adhesiveness in vitro (34).

In our study, light microscopy and immunohistochemical studies did not reveal any obvious differences in angiogenic activity, amount of pericellular matrix, or infiltration with lymphocytes or other stromal cells between 2G7-treated subcutaneous MDA-231 tumors and control tumors. However, the inhibition of mouse spleen NK activity in mice bearing MDA-231 tumors, the inhibition of lymphocyte-mediated NK function by MDA-231 CM and its reversal by 2G7, the increase in NK cell activity in animals treated with the tumor inhibitory anti-TGF- $\beta$  IgG, and the lack of an antibody-induced antitumor effect in NK-deficient mice all provided circumstantial evidence of the following. First, that in our animal model tumor cell TGF- $\beta$  may contribute to the tumorigenic process by suppressing mechanisms of immune surveillance; and second,

that in an intact animal, the endogenously produced TGF- $\beta$  regulates immune function in an autocrine fashion. Since 2G7 was probably blocking the autocrine/paracrine effects of TGF- $\beta$  on both tumor and host cells, it is difficult to dissect the contribution of each (or all) of these blocks to the net effect of tumor inhibition. Whether upregulation of NK function by blocking endogenous TGF- $\beta$  (with antibodies or other inhibitors) would be a practical approach to prevent or inhibit tumor progression, and whether this would only be effective against tumors expressing high levels of immunosuppressive cytokines, are speculations that require further testing.

Other genetically manipulated cell systems also support an association between tumor cell TGF- $\beta$  and immune suppression. UV-induced fibrosarcoma cells transfected with a mouse TGF- $\beta$ 1 cDNA exhibited enhanced tumorigenicity in nude mice and, different from the parental cells, were unable to induce cytotoxic mouse T lymphocyte responses (35). CHO cells transfected with a TGF- $\beta$ 1 expression vector encoding for latent TGF- $\beta$ 1 decreased NK cell activity and rapidly formed tumors in nude mice (36). Furthermore, MATLyLu rat prostate cancer cells (37) and MCF-7 human breast carcinoma cells (20) became more tumorigenic or metastatic after transfection with a mouse TGF- $\beta$ 1 expression vector. Of note, some of these cells were sensitive to TGF- $\beta$  inhibition in vitro (37) but still behaved more tumorigenic in the animal after transfection. Wu et al. (38) reported the opposite result in that FET human colon cancer cells transfected with an antisense expression vector for TGF- $\beta$ 1 escaped TGF- $\beta$ -mediated autocrine growth control and uniformly formed tumors in nude mice. In the last three studies, however, immunological functions on animals bearing TGF- $\beta$ 1-overexpressing or TGF- $\beta$ 1-antisensed tumors were not reported.

A more clinico-biological precedent to our findings is the identification of TGF- $\beta$ 2 as the glioblastoma-derived T cell suppressor factor, which explains the cellular immunodeficiency state exhibited by some patients with this common type of brain tumor (39–41). Obviously, the possible identity of the TGF- $\beta$  isoform(s) involved in MDA-231 tumor formation is beyond the scope of this report. Experiments with TGF- $\beta$ 1-specific neutralizing antibodies are currently in progress to address that question.

Because of our inability to assess the relative contribution of 2G7-induced effects on tumor cells vs. host cells to the net outcome of MDA-231 tumor growth inhibition, we examined the effect of MDA-231 cells on NK function in vitro. Concordant with the animal data, medium conditioned by the MDA-231 cells inhibited human lymphocyte-mediated NK activity, and this inhibition was reversed by the 2G7 IgG2b but not by a nonspecific IgG2 (Figs. 8 and 9). The biological implications of this observation in the context of breast carcinomas are unclear but suggest that TGF- $\beta$  secreted by tumor cells may have immunosuppressive effects in diseases other than glioblastoma. Future studies of TGF- $\beta$  isoform and receptor localization in tumor and especially in host cells along different stages of the progressive transformation of human mammary epithelial cells will shed light on some of these questions.

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## References

- Moses, H. L., E. B. Brantum, J. A. Proper, and R. A. Robinson. 1981. Transforming growth factor production by chemically transformed cells. *Cancer Res.* 41:2842–2848.
- Roberts, A. B., M. A. Anzano, L. C. Lamb, J. M. Smith, and M. B. Sporn. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc. Natl. Acad. Sci. USA.* 78:5339–5343.
- Roberts, A. B., and M. B. Sporn. 1990. The transforming growth factor- $\beta$ s. In *Handbook of Experimental Pharmacology*, vol. 95. M. B. Sporn and A. B. Roberts, editors. Springer-Verlag, Heidelberg. 419–472.
- Roberts, A. B., N. L. Thompson, U. Heine, C. Flanders, and M. B. Sporn. 1988. Transforming growth factor- $\beta$ : possible roles in carcinogenesis. *Br. J. Cancer.* 57:594–600.
- Zajchowski, D., V. Band, N. Puzie, A. Tager, M. Stampfer, and R. Sager. 1988. Expression of growth factors and oncogenes in normal and tumor-derived human mammary epithelial cells. *Cancer Res.* 48:7041–7047.
- Arrick, B. A., M. Korc, and R. Derynck. 1990. Differential regulation of expression of three transforming growth factor  $\beta$  species in human breast cancer cell lines by estradiol. *Cancer Res.* 50:299–303.
- Knabbe, C., M. E. Lippman, L. M. Wakefield, K. C. Flanders, A. Kasid, R. Derynck, and R. B. Dickson. 1987. Evidence that transforming growth factor- $\beta$  is a hormonally regulated negative growth factor in human breast cancer cells. *Cell.* 48:417–428.
- Arteaga, C. L., A. K. Tandon, D. D. Von Hoff, and C. K. Osborne. 1988. Transforming growth factor  $\beta$ : potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. *Cancer Res.* 48:3898–3904.
- Zugmaier, G., B. W. Ennis, B. Deschauer, D. Katz, C. Knabbe, G. Wilding, P. Daly, M. E. Lippman, and R. B. Dickson. 1989. Transforming growth factors type  $\beta$ 1 and  $\beta$ 2 are equipotent growth inhibitors of human breast cancer cell lines. *J. Cell. Physiol.* 141:353–361.
- Arteaga, C. L., R. J. Coffey, T. C. Dugger, C. M. McCutchen, H. L. Moses, and R. M. Lyons. 1990. Growth stimulation of human breast cancer cells with anti-transforming growth factor  $\beta$  antibodies: evidence for negative autocrine regulation by transforming growth factor  $\beta$ . *Cell Growth & Differ.* 1:367–374.
- Zugmaier, G., S. Paik, G. Wilding, C. Knabbe, M. Bano, R. Lupu, B. Deschauer, S. Simpson, R. B. Dickson, and M. Lippman. 1991. Transforming growth factor  $\beta$ 1 induces cachexia and systemic fibrosis without an antitumor effect in nude mice. *Cancer Res.* 51:3590–3594.
- Dickson, R. B., A. Kasid, K. K. Huff, S. E. Bates, C. Knabbe, D. Bronzert, E. P. Gelman, and M. E. Lippman. 1987. Activation of growth factor secretion in tumorigenic states of breast cancer induced by 17 $\beta$ -estradiol or v-Ha-ras oncogene. *Proc. Natl. Acad. Sci. USA.* 84:837–841.
- Kasid, A., C. Knabbe, and M. E. Lippman. 1987. Effect of v-ras<sup>H</sup> oncogene transfection on estrogen-independent tumorigenicity of estrogen-dependent human breast cancer cells. *Cancer Res.* 47:5733–5738.
- Daly, R. J., R. J. B. King, and P. D. Darbre. 1990. Interaction of growth factors during progression towards steroid independence in T-47-D human breast cancer cells. *J. Cell. Biochem.* 43:199–211.
- Barrett-Lee, P., M. Travers, Y. Luqmani, and R. C. Coombes. 1990. Transcripts for transforming growth factors in human breast cancer: clinical correlates. *Br. J. Cancer.* 61:612–617.
- King, R. J. B., D. Y. Wang, R. J. Daly, and P. D. Darbre. 1989. Approaches to studying the role of growth factors in the progression of breast tumors from the steroid sensitive to insensitive state. *J. Steroid Biochem.* 34:133–138.
- Welch, D. R., A. Fabra, and M. Nakajima. 1990. Transforming growth factor  $\beta$  stimulates mammary adenocarcinoma cell invasion and metastatic potential. *Proc. Natl. Acad. Sci. USA.* 87:7678–7682.
- Walker, R. A., and S. J. Dearing. 1992. Transforming growth factor  $\beta$ 1 in ductal carcinoma in situ and invasive carcinomas of the breast. *Eur. J. Cancer.* 28:641–644.
- Thompson, A. M., D. J. Kerr, and C. M. Steel. Transforming growth factor  $\beta$ 1 is implicated in the failure of tamoxifen therapy in human breast cancer. *Br. J. Cancer.* 63:609–614.
- Arteaga, C. L., T. Carty-Dugger, H. L. Moses, S. Hurd, and J. Pietenpol. 1993. Transforming growth factor  $\beta$ 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice. *Cell Growth & Differ.* 4:193–201.
- Lucas, C., L. N. Bald, B. M. Fendly, M. Mora-Worms, I. S. Figari, E. J. Patzer, and M. A. Palladino. 1990. The autocrine production of transforming growth factor- $\beta$ 1 during lymphocyte activation: a study with a monoclonal antibody-based ELISA. *J. Immunol.* 145:1415–1422.
- Sehested, M., and K. Hou-Jensen. 1981. Factor VIII-related antigen as an endothelial cell marker in benign and malignant diseases. *Pathol. Anat.* 391:217–225.
- Masson, P. 1929. Trichrome stainings and their preliminary technique. *J. Tech. Methods.* 12:75–90.
- Fraker, L. D., S. A. Halter, and J. T. Forbes. 1986. Effects of orally admin-

- istered retinol on natural killer cell activity in wild type BALB/c and congenitally athymic BALB/c mice. *Cancer Immunol. Immunother.* 21:114-118.
25. Fogh, J., and B. C. Giovanella. 1978. *The Nude Mouse in Experimental and Clinical Research*. Academic Press, New York. 502-521.
26. Rook, A. H., J. H. Kehrl, L. M. Wakefield, A. B. Roberts, M. B. Sporn, D. B. Burlington, H. C. Lane, and A. S. Fauci. 1986. Effects of transforming growth factor  $\beta$  on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *J. Immunol.* 136:3916-3920.
27. Roberts, A. B., M. B. Sporn, R. K. Assoian, J. M. Smith, N. S. Roche, L. M. Wakefield, U. I. Heine, L. A. Liotta, V. Falanga, J. H. Kehrl, and A. S. Fauci. 1986. Transforming growth factor type- $\beta$ : rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. USA.* 83:4167-4171.
28. Keski-Oja, J., F. Blasi, E. B. Leof, and H. L. Moses. 1988. Regulation of the synthesis and activity of urokinase plasminogen activator in A549 human lung carcinoma cells by transforming growth factor- $\beta$ . *J. Cell Biol.* 106:451-459.
29. Laiho, M., and J. Keski-Oja. 1989. Growth factors in the regulation of pericellular proteolysis: a review. *Cancer Res.* 49:2533-2553.
30. Postlethwaite, A. E., J. Keski-Oja, H. L. Moses, and A. H. Kang. 1985. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor  $\beta$ . *J. Exp. Med.* 165:251-256.
31. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakolew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor  $\beta$  by T lymphocytes and its potential in the regulation of T cell growth. *J. Exp. Med.* 163:1037-1050.
32. Kehrl, J. H., A. B. Roberts, L. M. Wakefield, S. Jakolew, M. B. Sporn, and A. S. Fauci. 1986. Transforming growth factor  $\beta$  is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* 137:3855-3860.
33. Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan. 1988. Deactivation of macrophages by transforming growth factor- $\beta$ . *Nature (Lond.)* 334:260-262.
34. Arrick, B. A., A. R. Lopez, F. Elfman, R. Ebner, C. H. Damsky, and R. Derynck. 1992. Altered metabolic and adhesive properties and increased tumorigenesis associated with increased expression of transforming growth factor  $\beta$ 1. *J. Cell Biol.* 118:715-726.
35. Torre-Amione, G., R. D. Beauchamp, H. Koeppen, B. H. Park, H. Schreiber, H. L. Moses, and D. A. Rowley. 1990. A highly immunogenic tumor transfected with a murine transforming growth factor type  $\beta$ 1 cDNA escapes immune surveillance. *Proc. Natl. Acad. Sci. USA.* 87:1486-1490.
36. Wallick, S. C., I. S. Figari, R. E. Morris, A. D. Levinson, and M. A. Palladino. 1990. Immunoregulatory role of transforming growth factor  $\beta$  (TGF- $\beta$ ) in development of killer cells: comparison of active and latent TGF- $\beta$ 1. *J. Exp. Med.* 172:1777-1784.
37. Steiner, M. S., and E. R. Barrack. 1992. Transforming growth factor- $\beta$ , overproduction in prostate cancer: effects on growth in vivo and in vitro. *Mol. Endocrinol.* 6:15-25.
38. Wu, S., D. Theodorescu, R. S. Kerbel, J. K. V. Willson, K. M. Mulder, L. E. Humphrey, and M. G. Brattain. 1992. TGF- $\beta$ <sub>1</sub> is an autocrine-negative growth regulator of human colon carcinoma FET cells in vivo as revealed by transfection of an antisense expression vector. *J. Cell Biol.* 116:187-196.
39. Wrann, M., S. Bodmer, R. de Martin, S. Siepl, R. Hofer-Warbinek, K. Frei, E. Hofer, and A. Fontana. 1987. T cell suppressor factor from human glioblastoma cells is a 12.5-kd protein closely related to transforming growth factor- $\beta$ . *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1633-1636.
40. de Martin, R., B. Haendler, R. Hofer-Warbinek, H. Gaugitsch, M. Wrann, H. Schlusener, J. M. Seifert, S. Bodmer, A. Fontana, and E. Hofer. 1987. Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor- $\beta$  gene family. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3673-3677.
41. Kuppner, M. C., M.-F. Hamou, S. Bodmer, A. Fontana, and N. De Tribollet. 1988. The glioblastoma-derived T-cell suppressor factor/transforming growth factor beta<sub>2</sub> inhibits the generation of lymphokine-activated killer (LAK) cells. *Int. J. Cancer.* 45:562-567.